8

High Throughput Formulation: Strategies for Rapid Development of Stable Protein Products

Rajiv Nayar and Mark C. Manning

INTRODUCTION

With the growing demand for new and innovative medicines, drug companies are spending record amounts of money on research and development. In the U.S. alone, research and development (R&D) investments are expected to exceed $24 billion in 1999, with investments in biotechnology totaling about $7 billion (Pharmaceutical Research and Manufacturers of America, 1999). As a result, new technologies are increasing the efficiency of the drug discovery process, and the drug pipelines have more products than ever in development. Nearly 350 biotechnology-related products are currently in clinical trials and over 50 are on the market (Pharmaceutical Research and Manufacturers of America, 1999). Pharmaceutical companies can now typically assess potential activities of up to 100,000 compounds a day using high throughput screening systems. In the biotechnology arena, recent advances in genomics, functional genomics, proteomics, bioinformatics and pharmacogenomics are facilitating the development of protein drug candidates at a much faster rate than was possible during the early years of the biotechnology industry. Couple these changes with the impending

Rajiv Nayar • HTD Biosystems, Hercules, CA. *Mark C. Manning* • Center for Pharmaceutical Biotechnology, University of Colorado Health, Sciences Center, Denver, CO 80262.

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Samsung Bioepis Exhibit No. 1002
Page 1324

publication of the complete sequence of the human genome by 2001 and there is a potential for an additional 15,000 protein drugs from the predicted 150,000 potential genes in the human genome.

This new wave of protein drugs will include compounds that can function as growth factors, act as specific stimulators or suppressors of certain functions or exhibit activities that have not been observed before. Many experts anticipate a golden age of protein-based therapeutics in the twenty-first century. As various research enterprises gear up to search for these new candidates using high throughput cellular and molecular screening assays, there undoubtedly will be a need for a corresponding ability to formulate these drugs much more quickly than in the past. In addition, there will be increased use of novel delivery systems to administer these protein therapeutics efficiently, making stabilization even more challenging. In short, the pharmaceutical biotechnology industry must find new ways to speed up the drug development process, in order to keep pace with the drug discovery process. We think that the limiting factor for introduction of new biotechnology-derived products will not be finding new drug candidates, but efficient and rapid formulation of these compounds into acceptable dosage forms for delivery and testing in humans.

The potential of many new drug candidates flowing through the pipeline will require implementation of an entirely new approach towards product development. The particular challenge addressed here is the demand that will be placed upon formulation groups, where there will be increased expectations to develop stable formulations of:

more proteins,
in a shorter period of time,
with less material, and
with little, if any, increase in personnel.

Therefore, one must consider new strategies for meeting such challenges.

This chapter is intended to serve as a forum for discussing and evaluating what factors must be considered in trying to implement new approaches to formulating protein pharmaceuticals in a rapid, rational fashion. As a comprehensive strategy for developing stable formulations of protein pharmaceuticals is not currently available, we have assembled what we consider to be the key elements necessary to accomplish this critical task. This chapter should not be viewed as a finished product, but as a work-in-progress, encouraging pharmaceutical scientists to comment on the concept and to modify the approach to their particular situations.

Again, the overarching goal is to possess a unified strategy for accommodating many more proteins at one time, while meeting shortening timelines. It should be expected that there would be a limited supply of well-characterized material and a fixed personnel head count. We envision a scheme that we term "high throughput formulation" or HTF. It is based upon our current

Samsung Bioepis Exhibit No. 1002
Page 1325

understanding regarding stabilization of proteins, but it also identifies areas where technical improvements must be made, especially in the area of analytical methodology and software. If the HTF strategy is successful, it should be possible to match the output of stable formulations by development groups with potential leads by discovery groups.

OVERALL STRUCTURE OF THE HTF APPROACH

The HTF concept includes four key elements, which are briefly outlined in Table 1. The heart of the HTF strategy is a decision plan, where the formulation scientist must decide on (a) the specific excipients to be used as well as (b) the final dosage form. Central to the HTF scheme is the use of a limited set of additives as well as dosage forms. Especially for lyophilized or freeze-dried formulations, our understanding of the behavior is such that some standard formulations can be envisioned (see Chapter 5). Also, the choice of basic solution conditions will be based upon preformulation studies that examine the behavior of the protein as a function of pH, ionic strength, and buffer type (see Chapters 1 and 3).

The second key aspect is an increased role for software and information databases. The preformulation studies could require a significant number of independent experiments to be conducted. Therefore, experimental design software should be used to reduce the number of experimental trials and optimize the results through statistical evaluation. In addition, there are a number of databases and associated programs for assessing physical and chemical properties based upon primary sequence of the protein through a number of websites (e.g. SWISS-PROT). These can provide insight into protein structure and other critical characteristics (e.g., pI, hydrophobicity and hydroflexibility plots and overall charge), and even identify residues that might be sensitive to chemical decomposition. We believe such capabilities will expand over time, and will play an increasingly important role in setting a rational plan for preformulation studies. Finally, we anticipate that specific protein stability databases will emerge, allowing scientists to gather data on specific development issues, without searching all of the open literature. Because information on protein stability comes from a multitude of disciplines (e.g., biochemistry, biophysics, food science, engineering, pharmaceutics, surface science and polymer science), open searches often miss crucial publications and documents. The challenge is not unlike Internet search engines trying to find a very specific subset of information on an ever-increasing world wide web. As these resources become more available and refined, they will emerge as invaluable tools for development of pharmaceutically acceptable formulations.

Samsung Bioepis Exhibit No. 1002
Page 1326

Table 1.
Key Elements of the HTF Approach

1. Decision Plan to Finalize Choice of Excipients and Dosage Form
   - A. Choice of Dosage Form
     1. Frozen Solution
     2. Liquid Formulation
     3. Lyophilized Formulation
     4. Protein Suspension
     5. Specialized Drug Delivery System
   - B. Choice of Excipients
     1. Proper pH/Buffer Species
     2. Salt
     3. Stabilizer
     4. Bulking Agent (for lyophilized formulations)
     5. Surfactant
     6. Chelators
2. Use of Software and Databases to Assist in Formulation Development
   - A. Experimental Design Software
   - B. Software and Databases for Analysis of Structure/Function/Properties/Stability
   - C. Protein Stability Databases
3. Availability of Essential Analytical Methods
4. Design of Appropriate Stability Testing Protocols

The third aspect of HTF is assembling the correct analytical methods to assay the stability, both chemical and physical, of a wide range of proteins. The mechanisms by which proteins degrade have been widely reviewed (Manning et al., 1989; Pearlman and Nguyen, 1992; Cleland et al., 1993), so they will not be discussed here. However, given this backdrop, we will describe certain key methods that must be available in order to conduct proper HTF studies. All of the required instruments do not necessarily need to be available in-house, as many can be accessed through contract organizations and through academic collaborations. In addition, it should be noted that there exists a tremendous opportunity to modify existing analytical techniques for use with smaller sample sizes and for rapid, parallel sampling. One of the expected limitations will be lack of well-characterized material. Whereas access to a minimum of 200–500 mg of protein for formulation studies is desirable, the genomic approach will tax the ability of process scientists to provide adequate amounts of purified proteins for all compounds under development. Therefore, in some cases, the total amount of protein available may be an order of magnitude less than the optimum. This situation should serve as an incentive to identify new approaches for gathering stability data. We will discuss below possible new methodologies that would be useful in the HTF scheme, but have not yet been applied to formulation development.

Samsung Bioepis Exhibit No. 1002
Page 1327

Finally, one must have a clear plan for stability testing, along with minimum criteria for a "stable" formulation. Certainly, that definition may change as a product matures. Minimum stability for the initial clinical studies may be quite different than the expectations for the final formulation tested in Phase III. Presently, there are debates within companies on how robust a preliminary formulation development or purification process must be in order to demonstrate efficacy in humans. Some companies will accept any minimal formulation in order to initiate clinical trials. However, even these formulations would likely have to have at least six to twelve months of shelf life. Our goal in the HTF approach is to develop a final formulation as early as possible in the drug development and testing process. If this goal can be accomplished, then fewer resources will need to be diverted to redesign the formulation later.

ROLE OF AN ESTABLISHED DECISION TREE FOR FORMULATION DESIGN

The central feature in the HTF scheme is a rational decision plan for choosing both the formulation components and the final dosage form. This decision tree must be flexible enough to accommodate a wide variety of proteins, it must allow for both liquid and solid dosage forms, and it must provide a rationale at each decision point. This framework allows the pharmaceutical scientist to decide what types of formulations are worth considering, both in terms of stability and the demands and desires of the marketing and clinical groups.

Often the design of a formulation of a protein pharmaceutical is not well developed. Companies attempt to expedite formulation selection by neglecting critical issues in formulation and stability. It is not uncommon to find sub-optimal formulations such as phosphate-buffered saline, possibly with the addition of human serum albumin as a ubiquitous protein stabilizer. For a few proteins, this approach has been sufficient, even resulting in commercially successful products, such as erythropoietin and granulocyte colony stimulating factor. However, there is a distinct danger in assuming that such successes with minimal formulations are generally applicable. Moreover, the current regulatory environment, and concerns in the general public surrounding the use of human-derived products in pharmaceuticals, makes the use of human serum albumin increasingly more difficult or even not acceptable. Therefore, there is pressure for any company selling a product that contains albumin to develop an albumin-free formulation. The same pressures are forcing companies to move from animal-derived surfactants to those obtained from vegetable sources. In addition, there is a realization that albumin, being a protein, is not indefinitely stable, and should have an expiration date above and beyond that of the active ingredient. All of these issues affect the

Samsung Bioepis Exhibit No. 1002
Page 1328

list of possible excipients that can be employed in a protein pharmaceutical product.

Constraints on a Pharmaceutically Acceptable Protein Formulation

Before we discuss the choice of dosage forms and excipients in the context of maximizing protein stability, we need to recognize the myriad of constraints placed upon the formulation by the rest of the company and by regulatory bodies. The first constraint, given the global nature of the pharmaceutical industry, is to have excipients that are acceptable in the major markets. This includes avoiding animal-derived additives, as discussed above. It also means that the list of possible additives is effectively limited to those already found in approved products in North America, Europe, and Japan. While this is not an immutable rule, few companies are willing to bear the added cost of getting a new excipient on the market while seeking approval for a new drug product. A major impact of this constraint is to move away from formulations containing albumin, which is still found in a number of protein products. One can anticipate that these older formulations will disappear in the coming years and be reformulated into second-generation products. A recent example has been the development of an albumin-free formulations for recombinant Factor VIII.

The second consideration is the tonicity of the final formulation. If a protein drug is to be administered by intravenous bolus injection or subcutaneously, rather than by continuous infusion, there are strict isotonicity and pH considerations that have to be met for a pain-free injection. Similarly, there are constraints on the choice of excipients, as some (e.g., citrate) have been shown to be irritating upon injection. This is one example of restrictions being set by the clinical groups, which should be clearly defined prior to the start of the formulation process.

Likewise, the eventual route of administration must be taken into account. A convenient administration system is highly desirable for ease of use in the clinical setting, so it is both a marketing and clinical issue. For example, in the case of a subcutaneous injection, there is a maximal volume (~1 ml.) that can be given to a patient without discomfort. Given the expected dose range, this will define the protein concentration that must be used. For other routes of administration, the ability to manipulate the protein concentration as a variable may be possible. Otherwise, it will be fixed, and the decision point regarding protein concentration is removed from the process described below.

The fourth issue is to work with a well-characterized bulk drug substance. This criterion will require cooperation and collaboration with the process science groups involved in fermentation and purification. Variation in the quality of the

Samsung Bioepis Exhibit No. 1002
Page 1329

starting material will compromise the validity of any preformulation or stability work done to date, as well as potentially invalidate data from toxicological tests.

The fifth constraint to be determined is the minimal stability required to complete proof-of-principle investigations, meaning animal studies and initial clinical trials. These investigations can extend for up to one year. Hence, at least a one-year shelf life for even the initial formulation may be required.

Finally, the manufacturing of the protein product must be possible at reasonable costs and allow adaptation to standard manufacturing processes. It is imperative that manufacturing economic analysis be conducted as soon as possible in the drug development process, including an assessment of whether sufficient manufacturing capability exists if the product is successful. Novel formulations that require special manufacturing setups and processes may not offer the advantages of speed which is necessary when one is confronted with development of numerous protein drug candidates. Most likely, specialized dosage forms and/or drug delivery systems will only be investigated with partners who possess the technology and capacity to solve these critical manufacturing issues. In addition, as the in-house pilot plants are challenged with manufacturing multiple products, with limited facilities and resources, there undoubtedly will be pressures to outsource the first clinical manufacturing because of both cost-effectiveness and timescale issues.

Proper Choice of Dosage Form

Considering all the therapeutic protein products on the market, it appears that three dosage forms are viable. The most preferred would be a solution formulation that is typically stored in the refrigerator and preferably in a pre-filled syringe. Second, a frozen solution loaded in a syringe or vial and thawed at the site of administration. Third, a lyophilized formulation, which can be stored at room temperature and reconstituted when needed. Each one of these dosage forms offers various advantages and disadvantages, in the speed of development, manufacturing, packaging and shipment logistics, and in administration of the product to the patient.

Generally, a frozen protein formulation offers the advantages of rapid development provided the protein is stable after multiple freeze/thaw cycles. It is also important to note that freezing can lead to selective precipitation of certain buffer species, producing possibly large pH shifts (van den Berg, 1959; van den Berg and Rose, 1959). This variation in pH, and the potential for damage at the water-ice interface (Chang et al., 1996; Hsu et al., 1995; Krielgaard et al., 1998), might limit the use of such a dosage form. Furthermore, there are disadvantages associated

Samsung Bioepis Exhibit No. 1002
Page 1330

with a frozen formulation concerning the packaging complexities involved in labeling, storage and maintaining the cold chain during transportation of a frozen product. These factors can add significantly to the development costs.

The second option for dosage form is a stable liquid formulation that can be stored in the refrigerator or at room temperature. This dosage form offers the advantages of potentially rapid development and ease of administration. However, for most proteins maintaining physical and chemical stabilities in aqueous solution for an extended period of time is extremely difficult. In addition, because the protein is free in solution there is potential for adsorption to the vial, whether it is made of glass or some type of plastic. A liquid product will have to be shown to be impervious to damage at the air-water interface during transportation, because agitation-induced denaturation and aggregation is a common problem with therapeutic proteins. There is also the possibility of leaching of metals and organics from the container and stopper, which is why some of the vials will need to be inverted during stability testing. These problems have been reported for drug products of all types (Airaudo et al., 1990; Nedich, 1983). Leached materials are particularly problematic with plastic vials and non-Teflon-coated stoppers. With glass vials the leachables could include ionic species that alter the pH and ionic strength of the solution during storage. Finally, the integrity of the container closure must be demonstrated, in order to ensure sterility of the product. For multi-use vials, preservatives can be added, but some are known to foster degradation of proteins (Lam et al., 1997; Rodrigues-Silva et al., 1999). Therefore, development of a stable liquid protein formulation has to be considered very carefully. Determination of the primary pathways responsible for decomposition of the active therapeutic protein is critical for developing this particular dosage form. All of these issues can add significant costs in terms of resources and analytical testing.

Most protein pharmaceuticals currently on the market are sold as lyophilized formulations. Placing proteins in the dried state significantly increases the shelf life, if the proper formulation and lyophilization cycle are used. The combined stresses of drying and freezing are inherently damaging to proteins, and only proper formulation will ensure that a stable product will be obtained. Fortunately, enough is understood now about freezing and drying of proteins that rational design of lyophilized formulations can be undertaken. Moreover, there are some formulations that seem to work well for a variety of proteins. We suggest that part of the strategy for rapidly identifying viable formulations is to make liberal use of some standard protein formulations. The rationale for these compositions has been described in detail in a number of review articles and book chapters, and is discussed in Chapter 5 (MacKenzie, 1976; Franks, 1990; Pikal, 1985, 1994; Carpenter and Chang, 1996; Carpenter et al., 1997). An approach towards developing a lyophilized formulation is described below.

Samsung Bioepis Exhibit No. 1002
Page 1331

As other formulation possibilities become more developed, one can modify the decision tree to accommodate them. For example, one can imagine alternatives to lyophilization for obtaining solid dosage forms. Both spray-drying and spray-freeze drying are being studied. However, it is expected that the formulation strategy for these processes will be quite similar to that used for lyophilization.

The most likely future additions to the dosage form list will be suspensions and specialized drug delivery systems. Protein suspensions can be produced by a number of different methods. The protein could be dried and suspended in a non-aqueous medium. Alternatively, the protein could be salted-out, using preferentially excluded solutes, such as ammonium sulfate or sucrose, leading to an aqueous vehicle containing most of the protein in its native state but in a solid form, because the solubility limit has been exceeded. The same effect might be accomplished by adjusting the pH to be near the isoelectric point of the protein. For the current version of HTF, we have chosen not to include these dosage forms until their use becomes more widespread and manufacturing facilities are developed to accommodate production of clinical batches.

The same can be said of specialized drug delivery systems, such as polymeric microspheres. Poly lactide/poly glycolide microspheres for sustained delivery of peptides have been in use for years. Recently, reports of their use for proteins, such as human growth hormone, have appeared. These systems bring their own specific stability issues (Cleland, 1998) in addition to making analysis of the stability of the active ingredient more challenging (Yang et al., 1999).

As discussed above, input from various departments, such as discovery research, pharmacokinetics, toxicology, purification, manufacturing, clinical and marketing, must be considered when deciding on the most appropriate dosage form for a given product. In the past, the drug development process has been more or less sequential, going from research to development to clinical, to manufacturing, and then to marketing. A change in strategy is required where there is more interactive, simultaneous communication between the various drug development departments. With the proper information from the other units, one should rapidly be able to decide on dosage form and develop candidate formulations.

Preformulation Studies

The exquisite sensitivity of protein structure, function, and stability to the primary sequence does not readily lend itself to a generic approach for protein formulation. Therefore, determination of the relative importance of various degradation pathways and elucidation of instability mechanisms for a given protein are essential. Even for closely related proteins, the relative stability and major

Samsung Bioepis Exhibit No. 1002
Page 1332

pathways for degradation might be quite different. Thus, the preformulation studies of new protein entities become very important and crucial tasks in the overall HTF strategy.

Preformulation refers to experimental studies designed at understanding the chemical and physical behavior of the protein or drug candidate. The major focus is on the solubility and stability as a function of a number of extrinsic factors, such as pH, protein concentration, ionic strength, buffer composition and temperature. This information will guide the appropriate choice of excipients, buffers and final protein concentration. Although a number of studies could be envisioned, HTF requires that these studies be very specific and focused. Any information that may narrow the choices would be essential for speedy formulation development. Relevant data can be gathered from the process-engineering group responsible for purification or literature studies on similar proteins, even if it is anecdotal. The final dosage form must also include constraints from marketing, clinical and regulatory groups addressing issues related to eventual approval of a pharmaceutically acceptable formulation for market. Ultimately, all of these data may be required to ascertain the most beneficial composition of excipients and the most appropriate dosage form for the protein product in hand.

Typical time scales for preformulation can range from one to three months depending on what assay systems have been established by the research groups and whether they can be utilized as stability-indicating. Therefore, early involvement of all of the formulation experts is critical for rapid development of lead formulations during the developmental phase. An additional driving force is also the fixed time required to generate the stability data to validate the formulation choice.

Proper Choice of Excipients

Liquid Formulations. If a solution dosage form is indicated, then there will be a finite set of possible excipients, restricting choices to those that are found in approved products and have been shown to be effective in protein formulations. For solution formulations, a list of possible excipients is given in Table 2.

Given that most protein formulations will exist at pH values between 4 and 9, there is a limited set of buffers that will exhibit sufficient buffering capacity. A number have been used in protein formulations and they are listed in Table 2. The main criteria for selection are good buffering capacity at the desired pH and lack of ability to accelerate specific chemical and physical reactions. Some proteins can preferentially bind certain buffer species, altering the conformational stability of the protein. In addition, some buffers (e.g., phosphate, Tris) have been

Samsung Bioepis Exhibit No. 1002
Page 1333

Table 2.
Possible Excipients for Use in Liquid Formulations

Excipient Class	Choices
Buffers	Histidine, Succinate, Acetate, Citrate Phosphate, Tris, Carbonate
Salts	Sodium Chloride, Calcium Chloride Magnesium Chloride
Non-Specific Stabilizers	Sucrose, Trehalose, other sugars, Amino acids (e.g., lysine, glycine)
Specific Stabilizers	depends upon the protein
Surfactants	Tween 20, Tween 80, Pluronic F-68 Sodium Dodecyl Sulfate
Chelators	EDTA

reported to catalyze certain hydrolytic reactions, such as deamidation (Patel et al., 1990).

Because proteins are polyelectrolytes, the ionic strength of the medium can often have an effect on structural stability. The most common salt used in protein formulations is sodium chloride. For an isotonic product, often it is advantageous to keep the concentration of salt as low as possible. Otherwise, the ability to employ stabilizing excipients will be limited.

Structural stabilization of proteins by specific ligands or cofactors in solution formulations can be important for formulation optimization. The mechanism by which these compounds increase thermodynamic stability is described by Timasheff (1992, 1995, 1998) and in other Chapters in this book. Several pharmaceutically important proteins have been stabilized by addition of specific ligands (including metals ions such as calcium), including fibroblast growth factor (Volkin et al., 1993) and DNAse (Chen et al., 1999).

To stabilize proteins (both in aqueous solution and during freezing) with non-specific compounds (e.g., sugars), relatively high concentrations (ca. > 0.2 M) of ligand (solute) are needed to affect protein stability. The mechanism of such stabilization is described in more detail in Chapters 2 and 3. The main point for the current discussion is that in the presence of nonspecific stabilizing excipients fewer protein molecules are unfolded at any point in time, either partially or completely, which reduces the amount of aggregation-competent species and the rate of aggregation (Kendrick et al., 1997, 1998). Addition of small amounts of a specific ligand should theoretically accomplish the same outcome. The most effective non-specific stabilizers tend to be disaccharides, such as sucrose and trehalose. However, certain salts, amino acids, and polymers are preferentially excluded as well. Still, unless there is evidence for advantage in use of a

Samsung Bioepis Exhibit No. 1002
Page 1334

particular compound from this group, sucrose and trehalose should remain the first-line choices.

Finally, one must decide on whether to include a surfactant in the formulation. Surfactants have been shown to inhibit protein aggregation during agitation (e.g., Bam et al., 1995; Krielgaard et al., 1998). The mechanisms by which surfactants stabilize proteins are discussed in a separate Chapter. The surfactant often will be most effective at or above the critical micelle concentration. In Table 2, only the surfactants that have been approved for use in parenteral products in the U.S. have been listed. Note that one potential drawback of including surfactants in the formulation are contaminants (e.g., peroxides) that could cause chemical degradation of proteins (e.g., oxidation).

Lyophilized Formulations. It can be assumed that most proteins will not exhibit sufficient stability in aqueous solution to allow a liquid formulation to be developed. Our understanding of the basic requirements for obtaining a stable lyophilized protein formulation is relatively well developed. The question then is what combination of excipients will allow such a formulation to be prepared. The minimal composition includes a buffer species, an additive capable of forming an amorphous glassy state and inhibiting lyophilization-induced unfolding in which the protein remains entrapped, a bulking agent to provide cake stability, and possibly a surfactant to retard surface-induced damage and/or promote refolding. The rationales for the minimal criteria for a stable lyophilized formation and the choice of appropriate excipients are described in detail in another Chapter.

While a comprehensive formulation development algorithm is yet to be published, following the HTF process outlined above, one can imagine obtaining a rational approach towards selecting final candidate formulation that can be used routinely, even with new classes of therapeutic proteins. Clearly, the details of each case will be guided by the outcomes of the preformulation studies. However, even without a complete data set from the preformulation experiments, implementation of a few standard formulations should be possible. Ultimately, we believe there will be a relatively small number (six of less) of generic, standard lyophilized formulations that will work for nearly all proteins. Each of these will have a corresponding lyophilization cycle that can be used to achieve optimal results. When the HTF process has evolved to this optimal state, the personnel demands be greatly reduced (in terms of full-time equivalents, or FTEs) and the time lines should shrink significantly from the current average of nearly one year.

Estimates of Resources Needed for Formulation Development

The current estimates of the resources required and the possible timelines for formulation development are given in Table 3. Specialized formulations

Samsung Bioepis Exhibit No. 1002
Page 1335

Table 3.
Estimated Resources Needed for Development of Final Stable Formulations of Protein Pharmaceuticals

Resource	Current Status	Initial HTF Targets	Optimal HTF Targets
• Personnel			
Standard Formulations	2 FTE	2 FTE	2 FTE
Complex Formulations	3–5 FTE 3–4 FTE	3 FTE	
• Time Scale			
Standard Formulations	12 months	6–9 months	3–6 months
Complex Formulations	12–24 months	12 months	6–9 months
• Minimal Amount of Material			
Standard Formulations	200–500 mg	100–200 mg	50–100 mg
Complex Formulations	400–2000 mg	200–500 mg	100–200 mg

(suspensions and controlled release dosage forms) will require more resources and less aggressive time lines (see Table 3). However, even this situation should improve as more of these types of products enter the marketplace. We anticipate that a significant number of these types of products will be approved over the next ten years. By then, most large pharmaceutical companies will have acquired the necessary expertise to develop them at rates comparable to the standard formulations of today. Once the HTF scheme has evolved to this stage, a company should be able to develop final formulations quickly and easily, even if personnel levels are relatively constant.

Interestingly, while a streamlined approach towards final formulation selection is the heart of the HTF strategy, it is the one aspect that can be introduced into any current product development scheme almost immediately, as it can be accomplished solely based on the current state of understanding of protein stabilization. Other aspects of the HTF package will demand more time to implement, as they will require significant advances to be made, particularly in the areas of analytical methodology and information technology. These two features of the HTF scheme are described below.

USE OF SOFTWARE AND DATABASES TO ASSIST IN THE HTF PROCESS

Unless formulation scientists have access to all pertinent information, critical choices for formulation components could be delayed or, even worse, incorrect. Therefore, we propose that a key aspect of the HTF process is to develop an integrated information system that can assist the scientist in making critical

Samsung Bioepis Exhibit No. 1002
Page 1336

decisions. Each and every step in the formulation development process is predicated upon having the most recent and comprehensive information on the physicochemical properties of the protein under development, as well as on the effects of additives on protein structure and stability. Clearly, the latter part of this goal is ever evolving as our knowledge of protein chemistry increases. Therefore, the final HTF scheme will involve a central database of essential information. While a large amount of this type of information is currently available, it appears in a multitude of sources, generated by researchers in disparate disciplines, ranging from materials science to biochemistry to food science to pharmaceutics. This makes access to the data difficult. Unless efforts are made to assemble this data in a cohesive package soon, the challenge will become even greater. Once the task is accomplished, sufficient information should be available to predict the performance of certain types of formulations.

In the absence of such resources, one can still make use of existing software and databases. These are summarized below. Briefly, they cover three areas: predictive protein algorithms based upon primary sequence, sequence homology analysis software, and programs aimed at predicting relative stability of proteins. Summaries of the locations and availability of these programs and databases has recently been published (Cook, 1999; Apweiler, 1999).

Predictive algorithms allow one to analyze a primary sequence and predict a variety of chemical and physical properties. For example, there has been enormous effort to develop schemes whereby one can predict the three-dimensional folding of a protein based solely on its sequence. Although there have been some successes, it has been demonstrated in double-blind studies that analysis of new sequences, when they have no clearly related partner in the existing database, fail to provide an accurate prediction of the folded conformation (Orengo et al., 1999; Vencloyas et al., 1999). When the analysis can be guided by sequence homology, the accuracy rises dramatically. Likewise, one can imagine developing structure-stability relationships, based solely upon primary sequence. Some studies of this nature have been reported. For example, Pandit and co-workers relate the frequency of certain dipeptide sequences to instability in proteins (Reddy, 1996; Guruprasad et al., 1990). The result is an algorithm that predicts the relative stability of a protein. Although the degradation criteria are based upon intracellular stability, this approach does provide researchers with an expectation of the overall fragility of the protein in question.

In another example of this type of sequence analysis, Roger et al. (1986) identified regions in proteins that were high in proline, acidic side chains (Asp and Glu), serine, and threonine. The presence of these regions was associated with more rapid degradation *in vivo*. Similar correlations should exist for *in vitro* stability. Even programs to predict likely deamidation sites (where Asn is converted into Asp-like residues) would be helpful, especially as our ability to remove those sites by recombinant DNA technology improves. There is now a

Samsung Bioepis Exhibit No. 1002
Page 1337

solid basis on which to predict both primary and secondary structure effects on deamidation rates (Wright, 1991; Xie and Schowen, 1999), which is considered in more detail in Chapter 4.

Given that numerous protein sequences, whether determined directly or extrapolated from nucleotide data, are available in databases that can be accessed via the world wide web, focused studies generating data on specific instabilities (e.g., deamidation, oxidation, aggregation) could lead to similar structure-stability relationships. The number of available sequences has been doubling every 18–24 months over the last ten years (Cook, 1999). Similarly, there has been an explosion in algorithms and software to analyze these data. Many focus on sequence homology and identification of functional and genetic relationships. Given these advances, it is not unreasonable to expect that stability-indicating profiles could be developed as well.

In addition to stability predictions, many programs are available to calculate important chemical and physical properties such as the isoelectric point, the hydrophobicity profile, the positioning of secondary structure elements, and the conformational flexibility. Knowledge of these properties would help the formulation scientist anticipate the behavior of a new molecule provided for development into a potential drug product. Furthermore, algorithms to predict correlations of these physical properties with physical and chemical stabilities of therapeutic proteins should be straightforward to design and implement with current programs.

ESSENTIAL ANALYTICAL METHODS

One must have access to certain equipment in order to characterize properly protein degradation pathways and effects of formulation variables on protein stability. In addition, we will discuss some areas where technological advancements must be made in order to allow formulation scientists to work with smaller amounts of protein. While a number of reliable and accurate stability-indicating assays exist, there needs to be efforts to miniaturize some of them. This will allow development of proteins where the amount of material is limited, e.g., less than 100 milligrams total.

The first analytical methods that are needed are those that can quantify the protein concentration. As determination of accurate extinction coefficients is straightforward, ultraviolet absorption spectroscopy is inexpensive and preferred over chemical methods, such as the Lowry method. One also needs to be able to monitor the global structure of the protein. Circular dichroism (CD) spectroscopy allows one to follow changes in both secondary and tertiary structure of proteins in solution (Manning, 1994). Infrared spectroscopy can also be used to detect

Samsung Bioepis Exhibit No. 1002
Page 1338

secondary structural changes (see below), and fluorescence and derivative U spectroscopies can be used to study tertiary structure.

The second category of required analytical methods are those that monitc retention of native primary structure, namely, chromatographic methods, suc as reversed phase HPLC and ion exchange HPLC, or alternative methods lik capillary electrophoresis. Among the most useful applications of these HPL methods is the generation of a "tryptic map", which is accomplished by digest ing the protein with a sequence-specific enzyme (e.g., trypsin) and separating th resulting peptide fragments by reversed phase HPLC. The pattern then become a sensitive fingerprint of the protein. Any chemical modification of the protei will cause one or more of the peaks to shift, indicating damage to the protein Mass spectrometry can be used to determine the specific chemical modificatio leading to the peak shift.

Among the most important degradation pathways for proteins is aggrega tion. A number of methods are available to characterize and quantify aggregatio levels in proteins, including polyacrylamide gel electrophoresis (PAGE), siz exclusion chromatography (SEC), light scattering techniques and analytical ultra centrifugation. Although each of these methods has its advantages and disad vantages, all four should be considered essential, as they can be complementary They allow both qualitative and quantitative characterization of the soluble an insoluble aggregates in a bulk substance or final formulation.

For lyophilized formulations, a certain set of equipment is considered to b the minimum required for conducting formulation development properly. Thes include an infrared (IR) spectrometer, a Karl Fischer titrator, a differential scan ning calorimeter (DSC), and x-ray diffractometer. The IR instrument will allow one to determine the extent of structural damage (at a secondary structure level in the solid state. In addition, it can be used to monitor the secondary structur composition in the original solution (prior to lyophilization), in the frozen soli and in the reconstituted product (Carpenter et al., 1998). X-ray diffraction c solids allows one to determine the crystallinity of a solid sample. In formulation where the bulking agent is crystallizing during the lyophilization process, on needs to know whether the crystallization is complete. Incomplete crystallizatio can lead to degradation during storage (cf. Carpenter et al., 1997). The Ka Fischer apparatus allows one to measure the amount of water in the final cak Typically, moisture contents need to be at 1–2 % in order for the product to hav the optimal glass transition temperature (T_g) and maximal stability. Finally, DS is required to measure the T_g of the final formulation. The storage temperatur must below the T_g. As the minimal acceptable storage temperature for lyophilize formulations is room temperature, a minimum T_g would be about 40° C. In add tion to these instruments, access to a microscope equipped with a lyophilizatio stage is valuable.

The issue of how to develop formulations with limited amounts of materi is a critical concern. One can imagine adapting a number of existin

Samsung Bioepis Exhibit No. 1002
Page 1339

technologies to assist in assessing the viability of a given formulation while using very small amounts of material. These would include:

1. microtiter plate assays,
2. robotics to assist in sample preparation and analysis,
3. ELISA or other antibody-based recognition assays for intact drug,
4. multi-sample adaptations of existing analytical techniques (e.g., DSC or analytical ultracentrifuge)
5. nanoseparation methodology (the so-called "lab-on-a-chip" approach),
6. surface-immobilized analytical methods, such as the BIAcore.

Each of these methods could provide significant increases in the capability to monitor the stability of a protein in a microformulation (less than 10 μg of active drug). For example, new chip technologies (either DNA- or protein-based) are becoming powerful tools that are increasingly used in all areas of drug discovery. They offer significant advantage over older technologies in terms of speed, sensitivity and cost. It is important to consider the use or implementation of these tools in formulation studies. One can envision protein chips doing for the study of proteins what DNA chips have done to the study of genes. That is, they can be used to identify critical parameters required for rapidly developing a stable formulation. Similarly, any of the other methods could be modified to assist the formulation scientist.

Finally, there has been a steady improvement in protein analysis tools such as mass spectrometry and more powerful and versatile biochemical techniques, which are not only more sensitive but offer the advantages of speed and low cost. So with the emerging technological advances in analytics and implementation of these enabling technologies in preformulation and formulation studies, one can envision a more time and resource efficient process for developing stable protein pharmaceuticals. Most of these technologies are being developed to meet the needs of efficient drug discovery research. The challenge here will be the speed at which the formulation scientists adapt these technologies into their programs. Unfortunately, these tools have not yet reached their maturity, so the implementation of micro-analytical methods for preformulation and formulation is crucial for developing HTF strategies for protein pharmaceuticals.

STABILITY PROTOCOLS

In addition to formal storage stability protocols that would be acceptable to regulatory agencies for real-time data, one must have standard approaches for assessing the possible long-term fate of the candidate formulations. Given the aggressive time line of the HTF strategy, one must rely, to some degree, upon accelerated storage studies to guide decision about suitable excipients. These

Samsung Bioepis Exhibit No. 1002
Page 1340

could be done as isothermal studies, storing the protein at room temperatu and 40° C, for example. In certain cases, exposure to higher temperature such as 50° C or even 60° C, may be warranted. However, it is important to no that at higher temperatures, there is the distinct possibility that the rat determining pathway may change compared to that at the projected stora conditions.

An alternative to isothermal testing that needs to be considered is the use non-iosothermal stress testing. In this procedure, the sample is exposed to a line increase in temperature over time (or approximately linear with respect to time Samples are taken at set intervals and assayed for protein damage. Using an alg rithm developed by Lee and Stavchansky (1998), it is possible to determine t Arrhenius parameters in a single experiment, which would otherwise require least three separate isothermal studies. This approach has been demonstrated f a pentapeptide (Lee and Stavchansky, 1998), and should be extended to larg proteins in the future.

UNIFIED STRATEGY FOR HTF

The requirements for HTF can be summarized in four points. First, integ tion of speed, sensitivity and cost can be achieved by having in place go analytical methodologies for monitoring both chemical and physical instabil mechanisms. These methods can serve as tools for elucidating the mechanism instability of protein drugs and also as stability indicating assays during analy of the final formulation. Second, identification of the instability mechanism proteins can lend itself to developing a fast-track stable liquid protein formu tion, although the disadvantages of addressing biocompatibility and transpor tion issues have to be addressed with such dosage forms. A third requirement HTF is to have obviously a safe and a convenient dosage form for administ tion. Coupled with this criterion should be the requirement for manufacturi the formulation using standard processes, so that if needed the production can out-sourced to a contract facility.

Finally, technological advances will be a major driver for HTF and in gene biotechnology development. The ability to work at low microgram to milligr scale of protein is essential for the ability to start the process before the d candidate achieves development status. In many cases, companies may initia neglect formulation and stability issues that will haunt them in later stages development. The development of successful formulations is dependent u the ability to study both the *in vitro* and *in vivo* characteristics of the prot drug, as well as its intended application in the clinic. Thus, it is essential the formulation group to work closely with the discovery research,

Samsung Bioepis Exhibit No. 1002
Page 1341

pharmacokinetics and the toxicology departments prior to the decision to move the drug into full-scale development. Resolving the difficulties in the early research and development stages will not only help in targeting the appropriate drug candidate for development, but also speed up the selection of a pharmaceutically acceptable formulation for clinical trials. What we propose is the involvement of the formulation group in a multi-disciplinary environment where interactions start early in the research environment. The appropriate lead candidates would be selected not only based on efficacy studies, but also on pre-formulation studies. We envision these studies to take one to three months to establish appropriate profiles for formulation development. As a result, the lead formulation can have the advantages of demonstrating efficacy and potential stability of the protein drug.

During the development process of a biotechnology drug we envision formulation to take typically three to six months. This period would encompass preformulation studies and evaluation of the formulation options determined by the planned clinical studies. Hence, protein formulation and purification can be developed concurrently using limited quantities of the product, and identification of the lead formulation could occur at the time of establishment of the purification process. What we propose is not a sequential development pathway for protein drugs, but a concurrent scenario where formulation design is initiated very early in the drug development pipeline. We are quite confident that the HTF can be coupled successfully with high throughput screening strategies of protein drugs and facilitate drug development within the inherent time and resource constraints of the pharmaceutical industry.

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Samsung Bioepis Exhibit No. 1002
Page 1345

Index

Acid-soluble spore proteins (SASP), 97–98
Acidic fibroblast growth factor (aFGF), 69
Administration, route of, 182
Adsorption, 13, 152, 162–164
Aggregation
  covalent and non-covalent, 13
  nonionic surfactants to inhibit, 127
Agitation, and stability, 71–72
Analytical instruments, 3
Analytical methods, essential, 180, 191–193
Antibodies: *see* Recombinant humanized anti-IgE monoclonal antibody
ArgHCl, 45–46
Arrhenius equation, 8
Asparagine (Asn) residues, deamidation of in peptide and proteins, 86–92

Bax, 169
Bioactivity, 49
Bovine pancreatic trypsin inhibitor (BTPI), 40
Bovine serum albumin (BSA), 154–155
Bovine somatotropin (BSTI), 91
Büchi laboratory spray dryer, 139–141, 146, 147
Buffers/salts, 14, 67
  choice of, 118–119
  debye screening, 67–68
  ionization enthalpy, 68
  lysis, 33
  preferential exclusion of salts, 67
Bulking agent, 14, 126
Carbohydrates, to stabilize spray-dried proteins, 149
Chemical modifications, 17
Chloramine T, 98
Chromatography, 192
  column, 7
Cleavages, 13
Commercial formulation, 19
  for early preclinical and clinical studies, 19
Commercial formulation development (process), 18
  decisions during, 18–19
  regulatory issues in, 20–22
  timeline, 18
Conformational stability, 51
Cosolvents, preferentially excluded, 66–67
Critical micelle concentration (CMC), 164, 165, 171
Cyclic imide, 13
Cytokine receptor homology (CRH), 30
Deamidation, 13; *see also under* Polypeptides, structure of
Decision tree, role of established for formulation design, 181–182
Degradation
  chemical and physical mechanisms, 160
  conditions used to accelerate, 6
  enzymatic, of non-ionic surfactants, 170
  minimization of routes of chemical, 116–117
Degradation products, qualification of, 17
Dehydration-induced unfolding, mechanism for inhibition of, 123–124
Delivery of products, 16
Denaturants, removal of, 41–43
  solid phase, 43
Denaturation, 13, 70–72
Deuterium, 96–97
Dialysis, 41–42
Dilution, 42–43
Disaccharides, 149
Disaggregation, high pressure, 48
Disulfide bond analysis, 50
Disulfide bond formation, 41

Samsung Bioepis Exhibit No. 1002
Page 1346

Dosage forms
  design of successful, vii–viii
  proper choice of, 183–185
  unconventional, 17
Drug delivery systems, specialized, 185
Drug design, rational, vii
Drying-air volumetric flow rate (v_{da}), 147
Electrophoresis, 7
Equilibrium unfolding, 62
Erythropoietin, recombinant human, 137
Escherichia coli (*E. coli*)
  deamidation of histidine-containing protein in, 90
  recombinant production of native proteins from, 27–28
    cell washing and lysis, 32–34
    distribution of expressed proteins, 28–31
    methods to analyze folded structures, 48–51
    purification and refolding of proteins from inclusion bodies, 36–37
    purification and refolding of soluble, misfolded proteins, 35
    purification of expressed proteins from inclusion bodies, 36–37
    purification of soluble, folded proteins, 34–35
    refolding mechanism, 38–40
      disulfide bond formation, 41
      effects of excipients, 44–47
      effects of tag sequences, 44
      high pressure disaggregation and refolding, 48
      removal of denaturant, 41–43
      response surface methodology, 47–48
Excipients, 21, 170
  animal-derived, 15
  choice of, 117, 120–121, 186–188
  effects on refolding mechanism, 44–47
  interactions with proteins, 65, 66–70
    preferentially excluded cosolvents, 66–67
  limiting solvent accessibility, 96

Fibroblast growth factor (FGF), 31, 32, 69
Fibronectin, 98
Formulation change/amendments, results required to file, 20–22
Formulation development, 1–2, 10; *see also specific topics*
  estimates of resources needed for, 188–189
Formulation development (*cont.*)
  financial ramifications of rapid *vs.* delayed, 110–111
  necessary studies for, 15–16
  preformulation development, 4–5
  preparation for
    resource requirements, 3–4
    useful information for designing formulations, 4
Formulation optimization studies, design of, 15
Formulation options for protein pharmaceuticals, 10
  liquid formulations, 10–11
  single- and multidose forms, 12
  solid dosage forms, 11–12
Formulation problems, strategies to overcome, 17
Formulation studies, information useful for, 4
Formulation variables, optimization of, 13–15
Formulations
  constraints on pharmaceutically acceptable, 182–183
  important components, 13–15
Free energy *vs.* extent of reaction, 73
Freeze-drying microscopy, 115
Freeze-thawing, and stability, 71, 125
Freezing protection, mechanism for, 121–123
Frozen formulations, 183–184
GdnHCl, 35, 36, 45
Gel filtration, 43
Gibbs adsorption equation, 162–164
Glass immobilization hypothesis, 149, 151
Glass transition temperature (T_g), 113–116, 120, 125, 126
Glutathione S transferase (GST), 44
Glycine, 116, 126, 150
Granulocyte colony stimulating factor (G-CSF) receptor, 29
Growth factor
  fibroblast, 31, 32, 69
  keratinocyte, 31, 32, 35
Growth hormone, recombinant human, 137, 143, 147–148, 152–154, 167, 170, 171
Growth hormone-releasing factor (GRF), 90
Handling of products, 16
High-pressure liquid chromatography (HPLC), 192
High throughput formulation (HTF), 178–179, 186, 188, 189
  concept, elements, and structure of, 179–181

Samsung Bioepis Exhibit No. 1002
Page 1347

High throughput formulation (HTF) (*cont.*)
requirements for, 194
software and databases to assist, 179, 189–191
unified strategy for, 194–195
Histidine-containing protein (HPr), 90
Hydrogen to deuterium (H-D) exchange rates, 96–97
Hydrolysis of peptides, 85; see also Deamidation
Hydrophobicity and hydrophobic interactions, 62–63, 72, 95, 161, 163, 166
Hydrophobicity reversal, 167–168
Hydroxyethyl starch, 126
Impurities, 14–15
Inclusion bodies (IBs), 29, 36–37
Infrared (IR) spectroscopy, 191–192
Interleukin-1$ (IL-1$), 28–30
Interleukin 6 (IL-6), 40, 50, 51
I(SH) and I(SS), 39, 41
Isothermal and non-isothermal stress testing, 194
Keratinocyte growth factor (KGF), 31, 32, 35
Lactate dehydrogenase (LDH), 138, 144, 145, 154, 155
Lactose, 149, 150
Ligands, 119–120
non-specific, 96
specific binding, 68–69
Light scattering/turbidity, 7
Liquid formulations, 10–11, 184
excipients for use in, 186–188
Lyophilization-induced protein unfolding, inhibition of, 112–113
Lyophilized products/formulations, 11, 109–110, 184, 188
minimal criteria for successful, 111–117
minimization of routes of chemical degradation, 116–117
storage below formulation glass transition temperature, 113–114
strong, elegant cake structure, 114–116
water content relatively low, 114
rational design of stable, 117–118
bulking agent, 126
choice of buffer, 118–119
ligands/pH optimizing thermodynamic stability of protein, 119–120
trehalose/sucrose to inhibit protein unfolding and provide glassy matrix, 120–126
Lyoprotectants, 149
Lysis buffers, 33
Maltose binding protein (MBP), 44
Mannitol, 116, 126, 137–138, 149–151
Manufacturing process, 21
Metal-catalyzed oxidation (MCO), 94–95
Metals, trace, 94
Methionines and methionine residues, 94–100, 116–117
Micelle concentration, critical, 164, 165, 171
Microcharacterization methods, 7
Monoclonal antibodies: *see* Recombinant humanized anti-IgE monoclonal antibody
Murine IFN-((mIFN-(), 37–38
Mutagenesis, site-directed, 17
OmpA, OmpG, and OmpT proteins, 28–29
Optimization of formulation variables, 13–15
Osmotic pressure, 165
Ovalbumin, 40
Oxidation (processes), 13; *see also* Polypeptides, structure of, role in protein oxidation
excluded effects on, 98–99
metal-catalyzed, 94–95
non-site-specific, 94–97
Oxygen, singlet ($^1O_2$), 93
Packaging material, 21
Parathyroid hormone, 95
Peptides: *see* Polypeptides
Periplasmic secretion strategy, 28–29
Peroxides, organic, 93
pH, 14, 186
Polyethylene glycol (PEG), 46
Polypeptides, structure of, 85–86, 101
deamidation rates and, 86–87, 92
primary structure effects, 87–89
secondary structure effects, 89–91
tertiary structure effects, 91–92
role in protein oxidation, 92–93, 100–101
conformational control of oxidation in aqueous solution, 97–99
effects of oxidation of methionines on protein structure, 95–96
limiting solvent accessibility of residues, 96–97
structural control of oxidation in lyophilized products, 99–100
types of oxidation processes, 93–95
Polysorbate concentration, critical, 153, 154

Samsung Bioepis Exhibit No. 1002
Page 1348

Preferential exclusion mechanism, 122–123
Preformulation development, 4–10
  development of analytical methods, 6, 7
  evaluation of the significance of problems, 7–8
    qualitative assessment, 9–10
    quantitative assessment, 8–9
  information obtained from, 5
Preformulation studies, 185–186
Preservatives, 12
Pressure, and stability, 72
Product development timeline, 18
Protein formulation: *see* Formulation
Protein pharmaceuticals, characterization of, 5
Proteins; *see also specific topics*
  maintenance of biophysical and biochemical properties of, 16
  typical methods used to characterize, 6, 7
Proteolysis, 85
  limited, 51
Purified protein, 3
Purity of raw materials, 14–15
Reaction, free energy *vs.* extent of, 73
Receptor binding, 49
Recombinant human erythropoietin (rhEPO), 137
Recombinant human growth hormone (rhGH), 137, 143, 147–148, 152–154, 167, 170, 171
Recombinant humanized anti-IgE monoclonal antibody (rhuMAbE25), 137–138, 148, 150–151
Regulatory applications, information included in, 20, 21
Regulatory documents, 22–23
Regulatory guidelines, 2
Regulatory license for drug product, results required to apply for, 20
Residues; *see also* Methionines and methionine residues
  asparagine (Asn), 86–92
  solvent accessibility, 96–97
Response surface methodology (RSM), 47–48
Salts: *see* Buffers/salts
SASP (acid-soluble spore proteins), 97–98
Sequence analysis, 190
Serine hydroxymethyltransferase (SHMT), 91
Sodium chloride (NaCl), 118
Sodium dodecyl sulfate (SDS), 160
Solid dosage forms, 11–12
Solubilizer, 14
Solutes, excluded, 99
Solvent accessibility of residues, 96–97
Spectroscopy, 7, 50–51, 191–192
Spray-drying of proteins, 156
  conditions, 140
    drying air volumetric flow rate, 147
    liquid feed rate and atomising air volumetric flow rate, 146–147
    temperature (T_{inlet}, T_{outlet}), 140–146
  developments in last 10 years, 136–138
  equipment, 139–140
  formulated systems, 149–151
  influence of formulation, 147
  pure proteins, 147–149
  reasons for, 135–136
  use of added surface active substances, 151–156
Stability (physical), protein, 61–62, 73, 181
  kinetic control of, 63–65
  physical factors affecting, 70–72
  self-stabilization, 69–70
  thermodynamic control of, 62–63
Stability problems, typical
  causes and solutions, 13
Stability protocols, 193–194
Stability studies, 3
  accelerated, 5–6
  guidelines for, 20
  storage, 15–16
Stabilization, structural, 187
Stabilizers, 14
  non-specific, 96, 187
Storage stability studies, 15–16
Stress testing, non-isothermal, 194
Sucrose, 66, 113, 114, 120–121, 138, 149, 150; *see also under* Unfolding
Sugar; *see also* Sucrose
  rationale for choice of stabilizing, 120–121
Surface denaturation, 13
Surface tension, 165
Surfaces, proteins and surfactants at, 161–166
Surfactant-protein interactions, 159–160
  in solution, 166–167
Surfactants, 188
  effects on protein assembly state, 167–169
  effects on proteins during freezing, freeze-drying, and reconstitution, 169–170
  non-ionic, 160, 161
    enzymatic degradation of, 170

Samsung Bioepis Exhibit No. 1002
Page 1349

Surfactants (*cont.*)
nonionic, to inhibit aggregation, 127
recommendations for protein formulation, 170–171
at surfaces, 161–166

Tag sequences, 44
Temperature, and stability, 70–71
Tetrapeptides, 90
Thermal analysis, 7
Thermodynamic box, classical, 77–78
Timasheff preferential exclusion mechanism, 73–78
Tonicity, 182
Tonicity modifier, 14
Transportation of products, 16
Trehalose, 113, 120–121, 149, 150, 154, 155; *see also under* Unfolding
Trypsinogen, 138, 148
Turbidity, 7
Tween 20, 164–170
a/b-type acid-soluble spore proteins (a/b-SASP), 97–98

Unfolding
dehydration-induced, 123–124
equilibrium, 62
lyophilization-induced, 112–113
trehalose/sucrose to inhibit, 120–126

Water replacement hypothesis, 149, 151
Water replacement mechanism(s), 123–124
Wide-angle X-ray diffraction (WAXS), 150
Wyman linkage function, 73–78

Pharmaceutical Biotechnology

Chronological Listing of Volumes

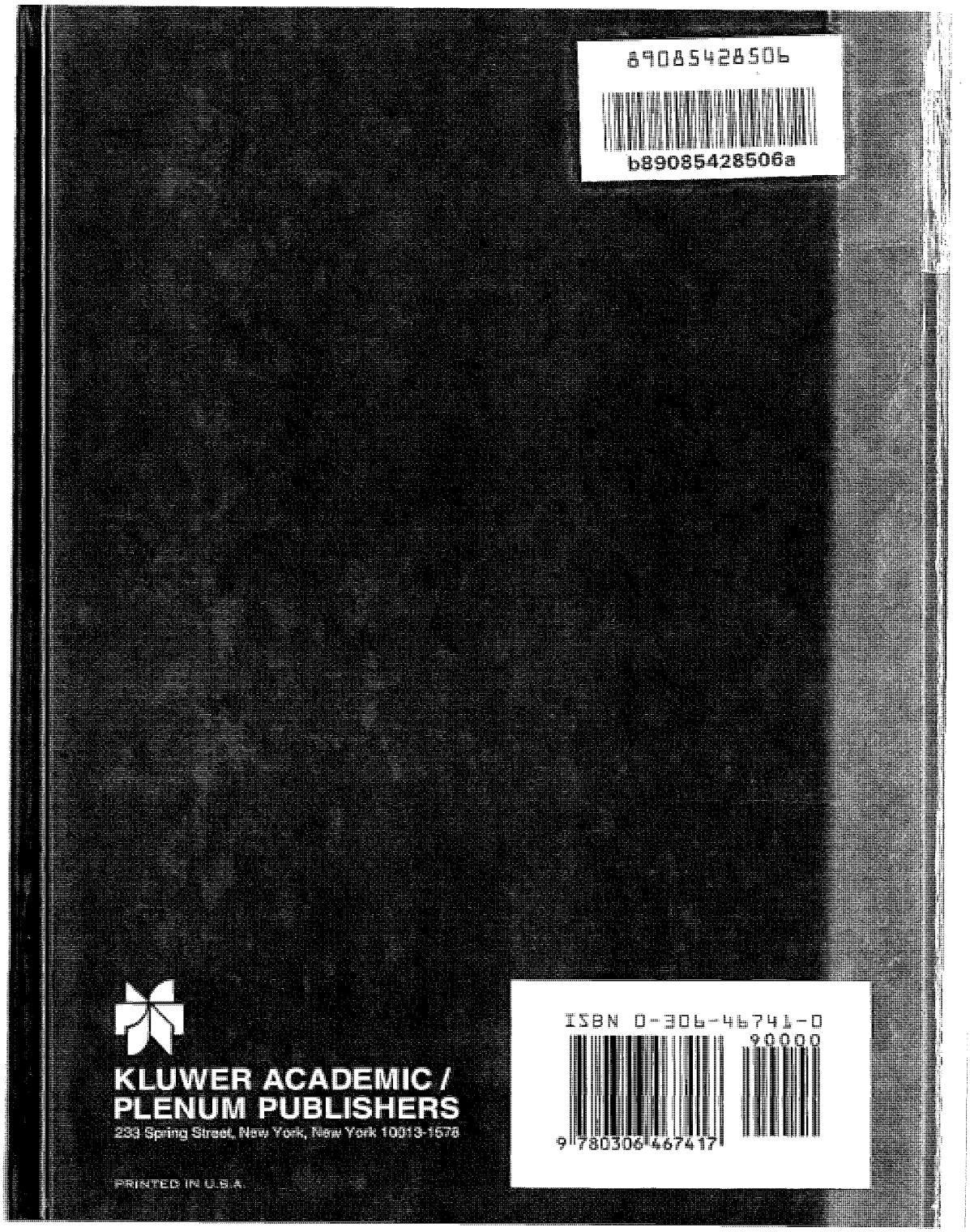
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RITUXAN™
Rituximab

DESCRIPTION

The RITUXAN (Rituximab) antibody is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen found on the surface of normal and malignant B lymphocytes. The antibody is an IgG_1 kappa immunoglobulin containing murine light- and heavy-chain variable region sequences and human constant region sequences. Rituximab is composed of two heavy chains of 451 amino acids and two light chains of 213 amino acids (based on cDNA analysis) and has an approximate molecular weight of 145 kD. Rituximab has a binding affinity for the CD20 antigen of approximately 8.0 nM.

The chimeric anti-CD20 antibody is produced by mammalian cell (Chinese Hamster ovary) suspension culture in a nutrient medium containing the antibiotic gentamicin. Gentamicin is not detectable in the final product. The anti-CD20 antibody is purified by affinity and ion exchange chromatography. The purification process includes specific viral inactivation and removal procedures.

RITUXAN is a sterile, clear, colorless, preservative-free liquid concentrate for intravenous (IV) administration. RITUXAN is supplied at a concentration of 10 mg/mL in either 100 mg (10 mL) or 500 mg (50 mL) single-use vials. The product is formulated for intravenous administration in 9.0 mg/mL sodium chloride, 7.35 mg/mL sodium citrate dihydrate, 0.7 mg/mL polysorbate 80, and Sterile Water for Injection. The pH is adjusted to 6.5.

CLINICAL PHARMACOLOGY

General

Rituximab binds specifically to the antigen CD20 (human B-lymphocyte-restricted differentiation antigen, Bp35), a hydrophobic transmembrane protein with a molecular weight of approximately 35 kD located on pre-B and mature B lymphocytes.[1,2] The antigen is also expressed on >90% of B-cell non-Hodgkin's lymphomas (NHL)[3] but is not found on hematopoietic stem cells, pro-B cells, normal plasma cells or other normal tissues.[4] CD20 regulates an early step(s) in the activation process for cell cycle initiation and differentiation,[4] and possibly functions as a calcium ion channel.[5] CD20 is not shed from the cell surface and does not internalize upon antibody binding.[6] Free CD20 antigen is not found in the circulation.[2]

Pre-clinical Pharmacology and Toxicology

Mechanism of Action: The Fab domain of Rituximab binds to the CD20 antigen on B-lymphocytes and the Fc domain recruits immune effector functions to mediate B-cell lysis *in vitro*. Possible mechanisms of cell lysis include complement-dependent cytotoxicity (CDC)[7] and antibody-dependent cellular cytotoxicity (ADCC). The antibody has been shown to induce apoptosis in the DHL-4 human B-cell lymphoma line.[8]

Normal Tissue Cross-reactivity: Rituximab binding was observed on lymphoid cells in the thymus, the white pulp of the spleen, and a majority of B-lymphocytes in peripheral blood and lymph nodes. Little or no binding was observed in non-lymphoid tissues examined.

Human Pharmacokinetics/Pharmacodynamics

In patients given single doses at 10, 50, 100, 250 or 500 mg/m² as an IV infusion, serum levels and the half-life of Rituximab were proportional to dose. In 9 patients given 375 mg/m² as an IV infusion for four doses, the mean serum half-life was 59.8 hours (range 11.1 to 104.6 hours) after the first infusion and 174 hours (range 26 to 442 hours) after the fourth infusion. The wide range of half-lives may reflect the variable tumor burden among patients and the changes in CD20 positive (normal and malignant) B-cell populations upon repeated administrations.

Rituximab at a dose of 375 mg/m² was administered as an IV infusion at weekly intervals for four doses to 166 patients. The peak and trough serum levels of Rituximab were inversely correlated with baseline values for the number of circulating CD20 positive B cells and measures of disease burden. Median steady-state serum levels were higher for responders compared to nonresponders; however, no difference was found in the rate of elimination as measured by serum half-life. Serum levels were higher in patients with International Working Formulation (IWF) subtypes B, C, and D as compared to those with subtype A. Rituximab was detectable in the serum of patients three to six months after completion of treatment.

The pharmacokinetic profile of Rituximab when administered as six infusions of 375 mg/m² in combination with six cycles of CHOP chemotherapy was similar to that seen with Rituximab alone.

Administration of RITUXAN resulted in a rapid and sustained depletion of circulating and tissue-based B cells. Lymph node biopsies performed 14 days after therapy showed a decrease in the percentage of B-cells in seven of eight patients who had received single doses of Rituximab ≥100 mg/m². [9] Among the 166 patients in the pivotal study, circulating B-cells (measured as CD19+ cells) were depleted within the first three doses with sustained depletion for up to 6 to 9 months post-treatment in 83% of patients. One of the responding patients (1%), failed to show significant depletion of CD19+ cells after the third infusion of Rituximab as compared to 19% of the nonresponding patients. B-cell recovery began at approximately six months following completion of treatment. Median B-cell levels returned to normal by twelve months following completion of treatment.

There were sustained and statistically significant reductions in both IgM and IgG serum levels observed from 5 through 11 months following Rituximab administration. However, only 14% of patients had reductions in IgG and/or IgM serum levels, resulting in values below the normal range.

CLINICAL STUDIES

A multicenter, open-label, single-arm study was conducted in 166 patients with relapsed or refractory low-grade or follicular B-cell NHL who received 375 mg/m² of RITUXAN given as an IV infusion weekly for four doses. Patients with tumor masses >10 cm or with >5,000 lymphocytes/μL in the peripheral blood were excluded from the study. The overall response rate (ORR) was 48% (80/166) with a 6% (10/166) complete response (CR) and a 42% (70/166) partial response (PR) rate. Disease-related signs and symptoms (including B-symptoms) were present in 23% (39/166) of patients at study entry and resolved in 64% (25/39) of those patients. The median time to onset of response was 50 days and the median duration of response is projected to be 10 to 12 months.

In a multivariate analysis, the ORR was higher in patients with IWF B, C, and D histologic subtypes as compared to IWF A subtype (58% vs. 12%), higher in patients whose largest lesion was <5 cm vs. >7 cm in greatest diameter (55% vs. 38%), and higher in patients with chemosensitive relapse as compared to chemoresistant (defined as duration of response <3 months) relapse (53% vs. 36%). ORR in patients previously treated with autologous bone marrow transplant was 78% (18/23). The following factors were not associated with a lower response rate: age ≥ 60 years, extranodal disease, prior anthracycline therapy, and bone marrow involvement.

In a second multicenter, multiple-dose study, 37 patients with relapsed or refractory B-cell NHL received 375 mg/m² of RITUXAN as an IV infusion once weekly for four doses.[10,11] The ORR was 46% with a median duration of response of 8.6 months (range 2.6 to 26.2+). Single doses of up to 500 mg/m² were well-tolerated.[9]

Twenty patients have received two courses and one patient has received three courses of RITUXAN as 4 weekly infusions of 375 mg/m² per infusion. The percentage of patients reporting adverse events upon retreatment was similar to that reported following the first course, although the incidence of specific adverse events differed (see ADVERSE EVENTS). All patients had obtained an objective clinical response (CR or PR) to the first course of RITUXAN™ (Rituximab); upon retreatment, 6 of 12 patients evaluable for response obtained a complete or partial remission.

Twenty-nine patients with relapsed or refractory, bulky (single lesion of >10 cm in diameter), low grade NHL received 375 mg/m² of RITUXAN as four weekly infusions. The overall incidence of adverse events and the incidence of Grade 3 and 4 adverse events was higher in patients with bulky disease than in patients with non-bulky disease (see ADVERSE EVENTS). Ten of 21 patients evaluable for response have obtained a complete or partial remission.

INDICATIONS AND USAGE

RITUXAN is indicated for the treatment of patients with relapsed or refractory low-grade or follicular, CD20 positive, B-cell non-Hodgkin's lymphoma.

CONTRAINDICATIONS

RITUXAN is contraindicated in patients with known Type I hypersensitivity or anaphylactic reactions to murine proteins or to any component of this product. (See WARNINGS.)

WARNINGS

RITUXAN is associated with hypersensitivity reactions which may respond to adjustments in the infusion rate. Hypotension, bronchospasm, and angioedema have occurred in association with RITUXAN infusion as part of an infusion-related symptom complex. RITUXAN infusion should be interrupted for severe reactions and can be resumed at a 50% reduction in rate (e.g., from 100 mg/hr to 50 mg/hr) when symptoms have completely resolved. Treatment of these symptoms with diphenhydramine and acetaminophen is recommended; additional treatment with bronchodilators or IV saline may be indicated. In most cases, patients who have experienced non-life-threatening reactions have been able to complete the full course of therapy. (See DOSAGE and ADMINISTRATION.) Medications for the treatment of hypersensitivity reactions, e.g., epinephrine, antihistamines and corticosteroids should be available for immediate use in the event of a reaction during administration.

Infusions should be discontinued in the event of serious or life-threatening cardiac arrhythmias. Patients who develop clinically significant arrhythmias should undergo cardiac monitoring during and after subsequent infusions of RITUXAN. Patients with preexisting cardiac conditions including arrhythmias and angina have had recurrences of these events during RITUXAN therapy and should be monitored throughout the infusion and immediate post-infusion period.

PRECAUTIONS

Laboratory Monitoring: Complete blood counts (CBC) and platelet counts should be obtained at regular intervals during RITUXAN therapy and more frequently in patients who develop cytopenias (see ADVERSE EVENTS).

Drug/Laboratory Interactions: There have been no formal drug interaction studies performed with RITUXAN.

HAMA/HACA Formation: Human anti-murine antibody (HAMA) was not detected in 67 patients evaluated. Less than 1.0% (3/355) of patients evaluated for human anti-chimeric antibody (HACA) were positive. Patients who develop HAMA/HACA titers may have allergic or hypersensitivity reactions when treated with this or other murine or chimeric monoclonal antibodies.

Immunization: The safety of immunization with any vaccine, particularly live viral vaccines, following RITUXAN therapy has not been studied. The ability to generate a primary or anamnestic humoral response to any vaccine has also not been studied.

Carcinogenesis, Mutagenesis, Impairment of Fertility: No long-term animal studies have been performed to establish the carcinogenic or mutagenic potential of RITUXAN, or to determine its effects on fertility in males or females. Individuals of childbearing potential should use effective contraceptive methods during treatment and for up to 12 months following RITUXAN therapy.

Pregnancy Category C: Animal reproduction studies have not been conducted with RITUXAN. It is not known whether RITUXAN can cause fetal harm when administered to a pregnant woman or whether it can affect reproductive capacity. Human IgG is known to pass the placental barrier, and thus may potentially cause fetal B-cell depletion; therefore, RITUXAN should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether RITUXAN is excreted in human milk. Because human IgG is excreted in human milk and the potential for absorption and immunosuppression in the infant is unknown, women should be advised to discontinue nursing until circulating drug levels are no longer detectable. (See CLINICAL PHARMACOLOGY.)

Pediatric Use: The safety and effectiveness of RITUXAN in children have not been established.

ADVERSE REACTIONS

Safety data are based on 315 patients treated in five single-agent studies of RITUXAN. This includes patients with bulky disease (lesions >10 cm), those who have received more than one course of RITUXAN, and patients receiving 375 mg/m² for eight doses.

Infusion-Related Events: An infusion-related symptom complex consisting of fever and chills/rigors occurred in the majority of patients during the first RITUXAN infusion. Other frequent infusion-related symptoms included nausea, urticaria, fatigue, headache, pruritus, bronchospasm, dyspnea, sensation of tongue or throat swelling (angioedema), rhinitis, vomiting, hypotension, flushing, and pain at disease sites. These reactions generally occurred within 30 minutes to 2 hours of beginning the first infusion, and resolved with slowing or interruption of the RITUXAN infusion and with supportive care (IV saline, diphenhydramine, and acetaminophen). The incidence of infusion-related events decreased from 80% (7% Grade 3/4) during the first infusion to approximately 40% (5% to 10% Grade 3/4) with subsequent infusions. Mild to moderate hypotension requiring interruption of RITUXAN infusion with or without the administration of IV saline occurred in 32 (10%) patients. Isolated occurrences of severe reactions requiring epinephrine have been reported in patients receiving RITUXAN for other indications. Angioedema was reported in 41 (13%) patients and was serious in one patient. Bronchospasm occurred in 25 (8%) patients; one-quarter of these patients were treated with bronchodilators. A single report of bronchiolitis obliterans was noted.

Immunologic Events: RITUXAN induced B-cell depletion in 70 to 80% of patients and was associated with decreased serum immunoglobulins in a minority of patients. The incidence of infection does not appear to be increased. During the treatment period, 50 patients in the pivotal trial developed 68 infectious events; 6 (9%) were Grade 3 in severity and none were Grade 4 events. Of the

6 serious infectious events, none were associated with neutropenia. The serious bacterial events included sepsis due to Listeria (n=1), Staphylococcal bacteremia (n=1) and polymicrobial sepsis (n=1). In the post-treatment period (30 days to 11 months following the last dose), bacterial infections included sepsis (n=1); significant viral infections included herpes simplex infections (n=2) and herpes zoster (n=3).

Retreatment Events: Twenty-one patients have received more than one course of RITUXAN™ (Rituximab). The percentage of patients reporting any adverse event upon retreatment was similar to the percentage of patients reporting adverse events upon initial exposure. The following adverse events were reported more frequently in retreated subjects: asthenia, throat irritation, flushing, tachycardia, anorexia, leukopenia, thrombocytopenia, anemia, peripheral edema, dizziness, depression, respiratory symptoms, night sweats, and pruritus.

Hematologic Events: During the treatment period (up to 30 days following last dose) severe thrombocytopenia occurred in 1.3% of patients, severe neutropenia occurred in 1.9% of patients, and severe anemia occurred in 1.0% of patients. A single occurrence of transient aplastic anemia (pure red cell aplasia) and two occurrences of hemolytic anemia following RITUXAN therapy were reported.

Cardiac Events: Four patients developed arrhythmias during RITUXAN infusion. One of the four discontinued treatment because of ventricular tachycardia and supraventricular tachycardias. The other three patients experienced trigeminy (1) and irregular pulse (2) and did not require discontinuation of therapy. Angina was reported during infusion and myocardial infarction occurred 4 days post-infusion in one subject with a prior history of myocardial infarction.

Table 1.

Adverse Events ≥5% of Patients (N=315)

	Incidence All Grades	
	N	%
Any Adverse Event	**275**	**87**
Body As A Whole		
Fever	154	49
Chills	102	32
Asthenia	49	16
Headache	43	14
Throat Irritation	19	6
Abdominal Pain	18	6
Cardiovascular System		
Hypotension	32	10
Digestive System		
Nausea	55	18
Vomiting	23	7
Hemic and Lymphatic System		
Leukopenia	33	11
Thrombocytopenia	25	8
Neutropenia	21	7
Metabolic and Nutritional System		
Angioedema	41	13
Musculo-Skeletal System		
Myalgia	21	7
Nervous System		
Dizziness	23	7
Respiratory System		
Rhinitis	25	8
Bronchospasm	24	8
Skin and Appendages		
Pruritus	32	10
Rash	31	10
Urticaria	24	8

Severe and life-threatening (Grade 3 and 4) events were reported in 10% (32/315) of patients. The following Grade 3 and 4 adverse events were reported: neutropenia (1.9%), chills (1.6%), leukopenia and thrombocytopenia (1.3% for each), hypotension, anemia, bronchospasm, and urticaria (1.0% for each), headache, abdominal pain, arrhythmia (0.6% for each), and asthenia, hypertension, nausea, vomiting, coagulation disorder, angioedema, arthralgia, pain, rhinitis, increased cough, dyspnea, bronchiolitis obliterans, hypoxia, asthma, pruritus, and rash (one patient each, 0.3%).

The following adverse events occurred in ≥1.0% but <5.0% of patients, in order of decreasing incidence: flushing, arthralgia, diarrhea, anemia, cough increase, hypertension, lacrimation disorder, pain, hyperglycemia, back pain, peripheral edema, paresthesia, dyspepsia, chest pain, anorexia, anxiety, malaise, tachycardia, agitation, insomnia, sinusitis, conjunctivitis, abdominal enlargement, postural hypotension, LDH increase, hypocalcemia, hypesthesia, respiratory disorder, tumor pain, pain at injection site, bradycardia, hypertonia, nervousness, bronchitis, and taste perversion.

The proportion of patients reporting any adverse event was similar in patients with bulky disease and those with lesions <10 cm in diameter. However, the incidence of dizziness, neutropenia, thrombocytopenia, myalgia, anemia and chest pain was higher in patients with lesions >10 cm. The incidence of any Grade 3 and 4 event was higher (31% vs. 13%) and the incidence of Grade 3 or 4 neutropenia, anemia, hypotension, and dyspnea was also higher in patients with bulky disease compared with patients with lesions <10 cm.

OVERDOSAGE

There has been no experience with overdosage in human clinical trials. Single doses higher than 500 mg/m² have not been tested.

DOSAGE AND ADMINISTRATION

Usual Dose:

The recommended dosage of RITUXAN is 375 mg/m² given as an IV infusion once weekly for four doses (days 1, 8, 15, and 22). RITUXAN may be administered in an outpatient setting. **DO NOT ADMINISTER AS AN INTRAVENOUS PUSH OR BOLUS. (See Administration.)**

Instructions for Administration

Preparation for Administration: Use appropriate aseptic technique. Withdraw the necessary amount of RITUXAN and dilute to a final concentration of 1 to 4 mg/mL into an infusion bag containing either 0.9% Sodium Chloride USP or 5% Dextrose in Water USP. Gently invert the bag to mix the solution. Discard any unused portion left in the vial. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration.

RITUXAN solutions for infusion are stable at 2° to 8°C (36° to 46°F) for 24 hours and at room temperature for an additional 12 hours. No incompatibilities between RITUXAN and polyvinylchloride or polyethylene bags have been observed.

Administration: DO NOT ADMINISTER AS AN INTRAVENOUS PUSH OR BOLUS. Hypersensitivity reactions may occur (see WARNINGS). Premedication, consisting of acetaminophen and diphenhydramine, should be considered before each infusion of RITUXAN™ (Rituximab). Premedication may attenuate infusion-related events. Since transient hypotension may occur during RITUXAN infusion, consideration should be given to withholding antihypertensive medications 12 hours prior to RITUXAN infusion.

First Infusion: The RITUXAN solution for infusion should be administered intravenously at an initial rate of 50 mg/hr. RITUXAN should not be mixed or diluted with other drugs. If hypersensitivity or infusion-related events do not occur, escalate the infusion rate in 50 mg/hr increments every 30 minutes, to a maximum of 400 mg/hr. If hypersensitivity or an infusion-related event develops, the infusion should be temporarily slowed or interrupted (see WARNINGS). The infusion can continue at one-half the previous rate upon improvement of patient symptoms.

Subsequent Infusions: Subsequent RITUXAN infusions can be administered at an initial rate of 100 mg/hr, and increased by 100 mg/hr increments at 30-minute intervals, to a maximum of 400 mg/hr as tolerated.

Stability and Storage: RITUXAN vials are stable at 2° to 8°C (36° to 46°F). Do not use beyond expiration date stamped on carton. RITUXAN vials should be protected from direct sunlight.

HOW SUPPLIED

RITUXAN is supplied as 100 mg and 500 mg of sterile, preservative-free, single-use vials.
Single unit 100 mg carton: Contains one 10 mL vial of RITUXAN (10 mg/mL).
NDC 50242-051-21
Single unit 500 mg carton: Contains one 50 mL vial of RITUXAN (10 mg/mL).
NDC 50242-053-06

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IDEC Pharmaceuticals Corporation
11011 Torreyana Road
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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

STUDY PROTOCOL

BB-IND 11075

***TRIUMPH*: A Hemoglobin Stabilization and Transfusion Reduction Efficacy and Safety Clinical Investigation, Randomized, Multi-Center, Double-Blind, Placebo-Controlled, Using Eculizumab in Paroxysmal Nocturnal Hemoglobinuria Patients**

PROTOCOL NUMBER: C04-001

Sponsor:	ALEXION PHARMACEUTICALS, INC. 352 Knotter Drive Cheshire, CT 06410 USA
Sponsor Contact:	Michael Bombara Clinical Project Manager Alexion Pharmaceuticals, Inc. 352 Knotter Drive Cheshire, CT 06410 Telephone: (203) 271-8213 Facsimile: (203) 271-8196
Medical Monitor:	Paul Shapiro, MD Associate Medical Director / Medical Monitor PPD Development 3151 South 17th Street Wilmington, NC 28412-6461 Telephone: (910) 772-6702
Version:	2.1
Date of Protocol:	02-June-2004
Amended:	not applicable
Proprietary Notice:	The concepts and information contained in this document or generated during the study are considered proprietary and may not be disclosed in whole or in part without the expressed, written consent of Alexion Pharmaceuticals, Inc.
Ethics Statement:	The study will be completed according to the guidelines of Good Clinical Practice. Compliance with this standard provides public assurance that the rights, safety, and well-being of trial patients are protected, consistent with the principles that have their origin in the Declaration of Helsinki.

Samsung Bioepis Exhibit No. 1002
Page 1359

[redacted] [redacted]

Confidential

SPONSOR SIGNATURE PAGE

PROTOCOL TITLE: *TRIUMPH:* A Hemoglobin Stabilization and Transfusion Reduction Efficacy and Safety Clinical Investigation, Randomized, Multi-Center, Double-Blind, Placebo-Controlled, Using Eculizumab in Paroxysmal Nocturnal Hemoglobinuria Patients

PROTOCOL NUMBER: C04-001

Christopher Mojcik, MD, PhD
Senior Vice President of Clinical Development
Alexion Pharmaceuticals, Inc.

3-June-04
Date

Michael Bombara
Clinical Project Manager
Clinical Operations
Alexion Pharmaceuticals, Inc.

3-June-04
Date

Samsung Bioepis Exhibit No. 1002
Page 1360

TABLE OF CONTENTS

PROTOCOL SUMMARY **6**

ABBREVIATIONS **9**

1. INTRODUCTION **11**

2. STUDY OBJECTIVES **13**

2.1. PRIMARY OBJECTIVE 13
2.2. SECONDARY OBJECTIVES 13
2.3 EXPLORATORY OBJECTIVES 13

3. STUDY PLAN **13**

3.1. OVERALL DESIGN 13

4. STUDY TREATMENT **14**

4.1. INVESTIGATIONAL PRODUCT DOSING 14
4.2. INVESTIGATIONAL PRODUCT ADMINISTRATION 15
4.3. PROHIBITED CONCOMITANT MEDICATIONS AND OTHER RESTRICTIONS 15
4.3.1. Transfusion Guidelines *16*
4.3.2. Warnings and Precautions *17*
4.5. INVESTIGATIONAL PRODUCT PACKAGING AND STORAGE 19
4.6. TEST ARTICLE ACCOUNTABILITY 19

5. PATIENT ENROLLMENT **20**

5.1. INCLUSION CRITERIA 20
5.1.1. Screening Period *20*
5.1.2. Observation Period *21*
5.2. EXCLUSION CRITERIA 21
5.3. RANDOMIZATION PROCEDURES 22
5.4. BLINDING PROCEDURES 22
5.5. BREAKING THE BLIND 22
5.6. PATIENT WITHDRAWAL 23
5.6.1. Discontinuation of Treatment *23*
5.6.2. Handling of Withdrawals *24*
5.6.3. Replacements *24*
5.6.4. Sponsor's Termination of Study *24*

6. STUDY VISITS **24**

6.1. ENROLLMENT PHASE 25
6.1.1. Visit 1 - Screening Period (2 weeks before Observation Period) *25*
6.1.2. Visit 2 – Observation Period (-3 to 0 months) *26*
6.2. TREATMENT PHASE - INDUCTION PERIOD 27
6.2.1. Visit 3 - Baseline *27*
6.2.2. Visit 4 and 6 – Induction Period (± 2 days) *28*
6.2.3. Visit 5 – Induction Period (± 2 days) *28*
6.2.4. Visit 7 – Induction Period (± 2 days) *29*
6.3. TREATMENT PHASE – MAINTENANCE PERIOD 30
6.3.1. Visit 8 (± 2 days) *30*
6.3.2. Visit 9 (± 2 days) *30*
6.3.3. Visits 10, 12, 14, and 16 (± 2 days) *31*

6.3.4. Visit 11 (± 2 days) *32*
6.3.5. Visit 13 and 17 (± 2 days) *32*
6.3.6. Visit 15 (± 2 days) *33*
6.4. UNSCHEDULED VISITS 34
6.4.1. General Visit *34*
6.4.2. Transfusion Visit *34*
6.5. VISIT 18 (±2 DAYS) OR EARLY TERMINATION VISIT 35
6.6. SAFETY FOLLOW-UP VISITS 36

7. STUDY ASSESSMENTS 37

7.1. DEMOGRAPHIC INFORMATION, MEDICAL HISTORY, AND PHYSICAL EXAMINATION 37
7.2. VITAL SIGNS MEASUREMENTS 37
7.3. ELECTROCARDIOGRAM 37
7.4. CLINICAL LABORATORY TESTS 37
7.4.1. Pregnancy test *38*
7.4.2. Laboratory measurements *38*
7.4.3. Specialty Tests *38*
7.5. PHARMACOKINETIC AND PHARMACODYNAMIC TESTING 39
7.6. QUALITY OF LIFE INSTRUMENTS 40
7.6.1. European Organisation for Research and Treatment of Cancer (EORTC QLQ C-30) *40*
7.6.2. Functional Assessment of Chronic Illness Therapy – Fatigue Subscale (FACIT-F SCALE) *40*

8. ADVERSE EVENTS 41

8.1. DEFINITIONS 41
8.2. SERIOUS ADVERSE EVENTS 41
8.3. ADVERSE EVENT REPORTING 41
8.3.1. Eliciting Adverse Event Information *42*
8.3.2. Study Specific Considerations *42*
8.3.3. Exposure in Utero *42*
8.4. ASSESSMENT OF CAUSALITY 43
8.5. ASSESSMENT OF SEVERITY 43
8.6. SERIOUS ADVERSE EVENT REPORTING 43

9. STATISTICAL METHODS 44

9.1. SAMPLE SIZE 44
9.2. POPULATIONS TO BE ANALYZED 45
9.2.1. Intent-to-treat Population *45*
9.2.2. Per-Protocol Population *45*
9.2.3. Safety Population *45*
9.3. STATISTICAL METHODOLOGY 45
9.3.1. Co-primary Endpoints *45*
9.3.2. Secondary Endpoints *46*
9.3.3. Exploratory Endpoints *47*
9.4. SAFETY ANALYSIS 47
9.5. PHARMACOKINETIC AND PHARMACODYNAMIC ANALYSES 48
9.6. DATA SAFETY MONITORING BOARD 49

10. DATA HANDLING AND QUALITY ASSURANCE 49

10.1. CASE REPORT FORMS 49
10.2. MONITORING OF THE STUDY 49
10.3. INSPECTION OF RECORDS 49
10.4. STUDY RECORD RETENTION 50

11. ADMINISTRATIVE CONSIDERATIONS 50

Samsung Bioepis Exhibit No. 1002
Page 1362

11.1. CONFIDENTIALITY 50
11.2. INSTITUTIONAL REVIEW BOARD/INDEPENDENT ETHICS COMMITTEE APPROVAL 50
11.3. MODIFICATION OF THE PROTOCOL 51
11.4. INFORMED CONSENT 51
11.5. PROTOCOL VIOLATIONS AND DEVIATIONS 51
11.6. STUDY REPORTING REQUIREMENTS 52
11.7. FINANCIAL DISCLOSURE AND OBLIGATIONS 52
11.8. INVESTIGATOR DOCUMENTATION 52
11.9. STUDY CONDUCT 53
11.10. PUBLICATIONS 53

12. INVESTIGATOR'S STATEMENT OF AGREEMENT 54

13. REFERENCES 55

14. APPENDICES 57

APPENDIX A: SUMMARY OF LABORATORY TESTS 58

APPENDIX B: EORTC QUALITY OF LIFE QUESTIONNAIRE (EORTC QLQ-C30) 59

APPENDIX C: FACIT-FATIGUE SUBSCALE VERSION 4 61

APPENDIX D: LIST OF MAJOR ADVERSE VASCULAR EVENTS (MAVE) 62

APPENDIX E: SCHEDULE OF EVENTS – LARGE VERSION 63

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4.5. Investigational Product Packaging and Storage

Investigational product will be released to the site upon receipt of all required essential documents based upon federal, state, and local regulations. Each kit will have a 2-panel label describing the contents and a place for the pharmacist to record the patient number and initials. The tear-off portion of the label will be affixed to the label sheet provided in the pharmacy binder.

When the investigational product arrives, it should be promptly removed from the shipping cooler and stored in refrigerated conditions at 2°C to 8°C. The pharmacy should immediately notify the distributor if vials are damaged and cannot be used or an error has been made in the randomization process (refer to pharmacy binder).

Eculizumab must be stored in a secure, limited-access storage area.

4.6. Test Article Accountability

When a drug shipment is received at the site, the pharmacist at each site shall verify the contents, sign the packing invoice provided with the shipment, and maintain the original

copy for review by the study monitor. The duplicate copy should be kept in the pharmacy binder.

Each shipment of investigational drug supplies will contain an accountability log to assist the pharmacist in maintaining current and accurate inventory records covering receipt, dispensing, and disposition of the investigational product. During the study, the following information must be noted in the accountability log: the identification number(s), initials of patient(s) who is dispensed investigational product, the date(s) that the investigational product is prepared and dispensed, and the initials of the pharmacist or designee who prepared the investigational product. Empty vials and vials with residual materials should be kept for inspection by the study monitor.

The study monitor will examine the inventory records during study conduct. Additionally, the inventory records must be readily available and may be subject to regulatory authorities or an independent auditor's inspection at any time. At the completion of the study, to satisfy regulatory requirements regarding drug accountability, all remaining investigational product inventory will be reconciled and retained or destroyed according to applicable local, state, and federal regulations.

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[redacted]

11. ADMINISTRATIVE CONSIDERATIONS

The following administrative items are meant to guide the Principal Investigator or Subinvestigator in the conduct of the trial but may be subject to change based on industry and government Standard Operating Procedures or Working Practice Documents or Guidelines. Changes will be reported to the IRB/IEC, but will not result in protocol amendments.

11.1. Confidentiality

All laboratory specimens, evaluation forms, reports, and other records will be identified in a manner designed to maintain patient confidentiality. All records will be kept in a secure storage area with limited access. Clinical information will not be released without the written permission of the patient or the patient's guardian, except as necessary for monitoring by the Sponsor, its designee, the FDA/regulatory authorities, or the IRB/IEC.

The Investigator and all employees and coworkers involved with this study shall not disclose or use for any purpose, other than performance of the study, any data, records or other unpublished, confidential information disclosed to those individuals for the purpose of the study. Prior written agreement from the Sponsor or its designee must be obtained for the disclosure of any said confidential information to other parties.

[redacted]

12. INVESTIGATOR'S STATEMENT OF AGREEMENT

I agree to conduct the study as outlined in the protocol entitled: *"TRIUMPH*: A Hemoglobin Stabilization and Transfusion Reduction Efficacy and Safety Clinical Investigation, Randomized, Multi-Center, Double-Blind, Placebo-Controlled, Using Eculizumab in Paroxysmal Nocturnal Hemoglobinuria Patients" and in accordance with the guidelines and all applicable government regulations. I have read and understand all sections of the protocol including Section 11, Administrative Considerations.

_____

Principal Investigator
(print name)

_____ _____

Principal Investigator (sign name) Date

CLINICAL STUDY PROTOCOL
CONFIDENTIAL

IND NUMBER:	BB-IND 8853
STUDY NUMBER:	C02-002
VERSION:	VERSION 1.0
USAN:	ANTI-C5 ANTIBODY
DOSAGE FORM:	PARENTERAL
STUDY TITLE:	A RANDOMIZED, THIRD-PARTY BLIND, PLACEBO-CONTROLLED, MULTI-CENTER, PHASE II STUDY ASSESSING THE SAFETY AND EFFICACY OF ECULIZUMAB [redacted] FOR THE TREATMENT OF SUBJECTS WITH DERMATOMYOSITIS
MEDICAL MONITOR:	ELLEN McCROSKERY, M.D.
AUTHOR:	JING JING WANG, M.S.
ADDRESS:	ALEXION PHARMACEUTICALS, INC. 352 KNOTTER DRIVE CHESHIRE, CT 06410
TELEPHONE NUMBER:	GENERAL INQUIRES-J.Wang 203-271-8348 EMERGENCIES- E.McCroskery, MD OFFICE: 203-271-8298 CELL: 203-858-2230
PROJECT SPONSOR:	ALEXION PHARMACEUTICALS, INC.
DATE:	SEPTEMBER 30, 2002

I have read the protocol and agree to conduct the study accordingly.

_____ _____
Principal Investigator Date

_____
Principal Investigator (Print)

Eileen M McCroskery M.D October 4, 2002
Alexion Clinical Director Date

Samsung Bioepis Exhibit No. 1002
Page 1368

TABLE OF CONTENTS

FIGURE 1 STUDY FLOW CHART 4
GLOSSARY OF ABBREVIATIONS 5
STUDY SYNOPSIS 7
1. INTRODUCTION 9
2. STUDY OBJECTIVES 16
3. OVERALL DESIGN AND PLAN OF STUDY 16
4. ETHICS AND GOOD CLINICAL PRACTICE 17
5. STUDY POPULATION 17
5.1 Primary Diagnosis 17
5.2 Enrollment Criteria 18
5.3 Exclusion Criteria 19
5.4. Number of Subjects 20
5.5. Method of Subject Assignment (Stratification and Randomization) and Blinding 20
6. DRUGS, DOSAGE AND DURATION OF TREATMENT 21
6.1 Test Material and Control Agents 21
6.2 Supply, Packaging, Labeling and Storage 22
6.3 Dosage and Administration 22
6.4 Duration of Treatment 23
6.5 Early Termination of Subjects from the Trial 23
6.6 Drug Accountability 24
6.7 Concomitant Medication 24
7. WARNINGS/PRECAUTIONS 25
8. PRETREATMENT PROCEDURES 26
8.1 Informed Consent 26
8.2 Observations and Measurements 27
9. DURING TREATMENT PROCEDURES 29
9.1 Observations and Measurements 29
10. POST-TREATMENT PROCEDURES 34
10.1 Observations and Measurements 34
11. ADVERSE EVENTS 36
11.1 Safety Definitions 36
11.2 Reporting Adverse Events 39
11.3 Exposure in Utero 40
11.4 Adverse Event Reporting Contacts 40
12. CRITERIA FOR THE EVALUATION OF STUDY RESULTS 41
12.1 Criteria for the Evaluation of Efficacy 41
12.2. Criteria for the Evaluation of Safety 41
12.3. Evaluation in Case of Discontinued Treatment 41
12.4. Removal of Subjects from Study or Analysis 41
12.5. Evaluation of Data for the Safety and Protection of Subjects 42
12.6. Stopping Rules 42
13. DATA PROCESSING AND ANALYSIS 42
13.1 Sample Size Estimation 42

Samsung Bioepis Exhibit No. 1002
Page 1369

13.2 Statistical and Analytical Plan 42
13.3 Interim Analysis 44
13.4 Use of Data and Publication 44
14. ESTIMATED DURATION OF THE STUDY 44
15. EARLY TERMINATION OF THE STUDY 45
16. PHARMACOKINETICS AND PHARMACODYNAMICS 45
17. MEDICAL MONITOR 46
18. STUDY TIMETABLE 46
19. REFERENCES 47
APPENDIX A 49
CRITERIA FOR THE DIAGNOSIS OF DERMATOMYOSITIS 49
APPENDIX B 51
DERMATOMYOSITIS SEVERITY AND ACTIVITY INDEX 51
APPENDIX C 52
SUMMARY OF LABORATORY TESTS 52
APPENDIX D 53
CUTANEOUS DERMATOMYOSITIS DISEASE AREA AND SEVERITY INDEX (CDASI) 53
APPENDIX E 54
FUNCTIONAL ASSESSMENTS 54
APPENDIX F 55
PROCEDURE FOR COLLECTING PK AND PD SAMPLES 55
APPENDIX G 56
SKIN BIOPSY PROCEDURES 56
APPENDIX H 58
SKIN PHOTOGRAPHIC PROCEDURES 58

Samsung Bioepis Exhibit No. 1002
Page 1370

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[redacted]

When study medication arrives, it should be promptly removed from the shipping cooler and stored in the refrigerator at $2^{\circ} - 8^{\circ}C$. The site should notify the sponsor immediately if vials are damaged and cannot be used or an error has been made in the randomization process (for procedures, please refer to Pharmacy Binder)

As with all investigational drugs, eculizumab must be stored in a secure, limited access storage area.

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Samsung Bioepis Exhibit No. 1002
Page 1371

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6.6 Drug Accountability

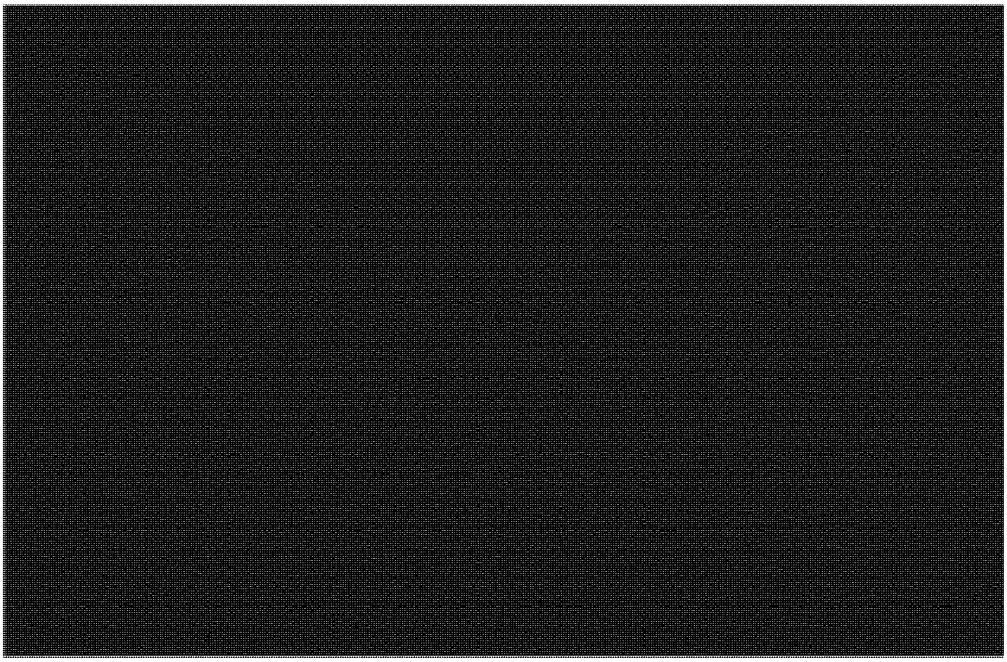
When a drug shipment is received at the site, the pharmacist at each site should verify the contents and sign the Drug Order Form provided with the shipment and maintain the original copy for collection by the Study Monitor/Research Associate. The duplicate copy should be kept in the Pharmacy Binder.

Each shipment of investigational drug supplies will contain a Drug Inventory / Disposition Form to assist the pharmacist in maintaining current and accurate inventory records covering receipt, dispensing, and disposition of the study drug. During the study, the following information must be noted in the Drug Inventory / Disposition Form: the identification number(s) and initials of subject(s) to whom drug is dispensed, the date(s) that the study drug is prepared and dispensed, and the initials of the pharmacist or his /her designee who prepared the study drug. Empty vials and vials with residual materials should be kept for inspection by the Study Monitor/Research Associate.

Because this study is third-party blinded, the inventory records may not be examined by the Study Monitor until the clinical database has been cleaned and locked. However, the inventory records must be readily available and may be subject to FDA, the local regulatory agency, or independent auditor's inspection at any time.

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Samsung Bioepis Exhibit No. 1002
Page 1372



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Samsung Bioepis Exhibit No. 1002
Page 1373

FDA Approves Alexion's Soliris(TM) for All Patients With PNH

- First Therapy Approved for This Rare and Life-Threatening Blood Disease -

CHESHIRE, Conn., March 16, 2007 /PRNewswire-FirstCall via COMTEX News Network/ -- Alexion Pharmaceuticals, Inc., (Nasdaq: ALXN) announced today that it has received marketing approval from the U.S. Food and Drug Administration (FDA) for Soliris(TM) (eculizumab). Soliris is the first therapy approved for paroxysmal nocturnal hemoglobinuria (PNH), a rare, disabling and life-threatening blood disorder defined by chronic red blood cell destruction, or hemolysis. Soliris is indicated for the treatment of patients with PNH to reduce hemolysis.

Hemolysis can cause one or more of the following symptoms in patients with PNH: severe anemia, disabling fatigue, recurrent pain, shortness of breath, pulmonary hypertension, intermittent episodes of dark colored urine (hemoglobinuria), kidney disease, impaired quality of life and blood clots (thromboses).(1,2) PNH often strikes people in the prime of their lives, with an average age of onset in the early 30's.(3) The estimated median survival for PNH patients is between 10 and 15 years from the time of diagnosis.(3,4)

Patients with PNH are missing a specific protein that normally protects red blood cells from destruction by a component of the immune system called terminal complement. Soliris, the first complement inhibitor approved in the United States for the treatment of any disease, prevents hemolysis by selectively blocking terminal complement.

"Soliris brings real hope to people who live daily with the devastating effects of PNH. With the approval of Soliris, we now have a therapy that dramatically improves the lives of patients suffering from this disease. Importantly, all patients with this life-threatening disease will be eligible for treatment," said Leonard Bell, MD, chief executive officer of Alexion Pharmaceuticals.

"Soliris directly targets the underlying disease process responsible for debilitating symptoms that may contribute to shortened life spans of PNH patients," said Wendell F. Rosse, MD, Florence McAlister Professor of Medicine Emeritus, Duke University. "Having cared for more than 300 patients with PNH over my career, I believe this is the most important advance that has been made in the treatment of this disease. Treatment with Soliris markedly decreases the hemolysis responsible for anemia, fatigue, poor patient functioning and blood clots in PNH patients."

Clinical Data

Soliris has proven to be a safe and effective therapy for PNH in three multi-national clinical studies: TRIUMPH, a placebo-controlled 26 week Phase 3 study involving 87 PNH patients,(5) SHEPHERD, an open-label 52 week Phase 3 trial involving 97 PNH patients,(6) and E05-001, a long term extension study.(7)

These studies showed that Soliris reduced hemolysis in every treated patient. Hemolysis was dramatically reduced from a baseline LDH of 2,032 U/L to 239 U/L at week 26 ($p<0.001$). The reductions in hemolysis occurred within one week of initiating treatment and were sustained for periods of up to 54 months with continued dosing of Soliris. The reduction in hemolysis expands the number of circulating PNH cells and, thereby, increases the hemoglobin level. Hemoglobin stabilization and the number of transfused packed red blood cell units, the pivotal study's co-primary endpoints, were both achieved; half of the Soliris-treated patients achieved hemoglobin stabilization compared with none of the patients in the placebo group, the median number of transfusions was reduced from 10 units/patient to 0 units/patient, respectively ($p < 0.001$ in both cases). Soliris patients reported less fatigue and improved health-related quality of life. There were fewer thrombotic events with Soliris treatment than during the same period of time prior to treatment.

"The Aplastic Anemia & MDS International Foundation (AA&MDSIF) is extremely pleased that PNH patients now have a treatment specifically for their disease. This is a tremendous step forward for all who suffer from PNH -- and for anyone with bone marrow failure," said Sherrie VanVliet, vice-chairperson, AA&MDSIF, and the mother of a child with aplastic anemia. "We also appreciate that Alexion, in cooperation with the National Organization of Rare Diseases, has established a program to ensure that all patients who need eculizumab have access to it."

"Patients with rare diseases face huge challenges -- first getting a proper diagnosis, then finding effective treatments and access to them," said Abbey Meyers, president, National Organization for Rare Disorders (NORD). "Now that Soliris has been approved, people with PNH will have an effective treatment, and through NORD's PNH Foundation, the uninsured and underinsured will receive help to assure they will have access. This is a sign of hope for others with orphan illnesses, that their needs will one day be met," she added.

Introducing Soliris OneSource(TM)

Alexion also today introduced Soliris OneSource(TM), a treatment support service for all PNH patients and their healthcare providers. Each patient enrolled in the program receives support from an Alexion Case Manager at OneSource. Alexion Case Managers are Registered Nurses and provide education about PNH and Soliris and facilitate solutions to help patients obtain Soliris. Alexion's goal is that all PNH patients who can benefit from Soliris will have access to it. Patients and their health care providers can learn more about OneSource by calling 1-888-SOLIRIS (1-888-765-4747) or visiting www.soliris.net.

Important Safety Information

Soliris is generally well tolerated. The most frequent adverse events observed in clinical studies were headache, nasopharyngitis (a runny nose), back pain and nausea.(8) Treatment with Soliris should not alter anticoagulant management because the effect of withdrawal of anticoagulant therapy during Soliris treatment has not been established.

The product label for Soliris also includes a boxed warning: "Soliris increases the risk of meningococcal infections. Vaccinate patients with a meningococcal vaccine at least 2 weeks prior to receiving the first dose of Soliris; revaccinate according to current medical guidelines for vaccine use. Monitor patients for early signs of meningococcal infections, evaluate immediately if infection is suspected, and treat with antibiotics if necessary. "Two out of 196 vaccinated PNH patients treated with Soliris experienced a serious meningococcal infection.

Prior to beginning Soliris therapy, all patients and their prescribing physicians will be enrolled in the Soliris Safety Registry which is part of a special risk management program that involves initial and continuing education and long-term monitoring for detection of new safety findings.

Please see full prescribing information at www.soliris.net.

Conference Call Information

Alexion will host a conference call/webcast to discuss FDA approval of Soliris. The call is scheduled for Monday, March 19th at 9:00 a.m., Eastern Time. To participate in this call, dial 913-981-4900, confirmation code 4700435, shortly before 9:00 a.m., Eastern Time. A replay of the call will be available for a limited period following the call, beginning at 12:00 p.m., Eastern Time. The replay number is 719-457-0820, confirmation code 4700435. The audio webcast can be accessed at: www.alexionpharm.com.

About PNH

PNH is an acquired genetic blood disorder defined by hemolysis, in which patients' red blood cells are destroyed by complement, a component of the body's immune system. PNH is a rare disease that affects an estimated 8,000 to 10,000 people in North America and Europe. Ten percent of all patients first develop symptoms at 21 years of age or younger.(2) PNH develops without warning and can occur in men and women of all races, backgrounds and ages. PNH often goes unrecognized, with delays in diagnosis often ranging from one to more than 10 years. PNH has been identified more commonly among patients with diseases of the bone marrow, including aplastic anemia (AA) and myelodysplastic syndrome (MDS). In patients with thrombosis of unknown origin, PNH may be an underlying cause.

Prior to approval of Soliris, there were no therapies specifically available for the treatment of PNH. PNH treatment was limited to symptom management through periodic blood transfusions, non-specific immunosuppressive therapy and, infrequently, bone marrow transplantations -- a high-risk and painful procedure used as a last resort.(2)

About Alexion

Alexion Pharmaceuticals is a biotechnology company working to develop and deliver life-changing drug therapies for patients with serious and life- threatening medical conditions. Alexion's lead product, Soliris(TM) (eculizumab), is indicated for the treatment of paroxysmal nocturnal hemoglobinuria (PNH). Alexion is engaged in the discovery and development of therapeutic products aimed at treating patients with severe disease states, including hematologic diseases, cancer and autoimmune disorders. In September 2006, Alexion applied for marketing authorization with the European Medicines Evaluation Agency for the use of Soliris(TM) (eculizumab) in PNH patients. This press release and further information about Alexion Pharmaceuticals, Inc. can be found at: http://www.alexionpharm.com.

This news release contains forward-looking statements, including statements related to medical benefits and commercial potential of Soliris(TM)(eculizumab), initiation and conduct of the PNH OneSource(TM) treatment support service, and estimates of the number of people living with PNH. Forward-looking statements are subject to factors that may cause Alexion's results and plans to differ from those expected, including requests by regulatory authorities for additional information following marketing approval, delays in arranging satisfactory manufacturing capability, inability to acquire funding on timely and

satisfactory terms, delays in or failure to establish internal sales and marketing capabilities, delays in developing or adverse changes in commercial relationships, the possibility that results of clinical trials are not predictive of the safety and efficacy of Soliris(TM)(eculizumab) when made available to larger number of people, the risk that third parties won't agree to license any necessary intellectual property to us on reasonable terms, the risk that third party payors will not reimburse for the use of Soliris(TM) at acceptable rates or at all, the risk that estimates regarding the number of people living with PNH are inaccurate, and a variety of other risks set forth from time to time in Alexion's filings with the Securities and Exchange Commission, including but not limited to Alexion's Annual Report on Form 10-K. Alexion does not intend to update any of these forward-looking statements to reflect events or circumstances after the date hereof, except when a duty arises under law.

(1) Rother R, Bell L, Hillmen P, Gladwin M. The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin. JAMA 2005; 293:1653-1662.

(2) Parker C, Omine M, Richards S, et al. Diagnosis and management of paroxysmal nocturnal hemoglobinuria. Blood 2005; 106:3699-3709.

(3) Socie G, Mary J Yves, de Gramont A, et al. Paroxysmal nocturnal haemoglobinuria: long-term follow-up and prognostic factors. Lancet 1996; 348:573-577.

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(8) Soliris(TM) (eculizumab) prescribing information. Alexion Pharmaceuticals, Inc., 2006.

SOURCE Alexion Pharmaceuticals, Inc.

Leonard Bell, M.D., Chief Executive Officer, of Alexion Pharmaceuticals, Inc., +1-203-272-2596; or Media, David Patti, of Makovsky + Company, +1-212-508-9623, dpatti@makovsky.com; or Investors, Rhonda Chiger, of Rx Communications Group, LLC, +1-917-322-2569, rchiger@rxir.com, both for Alexion Pharmaceuticals, Inc.

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News Provided by COMTEX

DECLARATION BY STEPHEN A. SAXE, PH.D., J.D.

Dear Sir:

I, Stephen A. Saxe, Ph.D., J.D., was Associate General Counsel, Intellectual Property at Alexion Pharmaceuticals, Inc. ("Alexion") from October 2001 through May 2014. In that role, I was the primary intellectual property counsel at Alexion responsible for handling matters related to Soliris®(eculizumab) from at least 2001 through 2009. I declare that:

1. In late March 2009, in the course of reviewing papers related to a matter in Australia, I noticed that eculizumab had a registry number assigned to it from the Chemical Abstracts Service ("CAS"). I inquired internally at Alexion and learned that in early 1999 CAS had assigned the molecule that would later be called "eculizumab" the CAS Registry Number 219685-50-4. However, I also learned at that time that incorrect information had been submitted to CAS in 1999 to support the CAS registry entry. In particular, there were numerous errors in the sequence of the molecule described in CAS Registry Number 219685-50-4.

2. On April 20, 2009, I emailed CAS and explained that it had recently come to our attention that the entry information submitted in 1999 was incorrect, and I proposed that the registry entry should be corrected. (See attached Exhibit A.)

3. Following up to that email, on April 28, 2009, I sent another email with an attached letter to CAS, detailing the errors and requesting correction of CAS Registry Number 219685-50-4. (See attached Exhibit B.) For example, I explained that the heavy chain is in fact 448 amino acids long, not 650 amino acids in length as had initially been submitted in 1999. (See Exhibit B letter, 3rd paragraph.) I also explained that there was an error in the sequence of the light chain that was submitted in 1999. (See Exhibit B letter, 3rd paragraph.) I provided complete and accurate amino acid sequences for both the heavy chain and the light chain of eculizumab.

4. On May 1, 2009, Ms. Lois Bishop at CAS responded to my April 28, 2009 communication and indicated that the changes I requested to CAS Registry Number 219685-50-4 had been made and that it would take a couple of days for these to appear on STN. (See attached Exhibit C.)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true.

Stephen A. Saxe — January 12, 2018

Stephen A. Saxe, Ph.D., J.D. — Date

Saxe, Stephen

From: Saxe, Stephen
Sent: Tuesday, April 28, 2009 3:03 PM
To: 'answers@cas.org'
Subject: CAS Registry Number 219685-50-4
Attachments: Letter to CAS 04-28-09.pdf

Dear Sir or Madam:

Please see the attached letter regarding correcting information for CAS Registry Number 219685-50-4. Please contact me if you need additional information. Thank you.

Stephen A. Saxe, Ph.D.
Associate General Counsel, Intellectual Property
Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410
U.S.A.
Tel. 203-271-8289
Fax 203-271-8195

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ALEXION pharmaceuticals

Stephen A. Saxe, Ph.D.
Associate General Counsel, Intellectual Property

saxes@alxn.com
Direct Tel. 203-271-8289

April 28, 2009

Dear Sir or Madam:

On February 8, 1999 Alexion Pharmaceuticals, Inc. submitted an application form for a new CAS registry number for Alexion's whole monoclonal antibody named 5G1.1. This was processed under your Order Number 41875. The antibody was assigned CAS Registry Number 219685-50-4. It recently came to Alexion's attention that incorrect information was submitted in 1999. At this time Alexion would like to correct the error that was made in 1999. Attached is information showing the correct sequence information. In the 1999 filing, the antibody was described as follows:

> 5G1.1 is a fully humanized monoclonal antibody (originally of murine origin) specifically reactive with the fifth component of human complement (C5). 5G1.1 specifically binds to the alpha chain of C5. It blocks the activation of C5 and thereby blocks downstream complement system function. It thus 1) blocks complement hemolytic activity, 2) blocks the assembly of the C5b-9 membrane attack complex of complement, and 3) blocks the generation of the anaphylatoxic complement C5 cleavage product, C5a. 5G1.1 consists of a heavy chain that is 650 amino acids long and a light chain that is 214 amino acids long. The amino acid sequence of the light chain is:

There is one error in the information provided in the above paragraph. The heavy chain is in fact 448 (not 650) amino acids long. The correct sequence is shown on the attached sheet. The light chain is 214 amino acids long and that information in the above paragraph is correct. However, there was a single error in the sequence of the light chain that was submitted in 1999 and the correct sequence is shown on the attached sheet. Both sequences on the attached sheet (labeled as Figure 3.2.S.1.2.2) are presented using the standard one-letter amino acid code. If you need more information than that, please let me know. We would like the heavy- and light-chain sequences shown on the attached sheet to be inserted into the CAS database in place of the two sequences that were erroneously sent to you in 1999.

Additionally, the disulfide bonds of the whole antibody are known. These bonds are as follow:

Samsung Bioepis Exhibit No. 1002
Page 1380

Disulfide Bonds Solely within a Light Chain
Cys23Light - Cys88Light
Cys134 Light - Cys194Light

Disulfide Bonds Solely within a Heavy Chain
Cys22Heavy - Cys96Heavy
Cys149Heavy - Cys205Heavy
Cys262Heavy - Cys322Heavy
Cys368Heavy - Cys426Heavy

Disulfide Bonds Connecting the Light and Heavy Chains
Cys136Heavy - Cys214Light

Disulfide Bonds Connecting Two Heavy Chains
Cys228Heavy - Cys228Heavy
Cys231Heavy - Cys231Heavy

From the above you see that each light chain has two intrachain disulfide bonds, each heavy chain has four intrachain disulfide bonds, there is a single disulfide bond connecting a light chain to a heavy chain, and there are two disulfide bonds connecting the two heavy chains to each other.

Additionally, there is one N-linked glycosylation site at Asn298 of the heavy chain.

We would like the same CAS Registry Number to be maintained for this molecule and to simply have the correct amino acid sequences for the light and heavy chains replace the incorrect sequences that were inadvertently submitted in 1999. Please let me know when the correction has been made. Please contact me if you need additional information.

Very truly yours,

Stephen A. Saxe

Stephen A. Saxe

Figure 3.2.S.1.2.2: Expected Amino Acid Sequence for h5G1.1-mAb

Heavy Chain

QVQLVQSGAEVKKPGASVKVSCKASGYIFSNYWIQWVRQAPGQGLEWMGEILPGSGSTEYTENFKDRVTMTRDTSTSTVYMELSSLR
SEDTAVYYCARYFFGSSPNWYFDVWGQGTLVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF
PAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVERKCCVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVV
VDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVY
TLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHY
TQKSLSLSLGK

N-Terminal = Pyroglutamate
C-Terminal = OH
Average Mass = 49,502 Da

Light Chain

DIQMTQSPSSLSASVGDRVTITCGASENIYGALNWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
NVLNTPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST
LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

N-Terminal = H
C-Terminal = OH
Average Mass = 23,135 Da

Samsung Bioepis Exhibit No. 1002
Page 1382

answers@cas.org
send new segs, reference old order #

Saxe, Stephen s-s disulfide bond change - let them know,

From: Saxe, Stephen Lois Bishop 614-447-3600, x2863
Sent: Monday, April 20, 2009 4:35 PM
To: 'help@cas.org'
Subject: Registry Number 219685-50-4

Dear Sir or Madam,

About 10 years ago my company (Alexion Pharmaceuticals) obtained a registry number (219685-50-4) for our antibody called eculizumab. We submitted amino acid sequences which we stated were for the two chains of eculizumab. It has recently come to our attention that the sequences we submitted to you 10 years ago were incorrect. How can we have this corrected? At present, I am uncertain whether the sequences we submitted are publicly available in a database. Whether publicly available or not, we feel that we should correct the sequences that were erroneously sent to you in 1999 and which we only recently realized were sent in error. Can we simply submit the correct sequences and keep the same registry number or is it necessary to obtain a new registry number? If the latter, we would like the original registry number deleted since the sequence associated with it is incorrect. Please let me know how we should proceed to get this matter corrected. Thank you.

Stephen A. Saxe, Ph.D.
Associate General Counsel, Intellectual Property
Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410
U.S.A.
Tel. 203-271-8289
Fax 203-271-8195

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Saxe, Stephen

From: Bishop, Lois [lbishop@cas.org]
Sent: Friday, May 01, 2009 8:18 AM
To: Saxe, Stephen
Subject: RE: CAS Registry Number 219685-50-4

Dear Dr. Saxe,

The changes to your sequence for CAS Registry Number 219685-50-4 have been made. It may take a couple of days before they appear on STN. Let us know if we can be of further assistance.

Best regards,
Lois Bishop
CAS Client Services
614-447-3600 x2863

From: Saxe, Stephen [mailto:SaxeS@alxn.com]
Sent: Tuesday, April 28, 2009 3:03 PM
To: answers
Subject: CAS Registry Number 219685-50-4

Dear Sir or Madam:

Please see the attached letter regarding correcting information for CAS Registry Number 219685-50-4. Please contact me if you need additional information. Thank you.

Stephen A. Saxe, Ph.D.
Associate General Counsel, Intellectual Property
Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410
U.S.A.
Tel. 203-271-8289
Fax 203-271-8195

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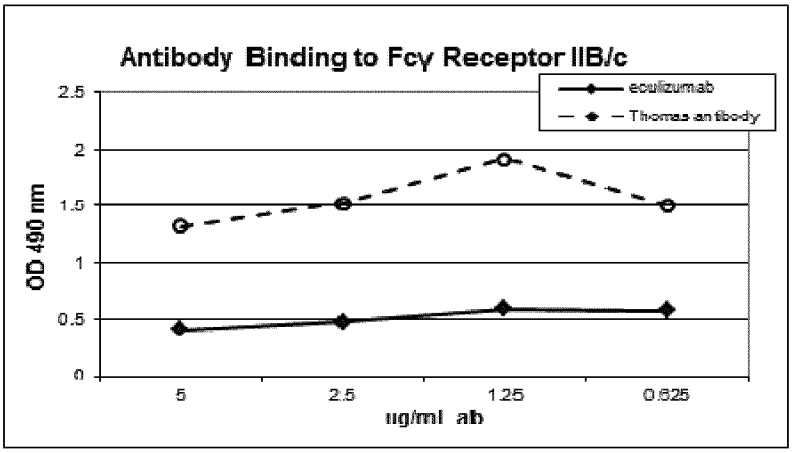
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Experimental Report

Methods

Ni-NTA pre-coated plates (Qiagen, Ca#35061) were incubated with 50 µL/well of His-tagged human FcγR IIb/c (R and D systems Cat# 1875-CD) at a receptor concentration of 5 µg/mL in PBS, overnight at 4°C. Following overnight incubation with receptor, the plates were washed 3 times with PBS buffer containing 0.02% Tween-20 using a multiwash advantage plate washer. After washing, 50 µL of complexed antibodies were diluted in PBS buffer containing 0.05% Tween-20 and incubated in the plate for 60 minutes at room temperature. Complexed antibody was prepared by prior incubation of antibodies (eculizumab and the anti-C5 IgG4 antibody described in Thomas et al. (1997) Mol. Immunol. 33: 1389-1401) overnight with a biotinylated $F(ab')_2$ fraction of goat-anti-human $F(ab')_2$ (Jackson Immunolabs Cat#109-066-006), at a 2:1 antibody: $F(ab')_2$ molar ratio in PBS. Following incubation of the plate wells with complexed antibody, the plates were washed as described above and followed by the addition of 50 µL/well of the secondary antibody streptavidin-HRP to detect biotinylated complexed antibodies (Zymed Cat#43-4323, diluted 1/2000 in PBS buffer containing 0.05% Tween-20). Incubation with secondary antibody was for 60 minutes at room temperature. Following this incubation, wells were washed and color development initiated with 100 µL/ well of *o*-phenylenediamine dihydrochloride (OPD, Sigma Cat#P 9187). Reactions were stopped with 25 µL/well 12.5% H_2SO_4 and the absorbance read at 490 nm.

Results



The Figure above shows the binding of the cross linked antibodies to Fcγ Receptors as immune complexes with $F(ab')_2$-anti-$F(ab')_2$, measured using an ELISA format. Concentration of antibody (µg/mL) is described on the X axis, and the Y axis reflects signal

as measured via optical density at 490 nm. The binding profile of the IgG4 subtype antibody (Thomas antibody) to FcγRIIB, the inhibitory FcγR, is markedly different from the binding profile exhibited by eculizumab. Binding of eculizumab to FcγRIIB is weaker than the binding of the IgG4 antibody to FcγRIIB.

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration
Rockville, MD 20857

MAR 16 2007

Our STN: BL 125166/0

Alexion Pharmaceuticals
Attention: Nancy Motola, Ph.D., RAC
Senior Vice President, Regulatory and Quality
352 Knotter Drive
Cheshire, CT 06410

Dear Dr. Motola:

We are issuing Department of Health and Human Services U.S. License No. 1743 to Alexion Pharmaceuticals, Inc., Cheshire, Connecticut, under the provisions of section 351(a) of the Public Health Service Act controlling the manufacture and sale of biological products. The license authorizes you to introduce or deliver for introduction into interstate commerce, those products for which your company has demonstrated compliance with establishment and product standards.

Under this license, you are approved to manufacture the product eculizumab, which is indicated for the treatment of paroxysmal nocturnal hemoglobinuria to reduce hemolysis.

You are approved to manufacture eculizumab drug substance at Lonza Biologics, PLC in Portsmouth, NH. The final formulated product will be manufactured, filled, labeled, and packaged at [redacted]. You may label your product with the proprietary name Soliris™ and will market it in 300 mg vials.

The final printed labeling (FPL) must be identical to the enclosed labeling. Marketing product with FPL that is not identical to the approved labeling text may render the product misbranded and an unapproved new drug.

The dating period for eculizumab drug product shall be 24 months from the date of manufacture when stored at 2-8°C. The date of manufacture shall be defined as the date of final sterile filtration of the formulated drug product. The dating period for your drug substance shall be 18 months when stored at 2-8°C.

You currently are not required to submit samples of future lots of eculizumab to the Center for Drug Evaluation and Research (CDER) for release by the Director, CDER, under 21 CFR 610.2. We will continue to monitor compliance with 21 CFR 610.1 requiring completion of tests for conformity with standards applicable to each product prior to release of each lot.

You must submit information to your biologics license application for our review and written approval under 21 CFR 601 12 for any changes in the manufacturing, testing, packaging or labeling of eculizumab, or in the manufacturing facilities.

We acknowledge your written commitments as described in your letter of February 22, 2007, March 12, 2007 and March 12, 2007 as outlined below:

Postmarketing Studies subject to reporting requirements of 21 CFR 601.70.

1. To evaluate long-term safety of eculizumab by analyzing outcomes in the Soliris Safety Registry for a time period of no less than five years. At the end of the five year period, a study report will be submitted to the Biological License Application (BLA) that describes the major safety findings from the registry program, including the specific items listed below and proposing labeling changes as appropriate. Additionally, annual interim reports will be submitted to the BLA, along with expedited reports as specified below. The protocol for addressing this PMC will be submitted to the IND by May 31, 2007, and the five year study report will be submitted by June 30, 2012. All patients within the registry will be followed for the occurrence of:

    a. Serious infections, defined as infections necessitating or prolonging hospitalization or resulting in death. Alexion commits to collecting follow-up information from these patients to assess the nature of the serious infection, the duration of hospitalization, the major features of the clinical course and the survival status. Expedited reporting (15 day telephone or facsimile Medwatch communication) will be provided for the occurrence of these serious infections.

    b. Malignancy, including the nature of the malignancy and the survival status of patients who develop a malignancy;

    c. Use of eculizumab among pediatric patients under 16 years of age, to include collection of eculizumab dosage information, as well as the same information being required for adult patients in the registry;

    d. Pregnancy, including the clinical course of each pregnancy and the detection of congenital abnormalities among babies born to the women exposed to eculizumab during the pregnancy.

    e. Thrombotic events, including the nature of the event, the clinical outcome as well as the anticoagulant management prior to and after the event.

2. To submit a comprehensive description of the Soliris Guardian Program Risk Minimization Action Plan (RiskMAP), including all items listed below. Fulfillment of this post-marketing commitment will be contingent upon FDA concurrence upon the expectations of the Soliris Guardian Program. Submission of all items listed below will occur no later than May 18, 2007. The submitted information will include:

   a. A final version of the Soliris Guardian Program document that, in addition to any other items, provides information fully consistent with the approved prescribing information.

   b. A copy of all educational materials to be provided as part of the program, including but not limited to all components of the Soliris Starter Kit, the Drug Fact Sheet and patient-health care provider documents relating to the Soliris Safety Registry.

   c. A commitment to develop and submit a protocol (with the understanding that this protocol may need modification based upon FDA review findings) that uses surveys of health care providers and patients to assess compliance with the vaccination requirements as well as their knowledge of the risks of eculizumab and the need for vaccination.

   d. A copy of the Soliris Safety Registry protocol and any supportive documents to be provided to health care providers and patients. These documents may need modification based upon FDA review findings. The submitted protocol will indicate that the Registry will continue until Alexion receives written concurrence from FDA to terminate the registry. The protocol will include collection of the occurrence of the following events:

      i. Death;

      ii. Meningococcal vaccination (type and dates of all vaccinations);

      iii. eculizumab administration dates, at designated time points to establish initiation and termination of therapy as well as to correlate eculizumab administration with the fatalities and the events listed below:

         - All meningococcal infections causing sepsis or meningitis
         - Other serious infections
         - Malignancy, including the nature of the malignancy and the survival status of patients who develop a malignancy;
         - Use of eculizumab among pediatric patients under 16 years of age, to include collection of eculizumab dosage information, as well as the required information for adult patients in the registry;
         - Use of eculizumab by indication;

- Pregnancy, including the clinical course of each pregnancy and the detection of congenital abnormalities among off-spring of the women exposed to eculizumab during a pregnancy and;
- Serious hemolysis, as defined by specific criteria.

e. A commitment to submit quarterly RiskMAP reports for the first year and annual reports thereafter (unless FDA provides written request for more frequent submissions) summarizing all information relating to the Soliris Guardian Program. The reports should include the following:

i. An analysis of all cases of the following, including a root cause analysis and factors that might have contributed to serious outcomes:
- Meningococcal sepsis or meningitis, including the timing of all vaccinations relative to administration of Soliris
- Other infections (with serious outcomes)
- All deaths
- Cases of serious hemolysis and all cases of hemolysis with serious outcomes

ii. Soliris use patterns including indication for use;

iii. Extent of compliance with RiskMAP requirements such as the percentage of patients that were vaccinated prior to receiving eculizumab and the percentage of patients that were re-vaccinated at 3-year or 10-year intervals (as applicable);

iv. Analysis of all cumulative data collected in the Soliris Safety Registry;

v. Results from all health care provider and patient surveys, including:

- Any known data about patients or physicians who refused to participate in the surveys
- Any known data about survey participants who are considered "lost" (drop-outs).

3. To conduct a randomized, controlled clinical study to assess the effects of anticoagulant withdrawal among PNH patients receiving eculizumab. This study will randomize at least 100 anticoagulated patients to either continue or discontinue anticoagulation therapy. The major outcomes will assess the safety of discontinuation of anticoagulant therapy while continuing eculizumab, especially with respect to providing important evidence regarding major bleeding and that this discontinuation does not increase the risk for occurrence of thrombotic events in these patients. A full study report and data from this study will be submitted to the BLA and may include a label revision, contingent upon the importance of the study results. The study protocol will be submitted to the investigational new drug application (IND) by June 30, 2007 and patient accrual completed no later than June 1, 2009. A final study report will be submitted no later than March 31, 2014.

4. To develop a validated and quantitative assay for the measurement of human anti-human antibodies (HAHA) for the detection of antibody formation to eculizumab. This assay will assess potential immune responses to the whole eculizumab molecule. Description of the validated assay will be submitted to the BLA as a CBE 30 by July 9, 2008.

5. To develop a validated and sensitive assay for the measurement of neutralizing HAHA to eculizumab. Alternatively, Alexion commits to submit documentation to FDA demonstrating with due diligence that a suitable assay could not be feasibly developed and that the assessment of serum lactate dehydrogenase (LDH) is a sufficiently sensitive indicator of the presence of neutralizing antibodies. This information will be submitted to the BLA by July 9, 2008.

6. To utilize samples from the ongoing E05-001 Phase III extension study (approximately 170 patients for at least 2 years) as test samples for the new validated HAHA assays. Alexion will continue to obtain serum samples from those patients who transition from E05-001 to the Soliris Safety Registry, at intervals of no less than one year, and continue this collection process for an additional three years. Sample collection will cease during this additional three year period for patients who terminate eculizumab administration. Additionally, serum samples will be obtained based upon physician reports of suspected loss of eculizumab bioactivity, based upon unanticipated alterations in serum LDH concentrations. All serum samples will be assayed at least annually and the results provided within an annual report to the BLA. Clinical data, to include the results of serum LDH concentrations, will also be obtained from any patients who show evidence of antibody formation. The protocol describing Alexion's plan for responding to this commitment will be submitted by May 1, 2007 and the final study report submitted by January 31, 2011.

Postmarketing Studies not subject to reporting requirements of 21 CFR 601.70.

7. To revalidate the linearity and accuracy of the Osmolality method across the full specification range using a combination of product samples diluted to lower osmolality and product samples spiked with osmolality standards. The revalidation plan will be submitted in advance to FDA for review and endorsement. This information will be submitted to the BLA as a CBE 30 by August 31, 2007.

8. To revalidate the linearity of the IEF method across a load range of [—] The revalidation plan will be submitted in advance to FDA for review and endorsement. This information will be submitted to the BLA as a CBE 30 supplement by August 31, 2007.

9. To revise the IEF method SOP to specify that the method is validated only for a [—] This information will be submitted to the BLA as a CBE 30 by August 31, 2007.

10. To improve and revalidate the existing hemolytic assay. Improvements include increasing the number of sample replicates and qualifying the chicken erythrocytes reagent. The revised method SOP and revalidation plan will be submitted in advance to FDA for review and endorsement. This information will be submitted to the BLA as a CBE 30 by August 31, 2007.

11. To develop a new quantitative biological activity assay to replace the existing hemolytic assay, or submit documentation to FDA demonstrating with due diligence that a suitable assay could not be feasibly developed. This information will be submitted to the BLA by February 29, 2008. Validation of the quantitative biological activity assay will be submitted by July 9, 2008.

12. To provide FDA with a completed drug substance and drug product container closure system leachables evaluation using end-of-shelf-life, long-term 2-8°C stability samples. This information will be submitted to the BLA as a CBE 30 by August 31, 2007.

13. To develop a suitable [redacted] assay and subsequently confirm [redacted] on three drug substance batches. This information will be submitted to the BLA as a CBE 30 by August 31, 2007.

We request that you submit clinical protocols to your IND, with a cross-reference letter to this biologics license application (BLA), STN BL 125166. Submit nonclinical and chemistry, manufacturing, and controls protocols and all study final reports to your BLA, STN BL 125166. Please use the following designators to label prominently all submissions, including supplements, relating to these postmarketing study commitments as appropriate:

- **Postmarketing Study Commitment Protocol**
- **Postmarketing Study Commitment Final Study Report**
- **Postmarketing Study Commitment Correspondence**
- **Annual Status Report of Postmarketing Commitment Studies**

For each postmarketing study subject to the reporting requirements of 21 CFR 601.70, you must describe the status in an annual report on postmarketing studies for this product. The status report for each study should include:

- information to identify and describe the postmarketing commitment,
- the original schedule for the commitment,
- the status of the commitment (i.e. pending, ongoing, delayed, terminated, or submitted),
- an explanation of the status including, for clinical studies, the patient accrual rate (i.e. number enrolled to date and the total planned enrollment), and
- a revised schedule if the study schedule has changed and an explanation of the basis for the revision.

As described in 21 CFR 601.70(e), we may publicly disclose information regarding these postmarketing studies on our Web site (http://www.fda.gov/cder/pmc/default.htm). Please refer to the February 2006 Guidance for Industry: Reports on the Status of Postmarketing Study Commitments - Implementation of Section 130 of the Food and Drug Administration Modernization Act of 1997 (see http://www.fda.gov/cder/guidance/5569fnl.htm) for further information.

Under 21 CFR Part 208, we have determined that this product poses a serious and significant public health concern requiring the distribution of a Medication Guide. Eculizumab is a product for which patient labeling could help prevent serious adverse effects and inform the patient of serious risks relative to benefit that could affect their decisions to use, or continue to use, the product. Therefore, a Medication Guide is necessary for safe and effective use of this product and FDA hereby approves the draft Medication Guide you submitted March 15, 2007. Please note that:

- this Medication Guide must be reprinted at the end of the package insert or accompany it [21 CFR 201.57(c)(18)];
- you are responsible for ensuring that this Medication Guide is available for distribution to every patient who is dispensed a prescription for this product [21 CFR 208];
- the final printed Medication Guide distributed to patients must conform to all conditions described in 21 CFR 208.24, including a minimum of 10 point text; and
- you are responsible for ensuring that the label of each container or package includes a prominent and conspicuous instruction to authorized dispensers to provide a Medication Guide to each patient to whom the drug is dispensed, and states how the Medication Guide is provided.

Please submit within 30 days content of labeling [21 CFR 601.14(b)] in structured product labeling (SPL) format, as described at http://www.fda.gov/oc/datacouncil/spl.html, that is identical in content to the enclosed labeling text dated March 16, 2007. Upon receipt and verification, we will transmit that version to the National Library of Medicine for public dissemination.

You must submit adverse experience reports under the adverse experience reporting requirements for licensed biological products (21 CFR 600.80). You should submit postmarketing adverse experience reports to the Central Document Room, Center for Drug Evaluation and Research, Food and Drug Administration, 5901-B Ammendale Road, Beltsville, MD 20705-1266. Prominently identify all adverse experience reports as described in 21 CFR 600.80.

The MedWatch-to-Manufacturer Program provides manufacturers with copies of serious adverse event reports that are received directly by the FDA. New molecular entities and important new biologics qualify for inclusion for three years after approval. Your firm is eligible to receive copies of reports for this product. To participate in the program, please see the enrollment instructions and program description details at www.fda.gov/medwatch/report/mmp.htm.

You must submit distribution reports under the distribution reporting requirements for licensed biological products (21 CFR 600.81).

You must submit reports of biological product deviations under 21 CFR 600.14. You should promptly identify and investigate all manufacturing deviations, including those associated with processing, testing, packing, labeling, storage, holding and distribution. If the deviation involves a distributed product, may affect the safety, purity, or potency of the product, and meets the other criteria in the regulation, you must submit a report on Form FDA-3486 to the Division of Compliance Risk Management and Surveillance (HFD-330), Center for Drug Evaluation and Research, Food and Drug Administration, 5600 Fishers Lane, Rockville, MD 20857. Biological product deviations sent by courier or overnight mail should be addressed to Food and Drug Administration, CDER, Office of Compliance, Division of Compliance Risk Management and Surveillance, HFD-330, Montrose Metro 2, 11919 Rockville Pike, Rockville, MD 20852.

Please submit all final printed labeling at the time of use and include implementation information on FDA Form 356h. Please provide a PDF-format electronic copy as well as original paper copies (ten for circulars and five for other labels). In addition, you may wish to submit draft copies of the proposed introductory advertising and promotional labeling with a cover letter requesting advisory comments to the Food and Drug Administration, Center for Drug Evaluation and Research, Division of Drug Marketing, Advertising and Communication, 5901-B Ammendale Road, Beltsville, MD 20705-1266. Final printed advertising and promotional labeling should be submitted at the time of initial dissemination, accompanied by a FDA Form 2253.

All promotional claims must be consistent with and not contrary to approved labeling. You should not make a comparative promotional claim or claim of superiority over other products unless you have substantial evidence to support that claim.

Please refer to http://www.fda.gov/cder/biologics/default.htm for information regarding therapeutic biological products, including the addresses for submissions.

Sincerely,

Richard Pazdur, M.D.
Director
Office of Oncology Drug Products
Center for Drug Evaluation and Research

Enclosure: Package Insert
Carton and Vial Labeling
Medication Guide
Patient Safety Card

Oral Communication

Paediatric and Red Cell Disorders

1

Thromboembolism in childhood: a prospective two-year BPSU study in the United Kingdom. February 2001–February 2003

B. E. S. Gibson,[1] E. A. Chalmers,[1] P. Bolton-Maggs,[2] D. J. Henderson[1] & R. Lynn[3]

[1]*Royal Hospital for Sick Children, Glasgow;* [2]*Alder Hey Children's Hospital, Liverpool;* [3]*British Paediatric Surveillance Unit, London, UK*

Registry data was collected over a 2-year period from children aged 1 month to 16 years with newly diagnosed, objectively documented venous and arterial thrombotic events. Children with stroke were excluded. The study aimed to identify incidence, epidemiology, risk factors and current diagnostic and therapeutic practices in childhood thrombosis. Presentation data was submitted on 309 of the 349 cases reported during the study period; 200 (64.7%) fitted the study criteria and 109 (35.3%) did not. Six-monthly follow-up data was collected on 170 cases (85%). The sites were venous 83.5%, cardiac 3.5%, arterial 6% and combined sites 7%; 88.6% of venous cases were DVT/PE, 35.8% in the upper and 64.2% in the lower limbs. The main risk factors were CVL (47.5%), sepsis (31.5%), immobility (28%), malignancy (26%); 63.5% had two or more risk factors. Only 17 cases (8.5%) had no risk factors reported. Thrombophilia screening was abnormal in 31 of the 100 cases appropriately investigated. Doppler ultrasound was the commonest diagnostic investigation (69%), but a third had additional investigations. Unfractionated/LMWH, followed by Warfarin was the commonest treatment (47.5%). Treatment duration varied from one to greater than 6 months. The outcome was as follows: 63 cases (31.5%) complete resolution, 70 (35%) partial and 56 (28%) either no resolution, or the outcome was unknown at the time of data collection. Eleven patients (5.5%) had extension/recurrence. Bleeding was minor in nine cases (4.5%) and major in two (1%). Overall mortality was 9%, with three deaths (1.5%) directly due to venous thrombosis and four (2%) in which it was a contributory factor. The first UK thrombosis registry in childhood has identified an annual incidence of 0.07/10 000 children, heterogeneity in events, diagnostic and management strategies, and a low mortality.

2

Outcome of Philadelphia positive (Ph^+) childhood acute lymphoblastic leukaemia (ALL) on the MRC ALL 97/01 trial

A. Roy,[1] M. Bradburn,[2] A. Moorman,[3] J. Burrett,[4] C. Mitchell,[5] S. Kinsey,[5] I. Hann[5] & V. Saha[5]

[1]*Cancer Research UK, Children's Cancer Group, Royal London Hospital, London;* [2]*Cancer Research UK, Medical Statistics Group, Institute of Health Sciences, Oxford;* [3]*LRF Cytogenetics Group, Cancer Sciences Division, University of Southampton, Southampton;* [4]*Clinical Trial Service Unit, Radcliffe Infirmary, Oxford, UK;* [5]*MRC ALL 97 Trial Coordinators, UK Children's Leukemia Working Party*

We report the outcome of children with Ph^+ ALL treated on the national protocol for childhood ALL, MRC ALL 97/01 between January 1997 and June 2002. A diagnosis of Ph^+ ALL was made by karyotyping and/or fluorescent *in situ* hybridization. Outcome was evaluated according to NCI risk criteria and treatment response. Statistical methods included Kaplan–Meier analysis, log rank and stratified log rank tests. Of the 1935 patients treated according to the MRC ALL 97/01 protocol, 1840 were successfully screened for Ph chromosome and 43 (2.3%) were positive. The median age at diagnosis and presenting white cell count were 6.9 years and 30×10^9/l respectively. Based on NCI risk criteria and early response to treatment, there were 10 standard, 10 intermediate and 20 high-risk patients; 37/43 patients (86%) achieved complete remission (CR1) by the end of a 4-week induction period; 28/43 patients (65.1%) underwent bone marrow transplantation (BMT) in CR1. The median follow up for the 43 patients was 24 months (range 5–69 months). The 3-year estimates for event-free survival (pEFS) and overall survival (pOS) (with 95% confidence intervals) were 52% (34–67) and 49% (30–66) respectively. The pEFS and pOS according to risk group stratification were as follows: for standard risk: 90% (47–99) and 90% (47–99); for intermediate risk: 53% (15–80) and 63% (22–87); for high risk: 33% (11–58) and 16% (1–48). Significant predictors for overall survival were age ($P < 0.5$), WBC count ($P = 0.002$) and risk stratification ($P = 0.02$). Steroid randomization, the addition of daunorubicin for induction or undergoing BMT in CR1 was not predictive of outcome. The results are comparable with those achieved by other groups. The outcome of high-risk Ph^+ childhood ALL remains poor even with BMT. Newer alternative treatment strategies, such as the use of imatinib mesylate are required for children with Ph^+ ALL.

3

Constitutional variants in the Fanconi anaemia group G gene (FANCG) in sporadic childhood acute myeloid leukaemia

S. Meyer,[1,2] L. Barber,[1] A. M. Will,[2] O. B. Eden[2] & G. M. Taylor[1]

[1]*Cancer Immunogenetic Laboratory, St Mary's Hospital;* [2]*Department of Paediatric Oncology, Royal Manchester Children's Hospital, Manchester, UK*

The high risk of acute myeloid leukaemia (AML) in children with Fanconi anaemia (FA) implies an important role for the intact cellular FA pathway in protection from AML. Abnormalities in the FA pathway resulting from variations in FA genes might therefore increase susceptibility to AML. Early haematological disease in some FA group G patients suggests that constitutional variations in Fanconi anaemia group G gene (FANCG) might also play a role for sporadic childhood AML. To investigate this we screened the entire coding sequence (14 exons) of the FANCG gene including the intron/exon boundaries for pathogenic mutations and polymorphisms in constitutional DNA from 90 children with AML, using single strand conformation polymorphism (SSCP). DNA samples with aberrant SSCP mobility patterns were sequenced to identify variants. For comparison, we screened 400 normal cord blood samples for the variants identified in AML patients, using isotope-labelled sequence-specific oligonucleotide probes. Of the children with AML, 4/90 (4.4%) carried the exon 12 variant R513Q, compared with 4/400 (1%) of cord blood controls (odds ratio: 4.64; 95% confidence interval: 1.1–18.9, $P = 0.04$). The 890C > T variant, initially identified in an FA group G patient, was found not only in two children with sporadic AML (2.2%) but also in 11 controls (2.7%). One child diagnosed with sporadic AML (1.1%) carried two FANCG pathogenic mutations, IVS2 + 2 and L589P, and appeared to be a cryptic FA patient. In addition, one silent variant was identified in a child with AML and three common intronic polymorphisms were identified in children with AML and controls. Pathogenic FANCG mutations do not play a major role in sporadic childhood AML and the frequency of cryptic FA-G patients presenting only with childhood

Samsung Bioepis Exhibit No. 1002
Page 1395

AML is approximately 1%. The higher frequency of the R513Q polymorphism in sporadic AML might imply an increased susceptibility to childhood AML in children carrying this variant.

4

Prodaptin-linked CD59, a membrane-targeted recombinant CD59, coats PNH red cells *in vitro* and protects murine red cells from human complement mediated lysis when given *in vivo*

A. Sah,[1] S. H. Ridley,[2] S. J. Richards,[1] M. J. Cullen,[1] R. Jones,[1] R. G. Oldroyd,[2] J. White,[2] D. Esser[2] & P. Hillmen[1]

[1]HMDS, Leeds Teaching Hospitals NHS Trust, Leeds; [2]Adprotech Ltd, Essex, UK

Paroxysmal nocturnal haemoglobinuria (PNH) is characterized by intravascular haemolysis, venous thrombosis and is associated with aplastic anaemia. PNH cells are deficient in glycosylphosphatidylinositol-linked antigens. The haemolysis and thrombosis result from increased complement sensitivity of PNH cells because they lack complement regulatory molecules, particularly CD59. CD59 deficiency from PNH red cells can be complete (type III) or partial (type II) cells. Patients with only type II cells do not haemolyse, indicating that low levels of CD59 control complement activity. Replacing CD59 on PNH cells should prevent complement-mediated haemolysis and possibly thrombosis. Prodaptin-CD59 is a recombinant human CD59, which has been modified using site-specific attachment of a membrane-interactive acylated, charged peptide instead of the GPI anchor (Prodaptin™ technology). Prodaptin-CD59 is effective in complement lysis assays significantly reducing haemolysis compared with unmodified recombinant sCD59 with comparable activity to native CD59. Whole blood from eight PNH patients was incubated with Prodaptin-CD59, at various concentrations and incubation times. PNH erythrocytes were coated with CD59 to levels similar to type II cells at concentrations of 100 nmol/l Prodaptin-CD59 in all patients. Normal levels of CD59 were achieved at concentrations of 1000 nmol/l. PNH type III cells were completely coated by CD59 after 1 min with persistent CD59 expression after 3 d storage at 4 and 37°C. In order to assess Prodaptin-CD59 *in vivo*, groups of CD1 mice were treated with various concentrations of Prodaptin-CD59 by i.v. injection. Blood was taken 15 min to 24 h after injection and exposed to human complement. The erythrocytes from mice given 10 mg/kg Prodaptin-CD59 were protected from lysis up to the 6-h time point. Erythrocytes from the 50-mg/kg group were protected from lysis up to and including the 24 h time point ($P = 0.002$). No toxicity was observed. Prodaptin-CD59 represents a potential novel therapeutic approach for patients with PNH and merits further investigation.

5

Eculizumab, a C5 complement-blocking antibody, controls haemolysis in paroxysmal nocturnal haemoglobinuria (PNH) with responses maintained over a prolonged period of therapy

P. Hillmen,[1] C. Hall,[1] J. C. W. Marsh,[2] M. Elebute,[2] S. J. Richards,[1] S. A. Rollins,[3] C. F. Mojcik[3] & R. P. Rother[3]

[1]Department of Haematology, Leeds Teaching Hospitals NHS Trust, Leeds; [2]Department of Haematology, St George's Hospital Medical School, London, UK; [3]Alexion Pharmaceuticals Inc., Connecticut, USA

The intravascular haemolysis of paroxysmal nocturnal haemoglobinuria (PNH) is caused by CD59 deficiency from PNH cells. Eculizumab, a humanized monoclonal antibody to C5, inhibits terminal complement activation. Eleven patients have now received eculizumab (900 mg infusion) on alternate weeks for over a year. Eculizumab is well tolerated with no dose limiting toxicity. The biochemical parameters of haemolysis improve within a week of starting therapy and persist over 12 months. Mean LDH decreased from 3017 U/l over 12 months prior to treatment to 547 U/l over 12 months on treatment ($P = 0.002$). Haemoglobinuria decreased from 3.0 to 0.2 paroxysms/patient-month with a significant improvement in quality of life measures. PNH type III red cells progressively increased during treatment from a mean of 36.7% to 67.1% at 12 months ($P < 0.001$). Transfusion requirement reduced from a median 1.8 units/patient/month prior to eculizumab to 0.2 units/patient/month on eculizumab ($P = 0.001$). Patients with purely haemolytic disease (macroscopic haemoglobinuria with a normal platelet count; $n = 6$) had the best response with cessation of haemolysis and a decrease in transfusions from a median of 2.0–0 units/patient-month. Four patients who continued to receive transfusions have been treated with subcutaneous erythropoietin in addition to eculizumab with improved haemoglobin and transfusion requirement. In most patients both the LDH and bilirubin (under 50 μmol/l) remain slightly elevated, haptoglobins remain undetectable and the reticulocytosis persists indicating a low level of well-compensated, extravascular, haemolysis. Two patients experienced transient breakthroughs in complement activity associated with increased LDH, haemoglobinuria and mild symptoms; these resolved immediately upon next eculizumab dose and further breakthroughs were prevented by a slight decrease in the dosing interval. This study demonstrates that eculizumab is well tolerated, dramatically decreases haemolysis and transfusion requirement as well as improving the symptoms of PNH. The effectiveness of eculizumab has been maintained for over 12 months of therapy and is ongoing in all patients.

6

A genetic interaction between chromosome 8q and the β-globin complex influences the switch from foetal to adult haemoglobin

N. Silver,[1] C. Garner,[2] S. Best,[1] S. Menzel,[1] C. Martin,[1] T. Spector[3] & S. L. Thein[1]

[1]Department of Haematological Medicine, GKT School of Medicine, King's College Hospital, Denmark Hill, London, UK; [2]Department of Medicine and Environmental Health, Science and Policy, University of California, Irvine, USA; [3]St Thomas' Twins Register Unit, St Thomas' Hospital, London, UK

The levels of HbF and F cells (FC) in adults are predominantly genetically controlled, influenced by several factors, including a common sequence variant (C to T) at position – 158 upstream of the Gγ-globin gene, referred to as the *Xmn*1-Gγ polymorphism. About 60% of the FC variance, however, is due to transacting factors. Three loci for FC levels have been mapped, on chromosome Xp22, 6q23, and most recently, on 8q in an extensive Indian kindred. The influence of the 8q quantitative trait locus (QTL) was shown to be conditional on the genotype at the *Xmn*1-Gγ site, suggesting a genetic interaction between the site and the 8q QTL. In an attempt to replicate the chromosome 8q linkage, 319 dizygotic (DZ) twin pairs of European descent were phenotyped for HbF and FC levels and genotyped for a set of 19 markers spanning 56 cM of chromosome 8. The effect of the *Xmn*1-Gγ genotype on the FC levels was estimated by linear regression and the effect of the genotype on the trait values was adjusted. The *Xmn*1-Gγ adjusted FC trait showed higher multipoint lod scores across the 56 cM interval than the unadjusted trait, with a maximum lod score of 2.76. The DZ twin sample was stratified according to the six possible unordered twin pair *Xmn*1-Gγ genotypes and linkage analysis was carried out with each subsample. The linkage between the chromosome 8q QTL and FC levels

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Samsung Bioepis Exhibit No. 1002
Page 1396

is shown to be present only when one or both of the twins is heterozygous at the *Xmn*1-Gγ site. The 8q QTL defines a new class of genetic determinants of HbF levels that are conditional on *cis*-acting sequences of the β-globin complex, which could explain some of the inconsistent associations of high HbF and FC levels with the *Xmn*1-Gγ site.

Oral Communication

Transplantation

7

Erythropoietin used early after allogeneic transplantation is effective at reducing anaemia and the need for blood transfusions

C. P. Fox, S. Pacey, E. Das-Gupta, N. Russell & J. Byrne
Department of Clinical Haematology, Nottingham City Hospital, Nottingham, UK

Anaemia is a common problem in the early months following allogeneic transplantation due to defective erythropoietin (Epo) production. Since blood transfusions may be particularly hazardous in this setting, we sought to avoid them by using Epo therapy. We treated 45 patients who had undergone allogeneic transplantation for haematological malignancy, between 2001 and 2003, with erythropoietin at a dose of 10 000 units, subcutaneously, twice weekly. Eighteen patients were transplanted for acute myeloid leukaemia/myelodysplasia, 10 for chronic myeloid leukaemia, 14 for lymphoproliferative disorders, one for myeloma and one for myelofibrosis. Thirty patients were male and 15 were female with a median age of 50 years (range 22–71 years). Twenty-three patients had received conventional transplants and 22 had received reduced intensity allografts, from 27 unrelated and 18 sibling donors. The median time of commencement of Epo was 65 d post-transplant (range 19–365 d) and the median haemoglobin (Hb) at introduction of Epo was 9.4 g/dl (range 7.0–10.7 g/dl). Twenty-two patients responded quickly to Epo and required no further blood transfusions and a further five patients had a partial response, requiring not more than 2 units of blood after starting Epo. Amongst the responders, the median rise in Hb after 2 weeks of therapy was 1.5 g (0.7–4.1 g/dl) and the Epo was discontinued at a median of 5 weeks after commencement. The median Hb at discontinuation of the Epo was 12.2 g/dl (range 10.0–14.3 g/dl). Fifteen patients required further courses of Epo after discontinuing therapy because of recurrent anaemia. Those patients who failed to respond to Epo often had additional reasons for anaemia including CMV reactivation and treatment, major ABO incompatibility or other transplant-related complications. We conclude that a short course of Epo is a highly effective treatment for anaemia arising after allogeneic transplantation and hence can avoid the need for blood transfusion in this setting.

8

Outcome of allogeneic stem cell transplantation in adults with high-risk myeloid malignancies using an alemtuzemab based reduced-intensity-conditioning regimen

S. Tauro, C. Craddock, K. Peggs, G. Begum, R. Chopra, T. Littlewood, A. Parker, J. Marsh, G. Cook, G. Hale, J. Snowden, D. Milligan, A. Hunter, N. Russell, A. Goldstone, P. Mahendra, D. Linch & S. Mackinnon
UK Collaborative Study

Allogeneic stem cell transplantation (SCT) is an important and potentially curative therapy in patients with high-risk acute myeloid leukaemia (AML) and myelodysplasia (MDS), but is precluded in most patients because of the toxicity associated with the use of a myeloablative preparative regimen. The development of reduced-intensity-conditioning (RIC) regimens has substantially decreased the toxicity of allografting, permitting its extension to patients in whom it would have previously been contraindicated on grounds of age or co-morbidity. However, the capacity of such regimens to exert a durable anti-leukaemic effect is unknown. Here, with a median follow-up of 840 d, we report the outcome of 57 patients with AML or MDS who were allografted using a fludarabine-based RIC regimen incorporating alemtuzemab. The median age of this cohort was 48 years (range 18–62 years). Thirty-one patients received a graft from an unrelated donor and 24 patients from a sibling donor. At the time of analysis, 20 patients were alive and in sustained complete remission (CR). The probability of overall survival (OS) and disease-free survival (DFS) at 2 years post-transplant was 44% and 35% respectively. In patients with relapsed AML who were allografted in second CR, the 2 years OS and DFS were 48% and 35% respectively. Non-relapse mortality at 100 d and 1 year was 12% and 23% respectively, and no patient developed greater than Grade 2 graft-versus-host disease. Since disease status at transplant was high-risk CR1 ($n = 12$), CR2 ($n = 20$) or relapsed/refractory disease ($n = 13$), the 2-year survival in our cohort of patients following intensive chemotherapy alone would have been expected to be approximately 10%. Thus, an alemtuzemab containing RIC regime can be used with minimal transplant related toxicity to achieve durable remissions in patients with AML and MDS who would otherwise be ineligible for allogeneic SCT.

9

Exploiting alloreactivity in relapsed and refractory Hodgkin Lymphoma: long-term follow-up of a T-cell-depleted reduced intensity transplant protocol

K. S. Peggs, K. Thomson, A. Hunter, R. Chopra, P. Mahendra, D. Milligan, A. Parker, R. Pettengell, C. Craddock, G. Hale, H. Waldmann, E. C. Morris, A. H. Goldstone, D. C. Linch & S. Mackinnon
UK Collaborative Group

Despite impressive primary response rates relatively few patients with multiply relapsed or refractory Hodgkin lymphoma (HL) are ultimately cured with conventional chemotherapy. The application of allogeneic stem cell transplantation (SCT) has historically been limited in this group by high transplant related mortality (TRM) rates. We investigated a reduced intensity SCT protocol incorporating fludarabine, melphalan and Campath-1H (50–100 mg) in 49 multiply relapsed and refractory HL patients. Median age was 32 years (range 18–51), number of prior treatment lines 5 (3–8), and time from diagnosis 4.8 years (0.6–14.8). Forty-four had progressed following autologous SCT. Disease status at transplant was CR in eight, PR in 27, untested relapse in one and refractory in 13. Thirty-one had matched related and 18 unrelated donors. Median follow-up is 967 d. Grade II–IV aGvHD occurred in 12%. Nineteen received donor lymphocytes from 3 months post-transplantation for progression ($n = 16$) or mixed chimerism ($n = 3$). Three received chemotherapy prior to DLI. One died of rapid disease progression and is not further evaluable for GvHD. Seven of 18 developed Grade II-IV aGvHD and five cGvHD, contributing significantly to mortality in 3. Nine of 16 demonstrated disease responses following DLI (7CR, 2PR), including two with prior chemotherapy. Of these, two died in CR, two progressed, and five

Samsung Bioepis Exhibit No. 1002
Page 1397

maintain responses (4CR, 1PR) at a median of 675 d (296–1038). TRM was 4% at day 100, 11% at 1 year and 24% at 3 years (incorporating DLI-related toxicity). Projected 4-year OS is 59% (66% for related donors, 45% for unrelated). Current PFS (incorporating DLI responses) is 41% (related) and 34% (unrelated) at 4 years. These data strongly support a clinically relevant graft-versus-HL effect and demonstrate the potential for durable responses even in this group of heavily pretreated HL patients. The relatively low procedural mortality may allow consideration of application earlier in the disease course.

10

The effect of conditioning therapy on Langerhans' cell density and phenotype in human bone marrow transplantation

M. P. Collin, T. Booth & A. M. Dickinson
Department of Haematological Sciences, University of Newcastle, Newcastle upon Tyne, UK

Graft-versus-host disease (GvHD) is mediated by donor lymphocytes. Their initial contact with the recipient is probably a recipient antigen-presenting cell (APC) but the kinetics of APC response to different conditioning regimes and their relationship with GvHD are not well defined. We have studied human Langerhans cells early post-transplant, using confocal scanning laser microscopy to image intact epidermal sheets. This provides more accurate information of the LC network than conventional immunohistochemistry, allowing up to 1 mm^2 epidermis to be examined in a horizontal plane for cell density and morphology. In normal skin, we find 705 ± 94 LC/mm^2. Transplant patients are similar, despite previous exposure to chemotherapy (645 ± 273/mm^2). At day 0, after completion of conditioning therapy most LC remain *in situ* (593 ± 143/mm^2) regardless of the preparatory regime, including CAMPATH and total body irradiation. Morphological activation is prominent at day 0, although this occurs without expression of immune activation antigens CD83 and CMRF-44 antigen. By 7 d post-transplant there is a progressive decline in LC numbers to reach a nadir at day 21 (11 ± 93/mm^2), significantly later than the nadir of blood myeloid components. Acute GvHD and steroid exposure is associated with a rapid loss of LC from the epidermis, as observed in previous studies, but by day 100 post-transplant, LC density is restored and may even exceed pretransplant levels (822 ± 332/mm^2). These studies illustrate that significant recipient antigen-presenting cells remain in the early post-transplant period, and that morphological changes are stimulated in the absence of expression of immune activation. Strategies to reduce APC numbers in the recipient may ameliorate acute GvHD.

11

Co-transplantation of murine mesenchymal stem cells and murine haemopoietic stem cells improves haemopoietic engraftment in an irradiated murine recipient

J. D. Grainger,[1] L. J. Fairbairn[2] & R. F. Wynn[1]
[1]*Stem Cell Research Group, Royal Manchester Children's Hospital;*
[2]*Patterson Institute Cancer Research, Manchester, UK*

In transplant experiments we co-infused murine MSC and murine haematopoietic cells (HSC) into lethally irradiated murine recipients and followed donor HSC and MSC engraftment. The MSC were derived from the bones of the Rosa26 murine strain, which expresses β-galactosidase. The MSC had the phenotype $CD45^-$, $CD31^-$, Sca-1 bright and could differentiate into osteocytes and adipocytes. Donor HSC were obtained from unmanipulated bone marrow of C57Bl/6J mice, which express the CD45 isotype CD45.2. Recipient animals were the PEP3b murine strain, expressing CD45.1. Following 10 Gy irradiation to the recipients 0.5–4 × 10^5 whole bone marrow cells were infused with half of each cohort also receiving 1 × 10^6 MSC. Haemopoietic engraftment in the peripheral blood (PB) and bone marrow (BM) was quantified by FACS analysis for CD45.1 and CD45.2. Only one-sixth of the recipients receiving the lowest dose survived. The recipients of 2 × 10^5 and 1 × 10^5 bone marrow cells combined with 1 × 10^6 MSC showed a haematopoietic engraftment advantage over those recipients not receiving MSC. MSC engraftment, demonstrated by LacZ staining of tissues and cells, was noted in the lung of the majority of recipients and may have been responsible for the cause of death of some recipients. Very low-level donor MSC engraftment was also noted following secondary isolation from recipient bone but was absent in sections from the spleen, liver, muscle, heart, brain and bone marrow. In conclusion, the co-infusion of MSC supports the engraftment of HSC and is maximal with lower donor HSC numbers but does not require substantial MSC engraftment.

Donor HSC dose (whole BM cells)	Donor BM at 6 weeks Mean (SEM)	Donor PB at 3 weeks	Donor PB at 6 weeks
4 × 10^5 alone	52% (15)	ND	63% (3)
4 × 10^5 plus MSC	47% (24)	ND	52% (15)
2 × 10^5 alone	32% (10.6)	35% (6.2)	43% (7.6)
2 × 10^5 plus MSC	50% (12.7)	39% (6.1)	56% (5.7)
	P = 0.14	*P = 0.33*	*P = 0.13*
1 × 10^5 alone	24% (15.1)	26.3% (6.0)	27% (10.2)
1 × 10^5 plus MSC	60% (15.5)	38% (6.5)	53% (9)
	P = 0.061	*P = 0.13*	*P = 0.045*

12

Reconstitution of cytomegalovirus (CMV)-specific $CD4^+$ T cells following allogeneic stem cells transplantation – impact of conditioning regimen, GvHD and source of stem cells

B. Pourgheysari, R. Bruton, K. Piper, J. Arrazi & P. Moss
Cancer Research UK Institute of Cancer Studies, University of Birmingham, Vincent Drive, Birmingham B15 2TT, UK

Cytomegalovirus (CMV) infection is one of the main causes of morbidity and mortality following allogeneic stem cell transplantation. Developing the most effective immunotherapy depends on a clear understanding of immune reconstitution following stem cell transplantation. CMV-specific $CD4^+$ T cells are important for the maintenance of the CMV specific CD8 T-cell response and have inherent anti-viral activity. We studied CMV-specific $CD4^+$ T-cell reconstitution following allogeneic stem cell transplantation in a cohort of 36 patients at risk of CMV reactivation. Patients had been treated with a myloablative (48%) or non-myoablative (52%) conditioning regimen for haematological malignancies. CMV antigenaemia was monitored by quantitative PCR. CMV-specific CD4 T cells were identified by intracellular cytokine staining before transplantation and every 2 weeks thereafter. CMV serology of recipient/donor was (+/+) in 61.6%, (+/−) in 23% and (−/+) in 15.4%. All (+/+) patients but only 40% of (+/−) and 25% of (−/+) patients developed CMV-specific $CD4^+$ T cells in the first 60 d post-transplantation. Patients with myeloablative conditioning had a lower frequency of CMV reactivation and lower CMV-specific CD4 numbers compared to the non-myeloablative

Samsung Bioepis Exhibit No. 1002
Page 1398

cohort (average 8000/ml compared with 24 000/ml in the first 100 d). Patients with acute GvHD (56.5%) with or without chronic GvHD had impaired reconstitution in both the first 100 d (8700/ml vs. 18 000/ml) and the second 100 d (13 600/ml compared with 28 300/ml, $P < 0.01$). Patients who had received a graft from a sibling (72%) had a higher CMV-specific CD4 T-cell count compared to patients with an unrelated donor ($P < 0.001$). There was a positive correlation between the absolute number of total $CD4^+$ T cells and the CMV-specific $CD4^+$ T-cell subpopulation. In conclusion, myeloablative conditioning, use of an unrelated donor, poor CD4 reconstitution and GvHD all had a negative impact on CMV-specific $CD4^+$ T-cell reconstitution following allogeneic stem cell transplantation.

Poster Presentation

Haematological malignancies

13

Use of serum-free light chain assays for determining complete remission in patients with multiple myeloma

S. D. Reid,[1] M. T. Drayson,[2] G. P. Mead,[1] B. Augustson,[2] S. Roberts[1] & A. R. Bradwell[2]

[1]The Binding Site Ltd, Birmingham, PO Box 4073, Birmingham; [2]Department of Immunology, The Medical School, University of Birmingham, B15 2TT, UK

Measurement of serum-free light chain (FLC) concentrations has recently been shown to be more sensitive than electrophoresis for the detection and monitoring of non-secretory multiple myeloma and light chain only multiple myeloma (LCMM). After treatment, however, complete response (CR) is currently defined as an absence of paraprotein in the serum and urine by immunofixation electrophoresis (IFE). The aim of this study was to determine whether the serum free κ/λ ratio is a more sensitive measure of CR in LCMM and intact immunoglobulin multiple myeloma (IIMM). FLC concentrations were measured in the sera of 85 patients from the MRC VI and VII Myeloma trials who were determined to have achieved CR by IFE. Of the 85, 31 had LCMM and 54 had IIMM; 11/31 LCMM and 37/54 IIMM patients, had normal serum FLC ratios, in agreement with the IFE results. The remaining 22/31 LCMM patients and 19/54 IIMM patients had abnormal FLC ratios indicating the presence of residual disease. Where available ($n = 44$), overall survival (OS) data, indicated that patients who had abnormal FLC ratios, had shorter OS than those who were IFE negative and had normal FLC ratios (mean OS of 990 vs. 1188 d for LCMM patients and 891 d vs. 1430 d for IIMM). A t-test analysis of the data revealed that the differences reached significance for the IIMM patients ($P < 0.03$) but not the LCMM patients. It has previously been shown that serum FLC assays are more sensitive than urine FLC tests for the determination of CR in LCMM and this increased sensitivity is due, in part, to normal renal function preventing light chains entering the urine. The current study indicates that serum FLC assays also identify residual disease in some IFE negative IIMM patients and that abnormal FLC ratios may be predictive of a reduced OS.

14

The ATR-p53 pathway is suppressed in non-cycling normal and malignant lymphocytes

G. G. Jones, A. R. Pettitt & P. D. Sherrington

Department of Haematology, Royal Liverpool University Hospital, Liverpool, UK

Chronic lymphocytic leukaemia is characterized by the accumulation of apoptosis-resistant clonal B cells arrested in G0/G1. An aggressive form of the disease is identified by an impaired p53 response to ionizing radiation. This is associated with inactivating mutations of either p53 or one of its upstream regulators, ATM. ATR is closely related to ATM but it activates p53 in the presence of DNA adducts of the type induced experimentally by ultraviolet irradiation (UVC). Thus, ATR pathway abnormalities also have the potential to cause p53 dysfunction. To test this theory, CLL cells and normal peripheral blood mononuclear cell (PBMC) were treated with UVC and assessed for p53 responses. Surprisingly, we were unable to detect p53 accumulation, its site-specific phosphorylation or its transcriptional consequences in response to UVC-induced DNA damage. The failure to activate p53 was explained by demonstrating that ATR was absent or undetectable in PBMCs and CLL cells, but readily detectable in lymphocyte cell lines. An obvious difference between the cell lines and the CLL cells/PBMCs was that the former were actively cycling while the latter were quiescent. We therefore hypothesized that the ATR-p53 pathway is selectively down-regulated in non-cycling lymphocytes. To test this, we induced cycling in the T-cell fraction of PBMC preparations and found that ATR protein expression was restored. Furthermore, p53 was up-regulated and phosphorylated in response to UVC in these cells and this response was abrogated by caffeine, an inhibitor of ATR. Our data support the conclusion that the ATR-p53 pathway is suppressed in non-cycling lymphocytes via ATR down-regulation. We tentatively suggest that this repressed DNA damage response may have evolved to protect quiescent lymphocytes from the potential for p53-dependent apoptosis in the face of some forms of endurable genotoxic stress but, as a consequence, it may facilitate the acquisition of potentially lymphomagenic mutations.

15

Evidence for a primitive cell origin of acute lymphoblastic leukaemia

C. V. Cox,[1] R. S. Evely,[2] D. H. Pamphilon,[1,3] N. J. Goulden[3,4] & A. Blair[1,4]

[1]Bristol Institute for Transfusion Sciences; [2]Bristol Haematology and Oncology Centre; [3]Bristol Royal Hospital for Children; [4]Department of Pathology and Microbiology University of Bristol, Bristol, UK

Characterization of ALL cells that proliferate to maintain the disease is essential for the development of targeted treatment regimens. We have used a serum free culture system and the NOD/SCID assay, to determine the phenotype and growth characteristics of ALL progenitors. Cells from 19 patients were sorted for expression of CD34/CD19 and cells from nine patients were sorted for expression of CD133/CD19 then evaluated in culture. The majority of cells at diagnosis were $CD34^+/CD19^+$ ($55 \pm 7\%$) or $CD133^-/CD19^+$ ($65 \pm 8\%$). However, after 6 weeks, the majority of proliferating cells were derived from the $CD34^+/CD19^-$ ($91 \pm 9\%$) and $CD133^+/CD19^-$ ($74 \pm 11\%$) subfractions. Cytogenetic analysis demonstrated the presence of the TEL-AML1 fusion gene in $CD34^+/CD19^-$ cells at sorting and confirmed the cultured cells had aberrant karyotypes. Subsequently we evaluated these sorted cells in the NOD/SCID assay. When cells from 6 patients were sorted for expression of CD34/CD19, engraftment was only detected in recipients of $CD34^+/CD19^-$ cells (3–95% $CD45^+$), using 5×10^4–2×10^5 cells. This sorting strategy leads to a 2-log purification of NOD/SCID leukaemia-initiating cells. Serial transplantation studies demonstrated that $CD45^+$ cells from NOD/SCIDs engrafted with $CD34^+/CD19^-$ cells could engraft secondary recipients, indicating these cells were capable of self-renewal. Engrafted $CD45^+$ cells had the same karyotype and characteristic immunophenotype of the patients' at diagnosis, with high levels of CD34, CD10 and CD19 and no evidence of myeloid engraftment. In the first of five experiments evaluating NOD/SCID repopulation in cells sorted for CD133/CD19, engraftment was achieved with the $CD133^+/CD19^-$ subfraction only (13% $CD45^+$ with 5×10^4 cells). These results demonstrate that ALL

Samsung Bioepis Exhibit No. 1002
Page 1399

cells capable of long term proliferation *in vitro* and NOD/SCID repopulation express CD34 and CD133 and lack expression of CD19. Our findings suggest that, in this group of patients, a primitive haemopoietic cell, rather than a committed progenitor cell was the target for the leukaemic transformation event.

16

Characterization of T-ALL progenitor cells

C. V. Cox,[1] R. S. Evely,[2] N. J. Goulden[3,4] & A. Blair[1,4]

[1]Stem Cell, Bristol Institute for Transfusion Sciences; [2]Department of Haematology, Bristol Haematology and Oncology Centre; [3]Department of Paediatric Oncology, Bristol Royal Hospital for Children; [4]Department of Pathology and Microbiology, University of Bristol, Bristol, UK

The malignant cells in T-cell acute lymphoblastic leukaemia (T-ALL) are thought to arise as a result of a transformation event in cells committed to the T lineage. Leukaemic cells have a limited proliferative capacity, suggesting only a small population have the ability to self-renew and maintain the disease. It is these cells that are most relevant to characterize and target. We have developed a serum free suspension culture (SC) system for the long-term growth of ALL cells and used this in parallel with the *in vivo* NOD/SCID assay to characterize T-ALL progenitor cells. Bone marrow cells from nine patients were sorted for co-expression of CD34 and CD4 and evaluated in SC. Cells at sorting were mainly $CD34^-/CD4^+$ ($33 \pm 7\%$) or $CD34^-/CD4^-$ ($46 \pm 6\%$). However, at weeks 3, 5 and 6, the majority of proliferating cells (>50%)were derived from the $CD34^+/CD4^-$ subfraction. Morphological analyses confirmed these cultured cells had blast morphology. Unsorted cells and cells sorted for the co-expression of CD34 and CD4, from six of these patients, were injected into sublethally irradiated NOD/SCID mice to evaluate their repopulating ability. In each case, engraftment was achieved using 5×10^7–10^7 unsorted cells (2.1–99% $CD45^+$) and in recipients of the $CD34^+/CD4^-$ subfraction only (0.4–89% $CD45^+$ using 1.5×10^4–10^6 cells). $CD45^+$ cells removed from primary NOD/SCIDs, which had been engrafted with $CD34^+/CD4^-$ cells were capable of repopulating secondary recipients with similar levels of engraftment (84–85% $CD45^+$), indicating they were capable of self-renewal. Immunophenotypic analyses of engrafted $CD45^+$ cells revealed high expression levels of CD34, CD4 and CD8 and no evidence of myeloid engraftment. These data demonstrate that ALL cells with long-term repopulating ability express CD34 but lack CD4. Our findings supports the hypothesis that, a primitive haemopoietic cell, was the target for leukaemic transformation and not a cell committed to the T-cell lineage.

17

Red cell autoantigen presentation by chronic lymphocytic leukaemia cells: a new mechanism underlying autoimmune disease

A. M. Hall, E. McLeod, R. N. Barker & M. A. Vickers

Department of Haematology and Medicine, University of Aberdeen, Aberdeen, UK

Chronic lymphocytic leukaemia (CLL) is frequently associated with autoimmune mediated disease, including haemolytic anemia (AIHA). Since these phenomena target constituents of the blood, which are broken down in close proximity to the malignancy, we hypothesized that CLL cells predispose to autoimmunity by acting as aberrant antigen presenting cells (APC). As a first step to testing this proposal, it was confirmed that all patients (7/7) with AIHA secondary to CLL harbor activated Th cells specific for epitopes on the dominant red blood cell (RBC) autoantigens in primary AIHA, the Rh proteins. Rh specific Th cells were also detected in a number of CLL patients who, although they did not suffer AIHA, had low levels of anti-RBC antibody in their serum. Fractionation of putative APC types from peripheral blood of patients by negative selection showed that $CD5^+$ CLL cells are the most effective population in processing and presenting purified Rh protein to autoreactive Th cells. This ability was confirmed using positively selected $CD5^+$ CLL cells. The study provides the first example of malignant cells driving an autoimmune response by acting as aberrant APC. This work describes a series of complex fractionation experiments coupled with several T helper cell assays to determine which cell types are most efficient at presenting autoantigen. Although CLL cells are inefficient at presenting other antigens, they comprise the major red cell autoantigen-presenting cell, providing an explanation for why autoimmune phenomena in CLL are restricted to constiuents of the blood.

18

Protein expression profiling confirms the close association between CLUS and CLL

F. L. Bennett, A. J. Dickinson, S. J. M. O'Connor, D. Gonzales, J. A. Fenton, M. J. Cullen, A. S. Jack, P. Hillmen & A. C. Rawstron

HMDS, Leeds General Infirmary, Leeds, UK

CLUS, defined as the presence of monoclonal CLL-phenotype (CD19+CD5+CD23+CD20wkCD79wk) lymphocytes at levels below the diagnostic criteria for CLL, is found in 5–6% of healthy adults over 60. The aim of this study was to determine whether it is possible to distinguish between CLUS and B-CLL on the basis of an extensive protein expression profile. Normal range full blood count samples were assessed from 211 male and 308 female patients with no history/suspicion of malignancy (median age 69, range 56–87). Samples were screened using CD19/CD5/κ/λ to assess clonality, of which 36/519 (6.9%) showed a $\kappa{:}\lambda$ ratio >5:1 or <0.2:1 in either total B-cell fraction or in the $CD19^+$ $CD5^+$ B cells. The samples were then assessed using CD19/CD5/CD20/CD79b, which identified CLUS in 23/36 and a non-CLL phenotype in 13/36. Samples from normal individuals ($n = 9$) and from patients with CLUS ($n = 8$), CLL ($n = 8$), or other B-cell disorders ($n = 26$) were assessed with a panel of 24 antibodies previously identified as potential markers to differentiate CLL from other normal/neoplastic B cells. Protein expression, assessed as geometric mean fluorescence intensity, was recorded for the $CD19^+5^+$ fraction in CLUS samples or for total $CD19^+$ fraction in the remaining samples. Hierarchical cluster analysis was performed using dChip software to determine the association between CLUS, CLL, normal B cells and other B-cell malignancies. Using an unsupervised analysis and excluding markers classically used to diagnose CLL (CD5, CD20, CD23 and CD79b), CLUS B cells and CLL cells formed a single cluster distinct from normal B cells and other B-cell malignancies. CLL and CLUS cells showed differential expression of CD24, CD81, CD82, and CXCR5 ($P < 0.05$). These markers are being tested on a larger series to determine whether they may provide prognostic information. Even excluding basic diagnostic markers, the close relationship between CLUS and CLL is confirmed by their protein expression profile.

19

The proliferating cells in CLL have a unique immunophenotype

P. Moreton, P. Hillmen & A. S. Jack

Haematological Malignancy Diagnostic Service, Leeds Teaching Hospitals NHS Trust, Leeds, UK

Proliferation centres (PC) exist in the lymph nodes and bone marrow in CLL. The morphology of these structures suggests that they are the source of cell proliferation. CLL cell survival and proliferation has

Samsung Bioepis Exhibit No. 1002
Page 1400

been reported to be influenced by a variety of cell types in culture systems, however, it is not known whether such signals are responsible for cellular proliferation *in vivo*. PCs may be the *in vivo* correlate of these experiments. Oct-2, Bob-1 and MUM-1 are transcription factors that are upregulated following stimulation with CD40 ligand and interleukin 4 in normal B cells. We hypothesized that MUM-1, Bob-1 and Oct-2 upregulation in PCs would suggest that accessory signalling such as that via CD40 ligation and interleukin 4 is actively occurring within PCs. Furthermore, accessory cells suggested by the *in vitro* data would be expected to be present: CD68 positive nurse-like cells, CD21 positive follicular dendritic cells, and CD57 positive germinal centre type T cells. Bcl-6 and CD10 were used to compare PCs to the phenotype of normal germinal centres. We analysed 53 lymph nodes and 35 bone marrow trephines for the presence of these antigens by immunohistochemistry. PCs were present in all lymph nodes and in 21 (60%) bone marrows. PCs exclusively expressed the immunophenotype: MUM-1 +ve, Oct-2 +ve, Bob-1 +ve, Ki-67 +ve, and CD71 +ve. The accessory cells present in all PCs were as follows: CD68 positive cells were found in a dendritic pattern in contact with the MUM-1 +ve CLL cells; CD57 +ve T cells were distributed evenly. PCs did not express CD21, Bcl-6 or CD10. The PC phenotype was equally prevalent amongst somatically hypermutated and non-mutated cases. This unique PC immunophenotype suggests that the most critical signals occurring within PCs are CD40 ligation and interleukin 4, and that CD68 +ve and CD57 +ve accessory cells are involved in this signalling.

20

Analysis of IgH rearrangements in class-switched IgG+ B-CLL

D. Gonzalez[1], A. C. Rawstron[2], S. J. Richards[2], G. J. Morgan[1] & J. A. L. Fenton[1]

[1]*Academic Unit of Oncology and Haematology, University of Leeds;* [2]*HMDS, Leeds Teaching Hospital NHS Trust, Leeds, UK*

B-CLL cells normally express a low amount of IgM on the surface, although IgG or IgA can instead be expressed following class-switch recombination. We have investigated the pattern of IgH mutations and gene segment usage in a series of 35 IgG+ B-CLL patients. Successful PCR amplification of recombined Sμ–Sγ switch regions was achieved in 33 patients confirming the presence of IgG+ class-switched B-CLLs. Mutational analysis of IgH genes revealed 29 (83%) contained more than 2% of somatic hypermutation (SHM). For IgH gene segment usage, a significant predominance of the VH4 family (51%) was observed, followed by VH3 (40%), with VH1 only used in two samples. VH4-34 was the most frequently used gene segment (29%), followed by VH3-07 (14%) and VH4-39 (11%). DH6-13 was the most frequently used DH segment (20%), followed by DH2-02 and DH2-15 (both 11%). JH gene segment usage did not differ from normal B cells, JH4 being the most frequently used, followed by JH6 and JH5. There was a significant association between DH6-13 and JH5 in three samples containing an unmutated VH4-39 rearrangement, this could be indicative of a different clonal history of these particular B cells in B-CLL. This data reveals a distinct pattern of IGH rearrangements in IgG+ B-CLL compared to classical IgM+ B-CLL. First, the rate of SHM in IgG+ B-CLL is significantly higher than the 50% observed in IgM+ B-CLL, probably due to a more mature origin of the clonotypic B-CLL in the IgG+ subgroup. Secondly, VH segment usage pattern differs with a significant underrepresentation of VH1 and an overrepresentation of VH4 family members in the IgG+ group. We conclude that class-switched IgG+ B-CLL contains different molecular features in the IgH genes compared with classical IgM+ B-CLL, the clinical implications of these differences should be further investigated.

21

Relationship between p53 dysfunction and karyotypic abnormalities in chronic lymphocytic leukaemia

A. Carter,[1] A. Burford,[2], M. Atherton,[3] K. Pearson,[3] A. Douglas,[3] R. Morilla,[2] P. D. Sherrington,[1] D. Catovsky,[2] E. Matutes[2] & A. R. Pettitt[1]

[1]*Department of Haematology, Royal Liverpool University Hospital, Liverpool;* [2]*Department of Haematology and Cytogenetics, Royal Marsden Hospital, London;* [3]*Department of Cytogenetics, Liverpool Women's Hospital, Liverpool, UK*

Adverse prognostic factors in CLL include deletion of p53 at 17p13, deletion of 11q23, trisomy 12, and p53 dysfunction due to inactivation of p53 or ATM – a kinase required for p53 activation in response to double-strand DNA breaks. p53 deletion is usually associated with point mutations of the remaining p53 allele and overexpression of mutant p53 protein owing to its prolonged half-life. Intriguingly, the minimum deleted region at 11q23 includes the ATM gene. Furthermore, MDM2 – the main physiological inhibitor of p53 – is encoded on chromosome 12. This suggests that suppression of the ATM-p53 pathway might be a shared mechanism through which each of the adverse chromosomal abnormalities in CLL could exert their deleterious effects. To examine this possibility, we probed the functional integrity of the ATM-p53 pathway in 129 CLL samples of known karyotype by measuring by flow cytometry the accumulation of p53 and p21 (a transcriptional target of p53) in response to ionizing radiation (IR). Compared with the group with a favourable karyotype ($n = 71$), the group with del 17p13 ($n = 20$) had markedly elevated basal p53 levels ($P < 0.001$) and showed a smaller proportional increase in both p53 ($P = 0.001$) and p21 ($P < 0.001$) in response to IR. The group with trisomy 12 ($n = 19$) also had increased basal p53 levels ($P = 0.005$) and showed a reduced proportional increase in p53 ($P = 0.0142$) but not p21. In contrast, the group with del 11q23 ($n = 19$) showed no significant impairment of IR-induced p53 or p21 accumulation despite having slightly increased basal p53 levels ($P = 0.011$). These findings indicate that del 17p13, del 11q23 and trisomy 12 are associated with distinct forms of p53 deregulation. Furthermore, the wide variation in basal p53 levels and IR-induced p53/p21 accumulation observed between individual patients within each cytogenetic group suggests that p53 functional analysis and karyotyping may provide complimentary prognostic information.

22

Cytomegalovirus (CMV) infection in patients treated with alemtuzumab and other non-transplant therapies – a manageable complication with early detection by pre-emptive screening

A. Sah,[1] P. Moreton,[1] P. E. Klapper,[2] A. D. Hale[2] & P. Hillmen[1]

[1]*HMDS, Leeds Teaching Hospitals NHS Trust;* [2]*Health Protection Agency, Leeds, UK*

CMV reactivation is well recognized following alemtuzumab therapy but not after other therapies for lymphoproliferative disorders (LPDs). We report 17 patients with LPD who developed CMV reactivation following non-transplant therapies. Twelve had chronic lymphocytic leukaemia (CLL), two had systemic marginal zone lymphoma (MZL), one each had angioimmunoblastic lymphoproliferative disorder (AILD), diffuse large B-cell lymphoma and mantle cell lymphoma. CMV reactivation occurred whilst receiving alemtuzumab in 14, fludarabine/cyclophosphamide in two and CHOP in one. Thirteen (76%) experienced fever, five (29%) hypoxia and eight (47%) cough. Six (35%) had radiological abnormalities. The median level of CMV DNA detected by quantitative PCR assay was 27 600 copies/ml (range 850–320 000). Sixteen (94%) received

Samsung Bioepis Exhibit No. 1002
Page 1401

intravenous ganciclovir. Five (29%) of those treated were resistant to ganciclovir, and three subsequently received foscarnet. Both patients who died on first-line ganciclovir were symptomatic prior to the diagnosis of CMV reactivation. The remainder (87%) had resolution of all evidence of reactivation. Eleven alemtuzumab patients had CLL, two had systemic MZL and one had AILD. Pre-emptive monitoring for CMV DNA in patients receiving alemtuzumab was introduced following the CMV-related death of a patient. The CMV reactivation rate was 11/91 (12%) CLL patients receiving alemtuzumab; 4/14 patients receiving alemtuzumab developing CMV reactivation were asymptomatic. CMV reactivation occurred a median of 5 weeks (range 2–8) after the start of therapy. Alemtuzumab was stopped in nine (64%) during CMV therapy, and restarted in seven after successful CMV eradication. All patients who developed CMV reactivation after pre-emptive screening was instituted during alemtuzumab were effectively treated. Alemtuzumab can be continued during asymptomatic CMV reactivation. The consideration of CMV reactivation following both alemtuzumab and other non-transplant therapies for LPDs, such as CHOP or purine analogues, is important. Pre-emptive screening for CMV reactivation during alemtuzumab permits the safe and effective management of this complication.

23

Routine analysis of IgV_H mutational status in CLL patients using BIOMED-2 standardized primers and protocols

C. Matthews,[1,2] M. A. Catherwood,[1] T. C. M. Morris[1] & H. D. Alexander[12]

[1]Department of Haematology, Belfast City Hospital, Belfast; [2]School of Biomedical Sciences, University of Ulster, Coleraine, UK

Although previously considered as a unique entity, a body of evidences has emerged indicating the biological heterogeneity of chronic lymphocytic leukaemia (CLL). Therefore a technique, suitable for routine use, that helps to discriminate between indolent and aggressive disease, would allow rational planning of CLL patient management. Current methods for the detection of IgV_H mutational status in CLL, which identifies two subgroups of patients with significantly different outcomes, are laborious, expensive and do not lend themselves to a routine diagnostic setting. With the introduction of BIOMED-2 primers, a rapid protocol is now available. This study used the protocol to examine DNA from 100 CLL patients. Conventional methods using RNA, and FISH analysis for recurring chromosomal abnormalities, were carried out on 10 and 60 of these patients respectively. This was designed to examine the reliability of the BIOMED-2 method and seek to confirm reported associations between unmutated IgV_H genes and chromosomal abnormalities associated with poor outcomes. There was complete concordance between the BIOMED-2 protocol and the RNA based method, in both mutational status and gene usage. Unmutated IgV_H genes showed association with 17p and 11q23 deletions, and trisomy 12, linked with poor and intermediate outcomes respectively. Abnormalities at 14q32 breakpoint were also observed in several patients, predominately with unmutated IgV_H genes. No surrogate marker for IgV_H mutational status has so far shown 100% concordance, there are technical complexities with the methods used and no gene usage information, which has been shown recently to be associated with prognosis in some populations, is available with this approach. This study demonstrates that IgV_H mutational status can be determined rapidly by the BIOMED-2 protocol and can be adopted as a clinical test in reference centres, for all patients presenting with CLL, obviating the need for detection of surrogate markers.

24

Detection of p53 dysfunction in chronic lymphocytic leukaemia cells: a comparison of Western blotting and flow cytometry

A. Carter, K. Lin, P. D. Sherrington & A. R. Pettitt

Department of Haematology, Royal Liverpool University Hospital, Liverpool, UK

Although p53 mutation/deletion occurs in only 10–15% of patients with CLL, we have shown that functional impairment of the p53 pathway is present in a further 15–20% of patients due to inactivating mutation of ATM – a kinase that phosphorylates p53 in response to double-strand DNA breaks. Both types of p53 dysfunction are readily detectable using a simple functional assay, in which CLL cells are exposed to ionizing radiation (IR), cultured overnight and examined by Western blotting for levels of p53 and p21 – a transcriptional target of p53 (*Blood* 2001;98:814). In the type A defect associated with p53 mutation, baseline p53 levels are increased (mutant p53 has a longer half-life than the wild-type protein), and IR-induced p21 accumulation is reduced. In the type B defect associated with ATM mutation, IR-induced accumulation of both p53 and p21 are reduced but there is no baseline overexpression of p53. In a series of 71 patients from the Royal Liverpool University Hospital, p53 functional analysis was found to be more effective than IgVH mutational status at predicting adverse clinical outcome [*Blood* 2002;100:1404]. To make the test more applicable to the routine clinical setting, we have evaluated a flow cytometric technique using 21 patient samples that had been previously analysed by Western blotting. In 20 of the 21 cases, the two methods gave identical results, with 12 cases showing no p53 dysfunction, five the type A defect and three the type B defect. One case, characterized as equivocal by Western blotting, was shown by flow cytometry to contain a subpopulation of cells with the type A defect, while the remaining cells displayed a normal response. Flow cytometry therefore appears to be superior to Western blotting since it can identify p53-dysfunctional sub-clones. Furthermore, double staining with CD19 allows CLL cells to be analysed separately from T cells.

25

The acquired CD40 ligand deficiency observed in patients with B-CLL is reversible and due to soluble leukaemia derived factor(s)

A. G. S. Buggins, J. Richards, G. J. Mufti & S. Devereux

Leukaemia Sciences, Department of Haematological Medicine, Kings College Hospital and GKT School of Medicine, London, UK

Patients with B-CLL have an acquired defect in T-cell CD40L expression, which contributes to the immunodeficiency characteristic of the disease. In this study we have investigated whether this is due to soluble leukaemia derived factor(s) of the type that we have previously shown to inhibit T-cell function in other leukaemias. Normal resting CD45RA positive and negative cells upregulated CD40L expression from a mean of 2% and 0% to 87.5% and 73% respectively in response to PMA and ionomycin ($n = 4$). Upregulation was reduced to a mean of 28.5% ($P < 0.0001$) and 54% (ns) in $CD4^+$ $45RA^+$ and $CD4^+$ $45RA^-$ T cells from four patients with B-CLL. Removal of T cells from the malignant clone by CD3 selection had a dramatic effect on CD40L upregulation, which returned to normal in both $CD45RA^+$ and $CD45RA^-$ CD4 subsets (16–66% and 5–57% before and after purification). This data indicates that the acquired CD40L deficiency in patients with B-CLL is determined by the presence of the malignant clone and is reversible. To investigate whether this phenomenon is due to cell contact or soluble mediators, B-CLL supernatants were incubated with normal donor T cells for 48 h and then stimulated for 4 h with PMA and Ionomycin. When compared with medium alone,

Samsung Bioepis Exhibit No. 1002
Page 1402

B-CLL supernatant reduced CD40L upregulation by a mean of 50.2% (range 14–123, $n = 14$, $P < 0.0005$). These findings indicate that the acquired CD40L deficiency observed in patients with B-CLL is reversible and due at least in part to soluble mediator(s) secreted by the leukaemic cells. This has important implications for the design of therapeutic strategies in B-CLL and underlines the fact that immunotherapy will be most effective in the context of minimal residual disease.

26

Do we need to monitor MGUS patients indefinitely?

A. C. Rawstron, J. A. L. Fenton, D. Gonzalez, S. J. M. O'Connor, A. M. Dring, R. G. Owen, S. J. Richards, F. E. Davies, J. A. Child & G. J. Morgan

HMDS, Leeds General Infirmary, Leeds, UK

MGUS is a predominantly benign disorder, and although approximately 1% of MGUS patients progress to myeloma per year, all patients are monitored indefinitely. The aim of this study is to determine an objective means of identifying the risk of progression from presentation features. Lack of CD19 expression reportedly identifies neoplastic plasma cells; however using four-colour flow cytometry approximately 15% of plasma cells in normal adult bone marrow were $CD19^-$, polyclonal, with high BCL-2 and low APO-1/Fas expression relative to $CD19^+$ plasma cells. This suggests that they represent a long-lived subpopulation, which we confirmed by longitudinal analysis of plasma cells in alemtuzumab-treated CLL patients. Myeloma patients in complete remission with normal immunoglobulin levels have only $CD19^+$ plasma cells for several years after high-dose therapy, suggesting a separate pathway for generation of $CD19^-$ plasma cells. We next used flow cytometric protein expression profiling with a panel of 66 antibodiesto identify markers could differentiate normal long-lived from neoplastic $CD19^-$ plasma cells. In combination with analysis of intraclonal variation and FISH-karyotyping, it was possible to demonstrate that the paraprotein in some MGUS patients is produced by an expansion of phenotypically and karyotypically normal long-lived plasma cells showing intraclonal variation consistent with a normal immune response. Analysis of 88 MGUS patients demonstrated that disease progression was virtually restricted to those with only $CD19^-$ neoplastic plasma cells whilst patients with normal plasma cells (either with or without a co-existing population of neoplastic plasma cells) had predominantly stable or regressing disease (26% vs. 2% progressive features at a median 31 months follow-up, $P < 0.0001$). The results have significant implications for studies into the initiating events in myeloma and MGUS. High-risk MGUS may be identified by a relatively simple analysis at presentation, whilst a significant proportion of the remaining MGUS patients do not have a haematological disorder.

27

The natural history of 'early' CLL

A. C. Rawstron, R. de Tute, S. J. M. O'Connor, S. J. Richards, A. S. Jack & P. Hillmen

HMDS, Leeds General Infirmary, Leeds, UK

CLL patients are increasingly diagnosed as an incidental finding. Furthermore, cells that are indistinguishable from CLL are detected in 3% of otherwise healthy adults. The aim of this study was to determine whether the detection of CLL cells below the levels required for a formal NCI-criteria diagnosis has any clinical relevance. During the period 1995–2000, 2228 blood samples were referred to the HMDS laboratory with a lymphocytosis or suspected B-cell disorder. A total of 1340 samples had a monoclonal $CD5^+23^+$ B-cell lymphocytosis with a typical CLL phenotype (i.e. weak surface immunoglobulin/CD20/CD79b/CD22) of which 344 (26%) had an absolute B-cell count below 5.0×10^9/l. We have assessed the outcome in a cohort of 150 of these patients who do not meet the NCI criteria for CLL. The median age at presentation was 73 years (range 42–96) with a slight female preponderance (male:female ratio 0.9:1). The presenting B-cell count was a median 3.1×10^9/l (range 0.3–5.0) with 44/150 (29%) having an absolute T-cell lymphocytosis (>2.7×10^9/l). At a median of 3.7 years (range 0.5–8.2) follow-up, 18/150 (12.0%) showed gradually decreasing levels of CLL cells, 59/150 (39.3%) had stable levels below 10×10^9/l, 11/150 (7.3%) had variable levels, whilst 62/150 (41.3%) had increasing levels with time. Patients progress to a lymphocyte count over 10×10^9/l after a median of 6 years, with 8/150 patients (5.3%) requiring treatment for progressive disease. CD38 expression did not predict poor prognosis in these patients, although relatively few (11/150, 7.3%) had >30% expression on the CLL cells. Of note, 7/150 patients showed bi-phasic progression kinetics, suggestive of a second oncogenic event. As such, longitudinal analysis of 'early' CLL may help our understanding of the events responsible for disease progression. Monitoring of these patients is clinically relevant as they progress to a level requiring treatment with an unpredictable disease course.

28

Soluble syndecan-1 modulates myeloma growth but not through FGF signalling

R. A. Lovell,[1,2] S. E. Guimond,[3] J. E. Turnbull,[3] P. A. H. Moss[2] & G. Pratt[12]

[1]Birmingham Heartlands and Solihull NHS Trust; [2]Institute for Cancer Studies, University of Birmingham, Birmingham; [3]School of Biological Sciences, University of Liverpool, Liverpool, UK

The serum level of syndecan 1 is an independent prognostic marker in multiple myeloma and soluble syndecan promotes tumour growth in a murine myeloma model. Syndecans are cell surface proteoglycans that can be actively shed and consist of a protein core with attached polysaccharide chains. Soluble syndecan-1 could influence myeloma biology by altering adhesion and by modulating the function of heparin binding molecules. Fibroblast growth factor (FGF) signalling is crucially dependent on the saccharide sequences of proteoglycan. In myeloma a stromal-tumour cell paracrine network between FGF2 and IL-6 has been described and 15% of myeloma cases have a t(4;14) translocation with overexpression of fibroblast growth factor receptor 3 (FGFR3). This study looked at the effects of soluble syndecan 1 on myeloma cell lines and FGF signalling. Soluble syndecan-1 was purified using a DEAE method from the supernatant of myeloma cell lines and levels measured using a commercial ELISA kit. Coomassie Blue staining of the extract showed a single band consistent with syndecan-1. BaF cells are a murine B-cell line that when transfected with an FGFR act as an assay for FGF signalling. Four myeloma cell lines that express no FGFRs, wild type or mutated FGFR3 were also examined. Cell growth was determined after incubation with syndecan-1 using a fluorescent method involving Alamar BlueTM. Soluble syndecan-1 did not modulate FGFR signalling in FGFR transfected BaF cells in direct contrast to heparin. In contrast, syndecan-1 does influence the proliferation of myeloma cell lines even in the absence of FGF2. The results were dose dependent with inhibition seen at the highest concentrations but enhanced proliferation at lower concentrations. Soluble syndecan-1 modulates the activity of myeloma cell lines but this effect does not act via FGF signalling in cell lines expressing FGFR3. By generating modified proteoglycans previous studies have identified a number of neoglycans with anti-cancer properties, which may be relevant to the treatment of myeloma.

Samsung Bioepis Exhibit No. 1002
Page 1403

29

Bcl-2 phosphorylation and up-regulation of Mcl-1 contribute to the drug resistant phenotype of B-CLL cells that survive *in vitro* exposure to chemotherapeutic drugs

A. Morgan,[1] D. P. Bentley,[1] C. D. Fegan[2] & C. J. Pepper[1,3]
[1]*Department of Haematology, Llandough Hospital, Penlan Road, Penarth, Vale of Glamorgan, CF64 2XX;* [2]*Department of Haematology, Heartlands and Solihull NHS Trust, Bordesley Green East, Birmingham, B91 1JR;* [3]*Department of Haematology, University of Wales College of Medicine, Heath Park, Cardiff, CF14 4XN, UK*

The drug resistance encountered in B-cell chronic lymphocytic leukaemia (B-CLL) is often pleiotropic and as such indicates the development of a fundamental inability to appropriately induce apoptotic cell death. Furthermore, it appears that treatment accentuates this drug resistance profile. In this study we investigated the protein expression pattern of Bcl-2 family and p53 in viable and apoptotic subpopulations of B-CLL cells following exposure to chemotherapeutic drugs. Lymphocytes from 32 patients with B-CLL (18 stage A, four stage B and 10 stage C) were cultured for 24 and 48 h with clinically relevant concentrations of chlorambucil or fludarabine. All the samples tested showed a time and dose-dependent increase in apoptosis with the previously treated patient group ($n = 13$) demonstrating a markedly higher mean LD50 value for both chlorambucil and fludarabine when compared with the untreated patient group ($P = 0.01$ and $P = 0.007$ respectively). The apoptotic cells showed a consistent reduction in Bcl-2 and Mcl-1 protein expression but the viable cells showed no significant difference in the total Bcl-2 protein expression ($P = 0.76$). However, there was a marked increase in the phosphorylation of Bcl-2 on serine residue 70 in viable cells following exposure to fludarabine ($P < 0.0001$). Similarly, we found no increase in total p53 protein expression ($P = 0.54$) but a significant increase in phosphorylated p53 (serine 15) in both viable and apoptotic cells exposed to fludarabine ($P = 0.02$ and $P = 0.003$ respectively). In contrast, total Mcl-1 protein expression was found to be up-regulated in viable cells following exposure to drug ($P = 0.008$). Taken together these results indicate that post-translational phosphorylation of Bcl-2 coupled with increased Mcl-1 expression points to a complex cellular response to chemotherapeutic drugs that is likely to contribute to the development of drug resistance in surviving sub-clones of B-CLL cells.

30

CD38$^+$ and CD38$^-$ B-CLL subsets from patients with bimodal expression of the CD38 antigen exhibit distinct cell cycle distribution and Bcl-2 family protein expression

R. Hambley,[1] T. Payne,[2] H. Taylor,[2] C. D. Fegan,[3] K. I. Mills[1] & C. J. Pepper[1]
[1]*Department of Haematology, University of Wales College of Medicine, Heath Park, Cardiff, CF14 4XN;* [2]*Department of Haematology, Llandough Hospital, Penlan Road, Penarth, Vale of Glamorgan, CF64 2XX;* [3]*Department of Haematology, Heartlands and Solihull NHS Trust, Bordesley Green East, Birmingham, B91 1JR, UK*

CD38 expression has been shown to have prognostic significance in B-CLL but to-date the biological rationale for this remains obscure. It might be postulated that if CD38 expression were a marker of poor prognosis in B-CLL then the clones that express this antigen would have a survival advantage, a proliferation advantage or both. Therefore, this study was designed to investigate the biological characteristics of CD38$^+$ and CD38$^-$ subclones derived from B-CLL patients. Twenty-five B-CLL patients with bimodal expression of the CD38 antigen were analysed for *ex vivo* protein expression of Bcl-2, Mcl-1, Bax, Bag-1, Bid and Ki-67 as well as cell cycle analysis. In addition, *in vitro* sensitivity to fludarabine was investigated in CD38$^+$ and CD38$^-$ populations. We found that Bcl-2 and Mcl-1 were significantly higher in the CD38$^+$ subclones in every patient ($P = 0.05$ and $P = 0.002$ respectively). None of the other proteins showed significant differences in mean protein expression between the two subsets although there was a trend towards a higher percentage of cells showing Ki-67 expression in the CD38$^+$ subclones. Cell cycle analysis corroborated this by showing elevated numbers of CD38$^+$ cells in S-phase and G2/M when compared with the CD38$^-$ cells from the same patient. The *in vitro* sensitivity assay gave variable results with some CD38$^+$ cells showing increased sensitivity to fludarabine whilst others showing increased resistance. The explanation for this is unclear but is the subject of on-going investigations in our laboratory. Taken together these data suggest that CD38 expression may be a poor prognostic marker in B-CLL because of both an increased anti-apoptotic phenotype and a higher proliferative index in the CD38$^+$ subclones. Insights gained about the biology of CD38-expressing B-CLL cells may yield mechanism-based strategies for disease control and provide novel targets for drug development.

31

Primary central nervous system lymphoma (PCNSL): a single centre experience of 55 unselected cases

D. J. Hodson,[1] K. M. Bowles,[1] S. L. Klager,[1] J. W. Grant,[2] M. Williams[3] & R. E. Marcus[1]
[1]*Department of Haematology, Addenbrookes Hospital;* [2]*Department of Histopathology, Addenbrookes Hospital;* [3]*Department of Clinical Oncology, Addenbrookes Hospital, Cambridge, UK*

The outcome of 55 consecutive patients diagnosed with PCNSL at a single centre is presented. Median age was 64 years (32–82). At diagnosis 45% of patients were considered unfit to receive treatment with high dose methotrexate (HDMTX). This was due to poor performance status or comorbidity. These patients had a median survival of just 46 d. The remaining patients were treated with one of three regimens containing HDMTX: CHOD/BVAM, MBVP or methotrexate and procarbazine. Patients who received at least one cycle of a chemotherapy regimen containing HDMTX had a median survival of 31 months; 40% did not complete planned chemotherapy due to toxicity, disease progression or death. The median survival of patients treated with HDMTX aged 60 years and older versus under 60 years was 26 vs. 41 months ($P = 0.07$). Younger patients treated with HDMTX who achieved a complete remission with chemotherapy had a median survival of 56 months. There was no difference in outcome between the three different treatment regimens although there was a trend to greater toxicity with CHOD/BVAM. We identified a high incidence of functional disability amongst survivors. This was due to a combination of the tumour itself, the neurosurgical procedure required for diagnosis and the late neurotoxicity of radiotherapy. Of 55 patients diagnosed with PCNSL only one recovered sufficiently to return to work. In conclusion the treatment of PCNSL is associated with significant toxicity both early and late. Further attempts to improve treatment should address mechanisms to reduce this toxicity. In particular the benefit of radiotherapy in patients who achieve complete remission with HDMTX will remain uncertain until it is addressed in a multicentre, randomized trial.

32

Clinical, immunophenotypic and cytogenetic features of 'unclassified', t(11;14) negative, CD5$^+$ B-cell leukaemia

N. Parry-Jones,[1] E. Matutes,[1] V. Brito-Babapulle,[1] A. Burford,[1] S. Maravelaki,[1] R. Morilla,[1] A. C.Wotherspoon[2] & D. Catovsky[1]
[1]*Academic Department of Haematology and Cytogenetics, The Royal Marsden Hospital and Institute of Cancer Research;* [2]*Department of Histopathology, The Royal Marsden Hospital, London, UK*

We studied 22 patients with CD5$^+$ B lymphocytosis unclassifiable by current criteria. Median age was 61 years and M:F 3:1. Fifty-two

Samsung Bioepis Exhibit No. 1002
Page 1404

per cent had splenomegaly and 45% lymphadenopathy; one had extranodal disease. Median Hb was 13.3 g/dl, WBC 22.4 × 10^9/l, lymphocytes 19.4 × 10^9/l and platelets 223 × 10^9/l. Lymphocytes were heterogeneous in size with moderate amounts of basophilic cytoplasm. Many had shallow clefts and small nucleoli; some had clumped chromatin. Immunophenotypic CLL scores (expected 4 or 5 in typical CLL) were 1 in 15 cases and 2 in seven cases; 6 were $CD23^+$ and 13/19 were $CD38^+$. All were negative by FISH for t(11;14) and t(14;18), thus excluding mantle cell lymphoma (MCL) and follicular lymphoma. Two cases had t(14;19), a rare translocation in lymphoproliferative disease. Histology was available in 15 including bone marrows (11), lymph nodes (five) and spleens (three). Cyclin D1 was negative in all cases. Molecular cytogenetics showed trisomy 12 in 41% and deletions at 13q14 (36%), 6q21 (13%), 11q23 (4.5%) and 17p13 (*TP53* locus) in 18%. Follow up was available in 20 patients (median 15 months; range 2–129); three have died 2, 65 and 129 months after diagnosis. Nine have not required treatment (median follow up 13 months; range 3–49). Two patients were splenectomized at diagnosis; one has not required further therapy for 5.5 years and the other remained treatment-free for 4 years. The remaining nine patients received CHOP (three), chlorambucil (four), FCM (one) and one patient who had simultaneous Hodgkin lymphoma had alternating ABVD/FCR. The clinical evolution, morphology, immunophenotype and histology of these cases do not fit with either MCL or CLL, and are unclassifiable by current criteria. They may represent a distinct form of B-cell leukaemia, or a very atypical form of CLL; gene expression studies may help define this group further.

33

A randomized study (WOSMM1) comparing the oral regimen Z-Dex with VAD as induction therapy for newly diagnosed patients with multiple myeloma

G. Cook,[1] R. E. Clark,[2] C. M. Morris,[3] M. Robertson,[4] N. P. Lucie,[5] S. Anderson,[6] J. Paul[6] & I. M. Franklin[5]

[1]St James's University Hospital, Leeds; [2]Royal Liverpool Hospital, Liverpool; [3]Belfast City Hospital, Belfast; [4]Royal Alexandra Hospital, Paisley; [5]North Glasgow University Hospitals Truts, Glasgow; [6]Clinical Trials Office, Beatson Oncology Centre, Glasgow, UK

Whilst infusional vincristine, adriamycin with dexamethasone is an effective treatment for patients with mulitple myeloma (MM) the administration via a central line is associated with considerable morbidity. The oral induction regimen, Z-Dex (Idarubicin & Dexamethasone) has been shown to be efficacious in MM and we therfore conducted a randomized study to compare Z-Dex with VAD as induction therapy in newly diagnosed patients with MM. One hundred ans six patients with a median age of 56 years (37–73) and Durie-Salmon stage II/II MM were randomized to receive 4–6 cycles of therapy. Grade 3/4 neutropenia occurred in 56% of the Z-Dex group compared with 42% in the VAD group ($P = 0.009$) although more infections were seen in the VAD group ($P = 0.001$). Central line complications were reported in 37 patients on 56 cycles, primarily due to infection. The response to therapy in evaluable patients was: CR/PR-VAD 74% cf. Z-Dex 58% with an estimated difference in response of 16% (95% CI–2–33%, $P = 0.075$). Forty-four patients have died: disease progression (Z-Dex $n = 13$, VAD $n = 10$), regimen-related toxicity (Z-Dex 2, VAD n2), infection (Z-Dex, $n = 0$; VAD, $n = 3$) and other causes (Z-Dex, $n = 10$; VAD, $n = 4$). No significant difference in overall and progression-free survival was demonstrated between the two groups. B2MG was the only progostic factor to influence the outcome from therapy. The data from this study indicates that Z-Dex is efficacious in MM, similar to VAD. Z-Dex demonstrates significant myelotoxicity although hospital admissions were more pronounced with VAD. Therefore Z-Dex may be a suitable alternative induction regimen, which avoids alkylating agents (stem cell toxicity potential) and vinca-alkaloids (neurotoxicity may prejudice thalidomide use) in newly diagnosed patients although definitive evidence of non-inferiority is not provided from this study.

34

Zap-70 expression in CLL. Correlation with clinical and biological features

I. Del Giudice, E. Matutes, A. Morilla, R. Morilla, A. Burford, S. Maravelaki, N. Osuji, K. Owusu-Ankomah, V. Brito-Babapulle & D. Catovsky

Academic Department of Haematology and Cytogenetics, Royal Marsden Hospital and Institute of Cancer Research, London, UK

The expression of the tyrosine kinase ZAP-70 in CLL cells has been proposed as a surrogate marker for the mutational status of immunoglobulin VH genes. Its overexpression has been suggested to be of prognostic value for shortened progression-free and overall survival. There is little information about correlation with genetic abnormalities. We analysed ZAP-70 expression using four colour flow cytometry and correlated it with stage, morphology, CD38 expression and chromosomal abnormalities detected by FISH in 142 CLL patients. Less than one third were ZAP-70 positive (+) (cut-off > 20% of $CD5/CD19^+$ cells). ZAP-70 positivity was associated with higher M:F ratio (see table), prevalence of stage B/C at diagnosis, CLL/PL or atypical morphology, CD38 positivity (regardless of the cut-off point), trisomy12+, del 6q21+ or no detectable abnormalities. ZAP-70 negative (−) cases were more often del 13q14+. There was no difference with respect to del 11q23 or p53 abnormalities. Of 142 patients, 83 patients (stage A progressive, B or C) were entered in the LRF CLL4 trial. Among these, 29 (35%) were ZAP-70+ and 54 (65%) ZAP–. Median time to progression from diagnosis in the two groups was 12 months and 21 months respectively. We confirm the correlation of ZAP70 overexpression with other adverse prognostic factors (such as CD38 and advanced stage) and provide new data concerning its association with cytogenetic abnormalities.

	ZAP-70 POS	ZAP-70 NEG	
Number of cases	38 (27%)	104 (73%)	
M/F ratio	**3.75**	2.3	
Stage A	32%	**62%**	
Stage B/C	**68%**	38%	$P^* = 0.002$
CLL/PL-atypical morph	**42%**	10%	
CD38+ (≥7%)	32 (**86%**)	58 (56%)	$P = 0.001$
del 13q14 (sole)	0/14 (0%)	9/27 (34%)	$P = 0.017$
+12	6/19 (31%)	7/56 (12%)	$P = 0.058$
del 11q22-23	3/16 (19%)	7/29 (24%)	$P = 1.00$
del 17p13 (p53)	3/20 (15%)	4/47 (8%)	$P = 0.41$
del 6q21	3/14 (21%)	2/29 (7%)	$P = 0.30$
No abnormalities	5/14 (36%)	2/27 (7%)	$P = 0.035$

*Fisher's exact test.

35

Diagnosis of $CD5^+$ lymphocytosis: correlation between t(11;14) by FISH and cyclin D1 staining on tissue sections

N. Parry-Jones,[1] E. Matutes,[1] A. C. Wotherspoon,[2] V. Brito-Babapulle[1] & D. Catovsky[1]

[1]Academic Department of Haematology and Cytogenetics, The Royal Marsden Hospital and Institute of Cancer Research; [2]Department of Histopathology, The Royal Marsden Hospital, London,UK

t(11;14)(q13q32) is detected by FISH in virtually all cases of mantle cell lymphoma (MCL). Juxtaposition of the CCND1 locus at 11q13 and the

Samsung Bioepis Exhibit No. 1002
Page 1405

immunoglobulin heavy chain enhancer at 14q32, results in overexpression of cyclin D1, a cell cycle protein important for G1-S phase transition. Cyclin D1 overexpression also occurs, by different mechanisms, in other haematological and non-haematological malignancies. Our group and others have shown that overexpression of cyclin D1 can be detected by flow cytometry, having up to 80% correlation with FISH results. Cyclin D1 protein staining on tissue sections correlates well with mRNA expression using Northern blot, the 'gold-standard' technique, but requires large amounts of m-RNA, and is unsuitable for routine diagnosis. Apart from a recent study by Hui *et al* (Leuk Lymphoma 2003), a direct comparison of t(11;14) by FISH on blood lymphocytes, and immunohistochemical staining on tissue sections has not been reported. We studied 36 patients with $CD5^+$ B-lymphocytosis and CLL score of 1 or 2, who had histological material available. Cases were analysed for t(11;14) by interphase FISH on methanol/acetic acid-fixed peripheral blood lymphocytes, and by immunohistochemical staining for cyclin D1 on tissue sections. Results were as follows:

Cyclin D1 by immunohistochemistry

		Positive	Negative	Total
FISH (on peripheral blood)	Positive	23	1	24
	Negative	0	12	12
	Total	23	13	36

Sensitivity of FISH compared with cyclin D1 = 100%; specificity of FISH compared with cyclin D1 = 92%.

We conclude that there is excellent correlation between results of FISH for t(11;14), and immunohistochemical staining for cyclin D1 protein. $CD5^+$ lymphocytosis can present diagnostic difficulty, particularly in cases without lymphadenopathy. FISH is an easy technique and can be performed on blood lymphocytes with results available in 24 h, making it a useful screening tool, particularly in cases without readily available histology.

36

The combined PET/CT scanner – an invaluable aid in the management of complex lymphoma patients from Northern Ireland

G. M. Benson,[1] A. P. Shiels,[2] J. Clarke,[3] R. J. G. Cuthbert,[1] M. Drake,[1] M. El-Agnaf,[4] D. Hull,[5] P. J. Kettle[1] & T. C. M. Morris[1]

[1]Department of Haematology, Belfast City Hospital; [2]Department of Radiology, Belfast City Hospital; [3]Department of Nuclear Medicine, Royal Victoria Hospital; [4]Department of Haematology, Ulster Hospital, Dundonald, Belfast; [5]Department of Haematology, Craigavon Area Hospital, Craigavon, UK

The availability of accurately aligned, whole body anatomical (CT) and functional (PET) images are having a significant impact on diagnosing and staging both Hodgkin and non-Hodgkin's lymphoma.

Aim: We set out to review the first cohort of haematology patients scanned from July 2002 to July 2003.

Results: Results on a total of 44 scans; 15 patients with Hodgkin's (34%) and 29 patients with non-Hodgkin's lymphoma (66%). The indications for scanning were prechemotherapy (4.5%); mid chemotherapy (6.8%) if conventional CT suggests a suboptimal response; postchemotherapy (77.2%) seven of whom had minimal residual disease on CT alone; and postautologous transplant (14.7%) assessment. A total of 38.6% was PET positive, while 61.4% had no uptake at all. A total of 28 patients were confirmed in remission. Of those PET positive, three patients had a repeat scan following BEAM autograft which was negative, while a further two patients whose mid chemotherapy scan showed poor response from first line therapy, had their treatment switched to salvage chemotherapy and gain had repeat PET/CT scans negative. Six of the seven patients with residual disease on CT had negative PET uptake. The seventh had PET positive residual disease and was transplanted with her subsequent scan PET negative.

Conclusion: PET/CT has been shown to predict at an early stage good prognosis patients who are likely to be 'cured' with standard chemotherapy from those patients with poorer prognosis who require alternative treatment. The future may necessitate it as the gold standard investigation at all stages of therapy for most types of lymphoma.

37

The role of flow cytometry on lymph node tissue as a rapid decision making tool for lymphoproliferative disease

L. Atkinson,[1] S. J. Couzens,[1] S. Phillips,[1] R. Attanoos,[2] S. Dojcinov,[3] C. Rowntree[1] & C. H. Poynton[1]

[1]Department of Haematology, University Hospital of Wales, Cardiff; [2]Department of Pathology, Llandough Hospital, Penarth, UK; [3]Department of Pathology, University Hospital of Wales, Cardiff, UK

Flow cytometry (flow) on fresh single cell suspensions from lymph nodes was performed within a few hours on 82 consecutive biopsies. This information was then used to inform selected diagnostic activity in cytogenetics/FISH, molecular diagnostics and immunocytochemistry (ICC). This study reviews retrospectively how often flow got it 'right'. The majority of samples were nodal excision biopsies (67) with other resections (six), trucut biopsies (five) and FNAs (four). The fiur colour flow included cell cycle analysis with the new cell-permeable DNA probe DRAQ5 (Biostatus Ltd). Viability was assessed by 7AAD exclusion [mean viability 77% (10–95) and lowest in trucut and FNA]. A specific diagnosis (equating to a WHO subtype) could be applied in 44 cases and a reactive non-malignant diagnosis in 27 cases. In 11 cases it was not possible to make a specific diagnosis. Flow was able to identify correctly 28 cases of the NHL subtypes (groups included follicular Grades 1 or 2, follicular Grade 3, mantle, marginal zone, SLL, DLBCL, Burkitt's, lymphoblastic, ALCL, high grade T-cell). Flow missed 10 cases of NHL (six called reactive and four inconclusive). Hodgkin's was correctly identified as were three of four other haematological malignancies and nine of 12 non-haematological malignancies, giving a very high specificity. Accurate distinction between follicular lymphoma (especially Grade 3) and diffuse large B-cell lymphoma (DLBCL) of follicular origin was aided by cell cycle analysis in conjunction with data on cell size from scatter properties. Flow is valuable in clonal detection of B cells in biopsies with minimal involvement (4% cases), distinction between tumours with high S-phase (blastic/Burkitt/mantle) (4% cases) and in its sensitivity of CD5 detection compared to ICC (CLL/SLL/MCL). Disadvantages include limited discrimination of cell size and morphology and the need for rapid processing. Flow provides a high level of specificity in lymphoma diagnosis, is rapid and focuses further testing appropriately.

38

Bone lesions at diagnosis in myeloma patients: age, subtype and β_2 microglobulin

E. Harris & B. Myers

Department of Haematology, Queen's Medical Centre, Derby Road, Nottingham, UK

Lytic bone lesions are common in patients with multiple myeloma, associated with significant morbidity. Bone lesions correlate with mass

Samsung Bioepis Exhibit No. 1002
Page 1406

of myeloma cells, having prognostic value. We evaluated 136 patients at diagnosis with respect to lytic lesions, and the relationship to age, β_2 microglobulin and type of immunoglobulin and light-chain excretion. Eighty-five patients were aged over 65 years. Of these, 70% had lytic lesions, compared with 63% of 51 patients who were 65 years or less. The adhesion molecule, NCAM (CD56) is overexpressed by myeloma cells, mainly of the κ subtype. NCAM is thought to bind homophilically to osteoblasts, hence decreasing osteoblast function. Conversely, λ light-chain subtype lacks/weakly expresses NCAM, and is therefore thought to have a lower osteolytic potential. In our group of patients lytic lesions were present at diagnosis in 60% of those with the κ subtype, in 62% of the λ and 62.5% overall of those patients with light-chain myeloma. In this latter group, lytic lesions predominated in the κ subtype, 78% as compared with 43% in the λ subgroup. Six of the patients in our study had non-secretory myeloma. All of this subgroup had lytic lesions at diagnosis. β_2 Microglobulin is an independent prognostic factor for myeloma. β_2 Microglobulin level at diagnosis was evaluable in 96 patients. Of those with a β_2 microglobulin of 3.5 mg/l or greater, 74% had lytic lesions at diagnosis when compared with 50% in the group with levels of less than 3.5 mg/l. All the patients with non-secretory myeloma had levels greater than 3.5. In conclusion, there is a clear correlation between the non-secretory group and presence of lytic lesions at diagnosis, and raised β_2 microglobulin. The size of the study may be insufficient to demonstrate differences between κ and λ subtypes, but there are other soluble factors variably secreted in the marrow microenvironment in myeloma, which may also confound this assessment.

39

Gene expression profiling identifies a sub-population of AML patients most likely to respond to the FLT3 inhibitor CEP701

S. Knapper, A. K. Burnett, A. F. Gilkes, K. I. Mills, L. N. Truran, V. Walsh & S. J. Austin

Department of Haematology, University of Wales College of Medicine, Cardiff, Wales, UK

Activating mutations of the tyrosine kinase receptor FLT3 are present in over 30% of cases of AML and are associated with adverse prognosis. FLT3 presents an attractive therapeutic target and a number of small molecule tyrosine kinase inhibitors have been developed. The range of AML patients who are likely to benefit from these agents remains unclear. CEP701 (Cephalon) is an indolocarbazole derivative that has been found to potently and selectively inhibit FLT3 phosphorylation. Blasts were obtained at presentation from 30 AML patients including 10 FLT-ITD mutants and 20 FLT3-wild type cases. An MTS-based assay was used to assess *in vitro* cytotoxic response to a range of concentrations of CEP701. The median IC50 value amongst the mutant cases was 110 nmol/l in comparison to 200 nmol/l for non-mutants: a non-significant difference. Additionally, no significant difference in cytotoxic response was seen at 50 nmol/l CEP701, a dose known to maximally inhibit the FLT3 receptor. Gene expression analysis was performed on pretreatment samples using the Affymetrix U133A gene chip. Statistical tests, including one-way analysis of variance and class predictor algorithms, were used to identify a common gene expression signature for cases that showed high *in vitro* sensitivity to CEP701. The signature showed no correlation with FLT3 mutation status. Genes involved in cell signaling and control of cell proliferation were amongst the most discriminatory. The possibility of using this gene set to predict *in vivo* patient responsiveness to CEP701 is being investigated in the context of a current phase II trial of the drug in newly diagnosed elderly patients considered unfit for intensive chemotherapy. Pretreatment gene expression profiling is being combined with clinical response data to validate the list of predictor genes. This study demonstrates the potential of using gene expression data in the identification of AML patients that may respond to specific targeted therapies.

40

Gene array profiling of the effect of FLT3 mutations in AML

K. I. Mills,[1] A. F. Gilkes,[1] V. Walsh,[1] S. J. Austin,[1] D. Linch[2] & A. K. Burnett[1]

[1]Department of Haematology, UWCM, Cardiff, Wales; [2]Department of Haematology, University College Hospital, London, UK

The presence of a mutation of the FLT3 receptor, either an internal tandem duplication (ITD) or a mutation within the activating loop domain (ALM) has been found to be the most common molecular abnormality in AML occurring in 30–35% of cases. Its incidence is not evenly distributed between FAB groups, but the presence of a mutation may act as prognostic modifier on the MRC cytogenetic based risk groups. The presence of an ITD mutation has the greatest effect within the Intermediate group, with 64% 5-year relapse rate for patients with an ITD compared with 48% without. Affymetrix U133A gene array analysis may explain the differential modification on prognosis within each risk group. In addition, molecular mechanisms activated by the different FLT3 mutations may be identified. Over 130 AML samples have been analysed, with approximately 26% having a FLT3 ITD mutation, 3.5% having an ALM mutation and one patient having both types of mutation. Data was analysed using GeneSpring 6.1 to identify gene lists representative of the various FLT3 mutations. These were refined using additional statistical tests including a Class Predictor algorithm in combination with Fischer's exact test and used to produce hierachical trees of gene expression signatures. Analysis of the total cohort did not show a clear cluster distinction between patients with wild type or mutated FLT3. However, when subgroups were analysed, clearer clusters of patients with or without FLT3 mutations could be identified; particularly within the intermediate risk group. Differential expression of genes in possible FLT3 signalling pathways was observed in patients within wild type, ITD and ALM groups. In conclusion, expression signatures are associated with the presence of mutations, but the signature is dependant on risk group and cytogenetic abnormality. Furthermore, pathway analysis offers possible indentification of novel molecular therapeutic targets.

41

Prognostic factors for response parameters and overall survival in patients with multiple myeloma following treatment with bortezomib

P. G. Richardson,[1] B. Barlogie,[2] J. Berenson,[3] S. Singhal,[4] S. Jagannath,[5] D. Irwin,[6] S. V. Rajkumar,[7] T. Hideshima,[1] B. Bryant,[8] A. Damokosh,[8] G. Mulligan,[8] H. Xiao,[8] D. Esseltine,[8] D. Schenkein[8] & K. C. Anderson,[1] for the SUMMIT CREST Investigators

[1]Dana-Farber Cancer Institute, Boston, MA; [2]University of Arkansas for Medical Sciences, Little Rock, AR, USA; [3]Cedars-Sinai Medical Center, Los Angeles, CA; [4]Northwestern Memorial Hospital, Chicago, IL; [5]St Vincent's Comprehensive Cancer Center, New York, NY; [6]Alta Bates Cancer Center, Berkeley, CA, USA; [7]Mayo Clinic Rochester, MN; [8]Millennium Pharmaceuticals, Cambridge, MA, USA

Bortezomib (VELCADE^{TM}) inhibits the proteasome, a key regulator of cellular protein homeostasis. SUMMIT, a phase II trial of bortezomib (1.3 mg/m^2 2 × /wk x2 q3wk) in 202 patients with advanced multiple myeloma (MM), demonstrated a 35% overall response rate and manageable toxicities. This analysis focused on identifying potential prognostic factors (PF) for time to response (TTR), duration of response (DOR), time to disease progression (TTP), and overall

Samsung Bioepis Exhibit No. 1002
Page 1407

survival (OS) with bortezomib. PF were evaluated by univariate (Cox proportional hazards model) and multivariate (stepwise model selection procedure) analyses, and included demographic, disease-related, and laboratory parameters. TTR, DOR, and TTP were assessed for bortezomib alone; OS was assessed for bortezomib with or without dexamethasone. Pharmacogenomic (PGx) analyses of plasma cells from pretreatment bone marrow were used to assess gene expression. By multivariate analyses, longer TTR was associated with increased age ($P = 0.009$), >50% bone marrow plasma cells (BMPC) ($P = 0.021$), and higher C-reactive protein ($P = 0.0501$). In CR/PR patients ($n = 53$), shorter DOR was associated with lower levels of albumin ($P = 0.003$) and a lower KPS ($P = 0.01$). Shorter TTP was seen with elevated C-reactive protein ($P = 0.011$) or abnormal cytogenetics ($P = 0.02$). OS was decreased with >50% BMPC ($P = 0.014$), lower platelet count ($P = 0.015$), lower albumin concentration ($P = 0.001$), and lower KPS ($P = 0.032$). Chromosome 13 deletion, β_2-microglobulin, and type or number of prior therapies were not significant predictors. PGx analyses identified a significant number of genes correlating with TTP, several of which were identified in earlier analyses of response to bortezomib; such approaches may ultimately offer useful prognostic markers. Overall, PF associated with increased TTR and decreased DOR, TTP, and OS were also associated with high tumor burden. Future analyses will assess the utility of PF incorporating PGx in making therapeutic decisions.

42

Interleukin-1β maintains an apoptosis-resistant phenotype in the blast cells of acute myeloid leukaemia via multiple pathways

J. Turzanski, M. Grundy, M. Pallis & N. H. Russell

Division of Haematology, University of Nottingham and Nottingham City Hospital, Nottingham, UK

Blast cell survival in suspension culture is associated with chemoresistance in acute myeloid leukaemia (AML). Autonomous production of IL-1β by AML blast cells is linked with cell growth, although its role in survival and hence apoptosis-resistance has not been examined in this disease. We have found an association between IL-1β production and an apoptosis-resistant phenotype in 48-h suspension culture of presentation blood and bone marrow samples from AML patients ($P = 0.012$, $n = 32$). Furthermore, the exogenous addition of rhIL-1β was found to significantly enhance survival in 24/28 blast cell samples, under these conditions ($P = 0.0001$, median increase in survival was 37.10%). IL-1 receptor ligation has previously been found to activate a PI-3 kinase mediated survival pathway, a novel IL-1 receptor-associated kinase (IRAK) and the ceramide metabolizing enzyme, ceramidase. To assess which of these pathways are involved in the IL-1β mediated survival of primary AML blasts, wortmannin, which inhibits PI-3 kinase, NN-dimethyl sphingosine (DMS), which inhibits the metabolism of ceramidase-generated sphingosine into survival promoting sphingosine-1-phosphate and the IRAK inhibitor, AG126 were used. An apoptosis- sensitive sample with a strong survival response to exogenous rhIL-1β was selected to study the IL-1β specificity and additive effects of these inhibitors. This sample was stimulated with rhIL-1β, followed by treatment with wortmannin, AG 126 and/or DMS. All three inhibitors down-modulated an IL-1β mediated increase in blast cell survival. Each combination of two inhibitors had a greater effect than single inhibitors, and only the combination of all three inhibitors totally eliminated viable leukaemic blasts. In apoptosis-resistant primary AML samples, the three inhibitors all increased apoptosis *in vitro* after 48 h. Exogenous rhIL-1β increased NF-κB activation in 5/15 blast cell samples. These findings suggest that IL-1β mediated survival pathways are a factor in resistance to apoptosis in primary AML blasts, and may contribute to chemoresistance in this disease.

43

Overexpression of total cellular phosphoserine in AML blasts with functional p-glycoprotein

S. Shang,[1] M. Grundy,[2] N. Russell[1,2] & M. Pallis[2]

[1]*Academic Haematology, University of Nottingham;* [2]*Nottingham City Hospital, Nottingham, UK.*

Reversible covalent modifications of protein by phosphorylation and dephosphorylation are key events in the regulation of protein activities involved in cellular growth, differentiation and apoptosis pathways. Multidrug resistance mediated by p-glycoprotein is one of the major obstacles in AML chemotherapy and is linked to cellular resistance to apoptosis as well as to drug efflux. Alterations of protein kinase and phosphatase expression have been noted in multidrug resistant cell lines. These include overexpression of protein kinase C (which increases p-glycoprotein activity) and a decrease in protein phosphatase 2A (which dephosphorylates bcl-2). In order to find out whether protein phosphorylation is altered in AML clones with an MDR phenotype, we developed a flow cytometric method to measure total phosphoserine and phosphothreonine. These were assayed in $CD34^+$ cells from normal donors and in 11 consecutive fresh primary AML samples from MRC trial patients. Reproducibility and peptide inhibition studies were used to validate the method. P-glycoprotein activity was measured using PSC-833-mediated modulation of rhodamine 123 accumulation. Mean total phosphoserine and phosphothreonine expression levels in normal CD34+ cells were 11.64 RFU (range 9.98–13.44) and 140.1 RFU (range 123.8–170.1) respectively. Compared with these values, phosphoserine was overexpressed in 10/11 patient samples (median 25.5 RFU, range 10.1–49.6) whereas phosphothreonine was only overexpressed in 2/11 samples (median 119.2, range 61.9–537.6). Five of the 11 patient samples had functional p-glycoprotein. These five cases all had higher phosphoserine than the p-glycoprotein negative cases, $P = 0.004$ by Mann–Whitney analysis (median 20.1, range 10.1–25.5 for the pgp negative cases; median 41.9, range 28.2–49.6 for the pgp positive cases). These experiments provide a rationale for the exploration of specific serine kinase and phosphatase activities as mediators of drug resistance in AML.

44

The CD4 T-helper response to b3a2 BCR-ABL is deficient in chronic myeloid leukaemia (CML) and normal subjects

H. M. Abu-Eisha,[1] N. M. Butt,[2] J. M. Rojas,[2] S. E. Christmas[1] & R. E. Clark[2]

[1]*Department of Immunology and* [2]*Haematology, University of Liverpool, Liverpool, UK*

BCR-ABL, the characteristic molecular lesion in CML, is leukaemia-specific and potentially immunogenic. We have previously demonstrated expression of a peptide from the b3a2 BCR-ABL junction on the CML cell surface in association with HLA-A3. BCR-ABL junctional peptides will elicit cytotoxic T-cell (CTL) responses in both healthy donors and CML patients. In contrast, information on whether BCR-ABL junctional peptides can elicit CD4 T helper responses is limited. Here we have used VHSATGFKQSSKALQRPVASDFE, a 23-mer sequence evenly spanning the b3a2 BCR-ABL junction, in an attempt to elicit *in vitro* T-helper responses. Responses at 7 d were assessed by a standard tritiated-thymidine incorporation assay. None of 14 healthy donors showed any proliferation [stimulation index (SI) < 1.5], despite adequate proliferation (SI > 3.8) to positive control recall antigens. Furthermore, none of 32 CML patients showed any proliferation, although 17 of these also did not respond to positive control antigens.

Samsung Bioepis Exhibit No. 1002
Page 1408

There was a non-significant trend for greater proliferation to positive control antigens in imatinib-treated patients than in α-interferon-treated cases. One of two donors and none of 12 patients proliferated when rechallenged with VHSATGFKQSSKALQRPVASDFE 7 d after primary exposure. In subsequent work using peptide pulsed PBMC or dendritic cells as antigen presenting cells, rechallenge on three to four occasions again produced no responses in either donors or CML samples, despite adequate positive control responses. The data suggest that in contrast to earlier reports using shorter peptides, BCR-ABL may not elicit an adequate CD4 T helper response in normal subjects. However, the present data underline earlier findings that CML patients do not have detectable circulating BCR-ABL specific T helper precursors. Vaccination of CML patients with BCR-ABL junctional peptides might elicit BCR-ABL specific T helper responses. This is now under evaluation at our centre (EPIC study), for chronic phase patients already stabilized on imatinib.

45

L3MBTL, a candidate 20q tumour suppressor gene is not mutated in patients with myeloid malignancies and displays monoallelic methylation

A. J. Bench,[1] J. Li,[1] B. J. P. Huntly,[1] G. S. Vassiliou,[1] E. Delabesse,[1] N. Fourouclas,[1] A. R.Hunt,[2] P. Deloukas,[2] A. C. Ferguson-Smith[3] & A. R. Green[1]

[1]Department of Haematology, University of Cambridge, Cambridge; [2]The Wellcome Trust Sanger Institute, Hinxton, Cambridgeshire; [3]Department of Anatomy, University of Cambridge, Cambridge, UK

Deletions of the long arm of chromosome 20 are a recurrent chromosomal abnormality associated with myeloid malignancies, particularly the myeloproliferative disorders, but target genes remain elusive. *L3MBTL* represents a strong candidate gene since it lies within the common region of deletion in patients with a 20q deletion, encodes the human homologue of a *Drosophila* tumour suppressor and is expressed within purified CD34 positive progenitor cells. L3MBTL is part of the growing family of Polycomb proteins, members of which regulate self-renewal of normal and malignant haematopoietic stem cells. We have determined the structure of *L3MBTL*, identified two putative promoters each associated with two CpG islands and characterized a complex pattern of 5' and 3' alternative splicing events. Analysis of all coding exons, 5' non-coding exons and splice sites from patients with a 20q deletion and cytogenetically normal patients with polycythaemia vera identified several polymorphisms but no acquired mutations. Methylation analysis demonstrated that the two CpG islands spanning promoter 2 undergo monoallelic methylation in normal haematopoietic cells. Leukaemic cells from patients with a 20q deletion retained either the methylated or unmethylated allele, but retention of the methylated allele did not correlate with complete loss of *L3MBTL* expression. Within normal haematopoietic cell types, monoallelic methylation of these 2 CpG islands correlated with transcriptional silencing of *L3MBTL*. These results demonstrate that *L3MBTL* represents a novel imprinted locus, a finding that has important implications for the pathogenesis of myeloid malignancies associated with 20q deletions.

46

Clonality of dendritic cells from chronic myeloid leukaemia patients treated with imatinib

L. Wang,[1] N. M. Butt,[1] M. G. Atherton[2] & R. E. Clark[1]

[1]Department of Haematology, Royal Liverpool University Hospital; [2]Merseyside and Cheshire Cytogenetics Laboratory, Liverpool Women's Hospital, Liverpool, UK

Chronic Myeloid Leukaemia (CML) cells express leukaemia-specific surface peptides derived from the BCR–ABL junction, in association with HLA Class I. Whether CML dendritic cells (DCs) can also do so is not known, although several studies have established that DC in patients with florid CML are both Ph and *BCR–ABL* positive. The clonality of DC in patients successfully treated with imatinib is unknown. We have investigated DC from 17 CML patients at varying stages of disease. DCs were generated from CD14 positive selection of peripheral blood mononuclear cells. Clonality was tested by PCR for *BCR–ABL* expression and by fluorescent *in situ* hybridization (FISH) for *BCR–ABL* fusion. DC from all eight cases in first chronic phase (CP1) and in complete cytogenetic remission (CCR) treated with imatinib were *BCR–ABL* negative by PCR although WBC from six of these cases remained *BCR–ABL* positive. All tested DC from these eight cases were also negative for BCR–ABL by FISH. In contrast, DC from all four cases treated with imatinib not in CCR and the CP1 case-treated solely by leukapheresis were *BCR–ABL* positive. DC generated from all four cases treated with INF-α (including one case who achieved CCR) remained *BCR–ABL* positive. Also we found that in all chronic phase patients DC consistently expressed a lower level of *BCR–ABL* than WBC. In conclusion, for CML CP1 patients achieving CCR on imatinib, normal DC may replace *BCR–ABL* positive DC. This may be advantageous since normal DC may be preferable for autologous DC-based immunotherapy. The disadvantage is that DC can no longer present leukaemia-specific endogenously derived BCR–ABL peptides to the immune system.

47

Bcr-Abl down-regulates CCN3 expression

L. M. R. Gilmour,[1] S. Price,[1] R. Burden,[1] A. Pierce,[2] A. D. Whetton,[2] B. Perbal[3] & A. E. Irvine[1]

[1]Department of Haematology, Queen's University, Belfast; [2]LRF Cellular Development Unit, UMIST, Manchester, UK; [3]Laboratoire d'Oncologie Virale et Moleculaire, UFR de Biochimie, Université Paris, Paris, France

Chronic myeloid leukaemia (CML) is characterized by expression of the constitutively active Bcr-Abl tyrosine kinase. We have previously shown that Bcr-Abl kinase activity decreases expression of CCN3 mRNA and protein in a murine cell line model for CML (ts-bcr-abl FDCP-MIX cells). CCN3, a member of the CCN family of growth regulatory molecules, has been associated with promoting cell adhesion, differentiation and quiescence. We now demonstrate reduced CCN3 expression in human CML cell lines and primary human CML cells. Real-time PCR was used to monitor expression of Bcr-Abl and CCN3 mRNA. The human CML cell lines K562, KU812 and LAMA showed strong expression of Bcr-Abl whilst CCN3 was barely detectable. Treatment of human CML cell lines with 1 μmol/l Imatinib for 2–4 d resulted in a reduction in Bcr-Abl expression and concomitant increase in CCN3 expression (K562 cells: mean Ct change Bcr-Abl 2.0 ± 0.6, CCN3 2.3 ± 0.3). Bone marrow was obtained from three normal donors and three CML patients at diagnosis and on entering haematological and cytogenetic remission. Primary human CML cells at diagnosis showed fourfold lower expression of CCN3 mRNA in comparison to normal bone marrow samples (NBM) ($P = 0.015$). Bcr-Abl expression fell significantly when patients entered haematological remission and there was a reciprocal increase in CCN3 expression ($P = 0.026$, CCN3 mean Ct change 1.0 ± 0.1). Confocal microscopy and Western blotting demonstrated analogous changes in CCN3 protein expression with a reversion to levels comparable with NBM upon entering remission. Dysregulation of CCN3 expression has not previously been recognized in CML, but has been associated with oncogenesis. Down-regulation of CCN3 expression is consistent with the CML phenotype.

Samsung Bioepis Exhibit No. 1002
Page 1409

48

GADD45 expression correlates with drug resistance phenotype and functional Pgp status in AML

L. Truran, H. Albertelli, A. F. Gilkes & K. I. Mills
Department of Haematology, Cardiff, Wales, UK

Treatment failure in AML is frequently associated with resistance to chemotherapeutic drugs. The active export of drugs from the cell, abnormalities of particular drug-metabolizing enzymes, efficient repair of drug-induced damage and failure of the apoptotic machinery may all contribute. The measurement of functional P-glycoprotein (Pgp) has been done previously by quantitative flow cytometry in over 300 patients, several of these have also been analysed using Affymetrix gene expression chips. We have previously reported that there was a direct correlation between Pgp gene expression levels and functional protein levels. A high Pgp level, by either method, could be correlated with an increased drug resistance. Further analysis of the expression data suggested that expression of the GADD45 (growth-arrest and DNA-damage 45) gene may also correlate with pGp levels and patient response to therapy. GADD45 RNA levels were determined in 47 AML patients using quantitative RT-PCR on the LightCycler. Functional Pgp and clinical data was available on approximately half of these patients. Normalized GADD45 gene chip expression values could also be directly correlated with standardized levels. Furthermore, a high standardized GADD45 level could be correlated with a high functional Pgp level and drug resistance. This study indicates that microarray analysis can be used to identify genes that may be related to drug resistance and which may also act as markers of a drug resistance phenotype whilst giving an insight into the molecular basis of resistance.

49

Comparison of signature gene profiles between cytogenetic risk group, FAB classification and WHO classification for AML

A. F. Gilkes,[1] D. Linch,[2] A. K. Burnett[1] & K. I. Mills[1]
[1]*Department of Haematology, Cardiff, Wales;* [2]*Department of Haematology, University College Hospital, London, UK*

AML has a high degree of heterogeneity at the morphological, cytochemical, immunological, cytogenetic and molecular level. Morphology is the basis of the French-American-British (FAB) classification in which leukaemia subtypes are based on the stage of myelo-monocytic differentiation. The MRC risk groups, favourable, intermediate and adverse, use cytogenetics as the major predictor of response. The more recent WHO classification takes into account both cytogenetics and morphology; with specific cytogenetic translocations defined prior to allocation of morphological grouping similar to the FAB classification. Gene expression profiling has been used to select genes to identify both similarities and differences between the risk groups, FAB or WHO classifications. We have analysed diagnostic samples from >130 patients entered into MRC AML trials using Affymetrix GeneChips. Within risk groups 39% were favourable, 52% intermediate and 9% adverse. All the FAB groups, with the exception of M6, were represented. Training and test sets were defined which combined with statistical analysis allowed gene lists representative of the risk groups, FAB groups and WHO categories to be identified and these were used to produce hierarchical trees corresponding to the different parameters. The assignment of FAB can, in some cases, be subjective and this may be the reason why expression signatures for FAB groups were not totally concordant. Although trends towards clusters of morphologically related FAB classes were identified. These trends were also observed when the morphology-based groupings in the WHO were examined. Some degree of overlap of statistically significant gene lists were observed between those for the risk and WHO groups or for the FAB and non-translocation morphology groups. This gene expression profiling study on AML patients reinforces the heterogeneity of the disease and may add a molecular basis to existing classifications of AML.

50

Identification of presentation acute myeloid leukaemia antigens in a testes cDNA library

B. A. Guinn, E. A. Bland, J. F. Collin, U. Lodi & G. J. Mufti
Department of Haematological Medicine, Guy's, King's & St Thomas' School of Medicine, London, UK

The serological analysis of antigens by recombinant expression cloning (SEREX) has been used to identify known and novel antigens in a broad range of tumour types. Such antigens may provide markers of disease, allow insights into the molecular mechanisms involved in the development of cancer and identify targets for vaccines that can induce immunotherapeutic responses. Using the SEREX technique we immunoscreened a phage-based cDNA library made from the testes of five normal donors. A testes library was chosen as these cells proliferate rapidly and express transcripts from a larger range of genes than most somatic tissues. We immunoscreened 1.2×10^6 pfu using sera from five newly diagnosed AML patient with either M4 or M5 disease subtypes and no cytogenetic abnormalities. We have identified 45 antigens of which 25 encode previously described or predicted genes. We identified TRIP11, MYH11, RHAMM, MAPK10 and BRAP which have previously been suggested to play a role in acute myeloid leukaemia and NAB2, ACVR2 and NY-BR-1 which have been shown to be involved in the pathogenesis of solid tumours. In addition we identified 20 novel genes including three zinc finger proteins and a protein that contains a bromodomain, a sequence motif often found in transcriptional co-activators. To date, we have immunoscreened all antigens with sera from 20 AML, 20 CML and 20 age- and sex-matched normal donors. We found that eight antigens were recognized solely by patient sera and not by normal donor sera. We also found eight antigens that were recognized preferentially by patient sera compared with normal donor sera. We will begin to characterize the expression of some of these leukaemia associated known and novel antigens including a novel X-linked gene which was only recognized by patient sera, as well as the previously defined SEREX antigen NY-BR-1.

51

Use of cDNA microarray analysis to identify cancer testes antigen expression in presentation acute myeloid leukaemia

B. A. Guinn,[1] A. F. Gilkes,[2] G. J. Mufti,[1] D. Linch,[3] A. K. Burnett[2] & K. I. Mills[2]
[1]*Department of Haematological Medicine, Guy's, King's & St Thomas' School of Medicine, London;* [2]*Department of Haematology, University of Wales College of Medicine, Cardiff, UK;* [3]*Department of Haematology, University College and Royal Free Hospital, London, UK*

Tumour antigens have been shown to be effective targets for cancer immunotherapy. To identify antigens which may be useful immunotheraupeutic targets for AML we used cDNA microarray to assess the transcription of 94 genes which encode tumour antigens for their expression in 106 presentation AML patients entered into the MRC AML12, 14 or 15 clinical trials prior to treatment. We analysed the present/absent calls of gene expression on the Affymetrix HG-U133A chip comparing expression in patients grouped according to cytogenetics risk. We found that none of the antigens were expressed only in the poor risk group and seven antigens were present in the good and standard risk group patients including TROP-2, CEACAM1 and SSX2IP. Seven antigens were only present in standard risk group patients including GAGE-3, GAGE-7B and MAGE-A12. Three antigens were only present in the good risk group patients including XAGE-1.

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Samsung Bioepis Exhibit No. 1002
Page 1410

We assessed present/absent calls in relation to FLT3 status, i.e. FLT3 wild type ($n = 63$) or those patients with the FLT3 internal tandem duplication (ITD) ($n = 25$). We found the only antigen present solely in patients with FLT3 ITD was MAGE-A12. However, nine genes were present only in patients with wild-type FLT3 including GAGE-2 and MAGE-C1. Most notable from these analyses was the decrease in the number of antigens expressed in the poor risk groups and in patients with FLT-3 ITD suggesting that targets in these groups will be more difficult to identify perhaps due to a diminished antigen expression. This may be one way in which the cells constituting the most aggressive forms of AML have avoided immune clearance. We are now comparing tumour antigen expression in patient samples with normal $CD34^+$ cells to determine which of these antigens may provide cancer specific targets for the immunotheraupeutic treatment of AML.

52

Internal tandem duplications of FLT3 and partial tandem duplications of MLL can provide further stratification of the intermediate risk category of acute myeloid leukaemia

S. J. Austin,[1] A. F. Gilkes,[2] D. S. Holt,[1] K. I. Mills,[2] L. Pearce[1] & A. K. Burnett[2]

[1]*Department of Haematology, Cardiff and Vale NHS Trust;* [2]*Department of Haematology, University of Wales College of Medicine, Cardiff, UK*

Acute myeloid leukaemia (AML) is a haematological stem cell malignancy with a generally adverse prognosis. The primary means for classifying and determining prognostic outcome of AML is through the determination of cytogenetic abnormalities, which stratify the patients into favourable, intermediate or adverse risk categories. Classification of AML in this manner has been successful apart from the fact that molecular aberrations have been observed in only 50% of all cases, leaving 50% to fall into the intermediate risk category because of the lack of a marker. Two genetic alterations recently documented are the FLT3 internal tandem duplication (ITD) and the MLL partial tandem duplication (PTD), which often occur in patients who would otherwise be classified as cytogenetically normal. Studies indicate these markers are associated with adverse prognosis. Using RT-PCR to detect FLT3-ITD mutations in unbiased AML patients revealed 28/141 (19.8%) positives. The MLL-PTD was demonstrated in 7/68 (10.3%) unbiased AML patients. In the FLT3 samples, 17/141 (12.1%) had either a t(8;21), t(15;17) or inversion 16 as detected by PCR, of those 17 only five (29.4%) also carried a FLT3 ITD. Nine of these translocations occurred in the 68 (13.2%) samples examined for MLL-PTD and none coincided with the MLL-PTD. In our hands the combination of these two assays produced information on 30/141 (21.3%) patients who would otherwise have had no information from PCR analysis. By including FLT3 and MLL-PTD assays, prognostic information from RT-PCR was provided for 24.8% of all AML patients. Their inclusion will provide greater information for patients who previously had no genetic markers and had been grouped into the intermediate risk category. This additional information can then be used to adjust the type and intensity of treatment in accordance to the severity of their disease.

53

Neutrophil PRV-1 gene expression in myeloproliferative, myelodysplastic and reactive blood disorders

J. Zhang, A. Pellagatti, L. Campbell, H. Eagleton, S. Gal, C. Fidler, J. Boultwood & J. S. Wainscoat

Nuffield Department of Clinical Laboratory Sciences, University of Oxford, Oxford, UK

There has been much interest in the use of the polycythaemia rubra vera 1 (PRV-1) gene as a molecular marker for the diagnosis of polycythaemia vera (PV). To assess the specificity of PRV-1 gene expression in PV, we have quantified neutrophil PRV-1 levels in 20 PV, 13 essential thrombocythaemia (ET), nine chronic myelogenous leukaemia (CML), 19 myelodysplastic syndromes (MDS), nine secondary erythrocytosis (SE), four idiopathic erythrocytosis (IE), and 12 secondary leukocytosis (SL) by TaqMan real-time RT-PCR. The relative expression of PRV-1 for each patient sample, in which the Ct values of PRV-1 were normalized to β_2-microglobulin and compared with healthy controls, was calculated as $2^{-\Delta\Delta Ct}$. Our results showed that the levels of PRV-1 expression were elevated in all groups. Among them, SL group had the highest expression of PRV-1 (mean 1260.98), and was significantly higher than any other groups ($P < 0.05$) except the PV group. There was a significant overlap in PRV-1 mRNA levels between PV and other polycythaemias (SE or IE) or other CMPD (CML or ET). Most MDS patients showed high levels of PRV-1 expression in their neutrophils although the levels were significantly lower than PV ($P < 0.05$). In conclusion, our data suggest that the PRV-1 gene expression is not a specific marker for the diagnosis of PV because elevated PRV-1 mRNA expression in neutrophils could be detected in various disorders ranging from myeloproliferative and myelodysplastic conditions to reactive conditions. The quantitation of PRV-1 cannot distinguish PV from any of the above conditions. It is clear that new molecular markers must be sought for the diagnosis of PV.

54

An audit of the use of imatinib in the treatment of chronic myeloid leukaemia in Scotland

P. R. E. Johnson,[1] C. Dhanapala,[2] C. Maguire,[3] J. White,[3] M. Drummond[4] & P. C. A. Shepherd[1]

[1]*Department of Haematology, Western General Hospital, Edinburgh;* [2]*Glasgow Area Medicines Evaluation Unit, Glasgow Royal Infirmary, Glasgow;* [3]*Scotland Leukaemia Registry, Public Health Sciences, University of Edinburgh, Edinburgh;* [4]*Department of Haematology, Glasgow Royal Infirmary, Glasgow, UK*

Chronic myeloid leukaemia has an incidence of approximately 0.7–1:100 000 per annum; in Scotland therefore, with a population of 5 million, 35–50 new cases are diagnosed yearly. Imatinib is an abl-kinase inhibitor, which specifically antagonizes the molecular pathway through which the Philadelphia chromosome causes leukaemia. Imatinib was first used in clinical trials in Scotland in March 2000, and was first approved by the Scottish Medicines Consortium in January 2002. The Scotland Leukaemia Registry (SLR) has been registering all cases of AML, ALL and CML since November 1998. Using SLR and pharmacy databases, we sought to audit the use, indications, effectiveness and trial involvement of patients treated with Imatinib throughout Scotland. All Imatinib usage since its first prescription in Scotland in Mar 2000 to July 2003 was audited. A total of 145 patients from 21 hospitals were identified; 102 chronic phase (CP), 35 accelerated phase (AP), five blast phase (BP), and three phase not specified. Eighty-six of 145 (59%) were treated in clinical trials. The initial starting dose was 400 mg in 97 (69%) of cases and 600 mg in 40 (28%). During continuing therapy, the dose was increased in 12 (8%) and reduced in 28 (19%). Time from diagnosis to initiation of therapy was 16.7 m CP, 31.2 m AP and 8.9 m BP. The percentage of patients remaining on treatment at 1 year was 90% CP, 73% AP and 33% BP. The median duration of therapy was not reached CP, 19 months AP and 6 months BP. Haematological response rates at 12 m were 92% CP and 67% AP. Major cytogenetic responses at 12 m were 64% CP and 43% AP. Overall survival for all CP patients from the start of Imatinib therapy was 96% at 12 m, 96% at 18 m, and 93% at 24 m. An audit of the effectiveness of the SLR as an auditing agency showed complete registration in 95.4% of cases.

Samsung Bioepis Exhibit No. 1002
Page 1411

55

Prognostic value of real-time quantification of XIAP and XAF-1 in AML

D. Livesey, A. Awan, K. Hyde, J. A. Liu Yin & K. Tobal

Molecular Oncology Group, University Department of Haematology, Manchester Royal Infirmary, Manchester, UK

Aberrant expression of apoptosis-associated genes has been detected in various leukaemias. We have developed real-time RT-PCR (RQ-PCR) protocols for the quantification of two apoptosis-associated genes, XIAP and XAF-1. XIAP is a member of the inhibitor of apoptosis proteins (IAP) family, while XIAP-associated factor-1 (XAF-1) is a pro-apoptotic gene recently isolated and shown to be an antagonist of XIAP. The expression level and prognostic value of these genes was examined in 25 AML patients at the presentation of their disease. Of these patients 10 relapse within 2 years of presentation and the other 15 patients remain in remission. All patients had a standard/good prognostic karyotype. ABL was used as a control and the level of these transcripts expressed as a ratio of their expression in NB4 cell line. XIAP transcript was detected in 17/25 patients, while XAF-1 transcript was detected in 21/25 patients. The mean XIAP and XAF-1 transcripts, in relapsed patients was 0.012206 and 0.048448 respectively, and in patients who continued in CR was 0.001264 and 0.429343 respectively. For prognostic value evaluation the level of 0.1 for both genes was considered as a cut-off point to discriminate between good and poor prognosis groups. A difference of a minimum of 1 log is considered significant. Four of seven patients with XIAP levels >0.1, while six of 18 patients with levels <0.1 relapsed within 2 years of presentation. Three of 14 patients with XAF-1 levels >0.1, and seven of 11 patients with levels <0.1 relapsed within 2 years of presentation. Patients with either XIAP levels higher than 0.1 or XAF-1 levels lower than 0.1 had worse overall survival than the other patients in this cohort ($P = 0.0036$ and $P = 0.01$ respectively). These data show that both genes are widely expressed in AML patients, and that their level at presentation may have a strong prognostic significance in AML patients especially those with standard/good karyotype.

56

Mechanism of eosinophilia in 26 cases of persistent unexplained eosinophilia. Low incidence of FIP1L1-PDGFRA, TEL-PDGFRB and rare variant BCR ABL

F. Brito-Babapulle, T. Vulliamy, H. W. Habboush, K. Higley, J. Baxter & N. C. P. Cross

Royal Berkshire Hospital, Reading; Imperial College; Nevill Hall Hospital; Wessex Regional Genetics Laboratory

We have studied the causation of eosinophilia in 26 cases of persistent unexplained eosinophilia (eosinophil counts greater than 1.5 × 109/l) where the cause was unclear from case history, physical examination, laboratory testing, and chest X-ray. These cases presented with diverse clinical symptoms, which led to the full blood counts being performed. There were 11 men and 16 women aged from 18 to 77 (median 53). The eosinophil count was 1.5–40 (median 2.6) IgE levels were normal indicating that eosinophilia was not produced by allergy. BM aspirate, trephine biopsy and cytogenetics revealed that four patients had chronic myeloproliferative diseases and eosinophilia. Two of these patients had trisomy 8 in all of 20 metaphases analysed. In another male patient a non-random clonal abnormality was detected. In all other patients cytogenetic analysis was normal. In one female patient a T-cell clone was identified by T-cell-receptor-gene rearrangement studies in the absence of any other evidence of lymphoma. In three other patients a pancreatic, colonic and rare skin tumour (Kimura–Weils disease) was present. Another patient had eosinophilic pseudotumours of the peritoneum. Mast cell tryptase was elevated in one patient in whom no other features of cutaneous or systemic mastocytosis could be found. Of 21 patients screened by RT-PCR for TEL-PDGFRB, rare variant BCR-ABL and FIP1L1-PDGFRA only one tested positive for FIP1L1-PDGFRA. One of the patients with hypereosinophilia due to a myeloproliferative disease was treated with STI-571 but died shortly in blast transformation. All the other patients are alive. Investigation of patients with persitant unexplained eosinophilia shows a low incidence of clonal eosinophilic disorders including the fusion genes known currently to cause eosinophilia. We conclude that hypereosinophilia is produced by a wide variety of pathogenetic pathways and provides a further clue to the understanding of eosinophilic end-organ damage and the idiopathic hypereosinophilic syndrome.

57

Hsp90 as a target molecule for selective therapy in FLT3 positive AML

L. M. Al-Shaer, C. J. Rowntree, K. I. Mills, A. Tonks, R. L. Darley & A. K. Burnett

Department of Haematology, UWCM, Cardiff, Wales, UK

FMS-like receptor tyrosine kinase 3 (FLT3) is expressed on leukaemic blasts in most cases of acute myeloid leukaemia (AML). Internal tandem duplication (ITD) of the FLT3 receptor occurs in 25–30% of cases of AML and is the most frequently reported molecular defect in AML. ITD mutations of FLT3 are associated with a poor prognosis. It has been postulated that FLT3 mutations play a critical role in the pathogenesis of AML. Consequently FLT3 and downstream signalling molecules are targets for inhibition as a novel therapeutic approach in AML. The mutant FLT3 is a client of the chaperone heat shock protein 90 (Hsp90), which is inhibited by 17-allylamino-17-demethoxygeldanamycin (17AAG). In this study, we tested the hypothesis that inhibition of Hsp90 may be a useful therapy for FLT positive leukaemias by exposing a cellular model to 17AAG. We stably transfected 32D cells, a murine myeloid IL-3 dependent cell line, with either full length human $FLT3^{WT}$ or $FLT3^{ITD}$. Transforming activity of the constructs was observed with and without IL-3. In addition, cell proliferation was studied in the $FLT3^{WT}$ and $FLT3^{ITD}$ 32D cells before and after exposure to 17AAG. Results showed abrogation of IL-3 dependence in the transfected $FLT3^{ITD}$ cells. This was not seen in the $FLT3^{WT}$ transfected cells. 17AAG specifically inhibited the $FLT3^{ITD}$ cells in a dose-dependent fashion with an inhibitory concentration of 50% of approximately 300 nmol/l in the absence of IL-3. However, presence of IL-3 rescued the cells from inhibition by 17AAG, suggesting that the IL-3 intracellular signalling pathway remained intact after treatment. Further work looking at the effects of 17AAG is now being done with primary AML blasts. These results suggesting that inhibition of the chaperone protein Hsp90 with 17AAG may have a role in the therapy of AML.

58

An investigation of the pro-apoptotic effect of the MEK/ERK inhibitor U0126 alone and in association with Ara-C in acute myeloid leukaemia

A. H. J. Kerr,[1] M. A. Smith,[1] J. A. James,[2] C. Willson[1] & J. G. Smith[3]

[1]*Centre for Research in Biomedicine, University of the West of England;* [2]*Department of Haematology, Frenchay Hospital, Bristol;* [3]*Department of Haematology, Frimley Park Hospital, Surrey, UK*

Constitutive activation of the Ras/Raf/MEK/MAPK signalling pathway at the MAPK level in acute myeloid leukaemia (AML) has been associated with poor clinical outcome. Interruption of the pathway with MEK inhibitors can induce pro-apoptotic signals. This study examined the pro-apoptotic potential of the Mek inhibitor U0126 on AML cell lines (KG1a, HEL, TF-1, M-07e, THP-1, MV411, ML-2,

Samsung Bioepis Exhibit No. 1002
Page 1412

HL60), marrow samples from 12 newly diagnosed AML patients and patients with non-leukaemic neoplastic disorders (NHL, $n = 7$; myeloma, $n = 5$; other $n = 2$). Normal marrow samples ($n = 5$), from femora of hip replacement patients were also included. Cells were exposed to U0126 (50 μmol/l) and alone or in combination with cytarabine (Ara-C) (10^2 mol/l) to determine whether these agents might act synergistically. Apoptosis was detected flow cytometrically using monoclonal antibodies Apo 2.7-RPE directed against the mitochondrial protein 7A6 and AnnexinV-FITC, which has high affinity for exposed phosphotidylserine on the outer cell surface membrane. The pro-apoptotic effect of U0126 was inconsistent in cell lines however a significant correlation between sensitivity and CD34 positivity was noted. This was particularly apparent in the primitive M0 cell line KG1a. Ara-C induced apoptosis was variable but with no evidence of enhancement of pro-apoptotic effect with U0126. Leukaemic patient samples exhibited inconsistent sensitivity to U0126 and a similar association with CD34 expression but no synergy with Ara-C. In non-leukaemic samples a varied response to U0126 was apparent whilst normal marrow samples showed no significant sensitivity to the agent and again no enhancement of the apoptotic effects of Ara-C. These results suggest that U0126 may have potential as a targeted therapeutic agent particularly in primitive AML and that there is no synergism between the inhibitor and Ara-C. Further work is indicated to further characterize differential sensitivity of AML, non-leukaemic neoplastic and normal marrow progenitors to U0126 induced apoptosis.

59

Dendritic cell-leukaemic blast fusion hybrids stimulate anti-leukaemic cytotoxicity in AML

B. T. Pottinger, J. A. Adams, M. A. Briggs & J. A. Liu Yin

University Department of Clinical Haematology, Manchester Royal Infirmary, Manchester, UK

In any cellular immunotherapy strategy dendritic cells (DC) are pivotal to an immune response as they present tumour antigens with appropriate co-stimulatory molecules to T cells (TC). We have cultured DC from remission material and explored a poly-ethylene-glycol (PEG) fusion method to introduce leukaemic antigens with the aim of inducing an autologous, anti-leukaemic TC response. Monocytes obtained by overnight plastic adherence of remission PBPC/PBMNC were cultured in McCoy's medium with 15% FCS, GM-CSF (100 ng/ml) and IL4 (20 ng/ml). TNF-α (10 ng/ml) was added on day 5. At day 7, DC content was assessed by morphology and immunophenotyping. These cells were then fused 1:1 with blasts using PEG (50% in Hepes). Controls contained the cell mixture without PEG. After fusion, hybrids were identified by dual immunofluorescence using membrane dyes or monoclonal antibodies. Fusion hybrids or control mixtures were irradiated then co-cultured with TC with IL-2 (20 units/ml). Further DC were fused and added 7 d later as a second round of TC stimulation. Following 14 d stimulation cytotoxicity was assessed by exposing leukaemic blasts (stained green) to stimulated TC for 14–18 h. Cytotoxicity was measured by propidium iodide uptake/exclusion. In a series of 12 patients using this technique we found mean fusion rates of 21.2% (range 5.4–42.5%), significantly higher ($P < 0.0001$) than paired, control cell mixtures, mean 11.6% (range 2.4–24.6%). Initial cytotoxicity experiments on four patients showed 39.8% mean cytotoxicity (range 29.9–68.4%) at TC:target ratio of 10:1, 40.87% (22.0–74.7%) at 20:1 and 52.2% (21.2–78.9%) at 50:1 against 17.4% (6.0–34.2%) for controls. These results indicate that PEG fusion may be an effective method of loading leukaemic antigens onto monocyte derived DC cultured from remission blood. Preliminary cytotoxicity data indicate that these fusion hybrids can stimulate TC to produce specific anti-leukaemic cytotoxicity, providing a basis for this form of immunotherapy in AML.

60

Toxic megacolon due to invasive aspergillosis localized to the colon

U. S. Mohite,[1] J. Kell,[1] S. Kundu,[1] M. A. Haj,[1] A. Goringe[1] & A. K. Burnett[2]

[1]*Department of Haematology, University Hospital of Wales, Cardiff;* [2]*Department of Haematology, University of Wales College of Medicine, Cardiff, UK*

Primary gut involvement by *Aspergillus* is a rare and often fatal complication of intensive chemotherapy in patients with acute leukaemia. We report a patient with toxic mega colon due to invasive aspergillosis of colon postchemotherapy. A 46-year-old patient presented with progressively increasing testicular swelling and following an initial scan he underwent left orchiedectomy. The pathology was consistent with granulocytic sarcoma of testis. The bone marrow was normal with no cytogenetic or molecular marker. In view of aggressive nature of granulocytic sarcoma, he underwent bone marrow harvest followed by ADE induction chemotherapy. During chemotherapy he developed neutropenic fever and was treated with antibiotics and antifungals according to protocol, without any response. Repeated blood culture did not grow any organism. Later he developed clostridium difficile negative diarrhoea. He became extremely unwell and developed massive abdominal distention. The CT scan did not show any pulmonary, liver, splenic or kidney pathology, however, there was diffusely abnormal thickening of bowel wall throughout colon, the appearances resembling pseudomembraneous colitis. He had emergency total colectomy with ileostomy. The pathology showed ischaemic mucosal necrosis of colon and *Aspergillus* fungal hyphae infiltrating into the bowel wall with evidence of immunosuppression suggested by reduced lymphocytes in mesenteric nodes. Postsurgery he was treated with 2 weeks of caspofungin. The patient recovered and is well. The largest series about deep mycotic infections affecting lower intestinal tract in patients with malignancies has reported an incidence of 1.6% including candida and *Aspergillus*. Of these patients isolated *Aspergillus* was seen in only 0.5%. Endoscopic biopsy may help in early diagnosis. Increased awareness of unusual presentation and early surgical intervention with aggressive antifungal treatment, has a key role in the management of these rare and often fatal cases.

61

Should patients who have spontaneous remission of acute leukaemia be treated with chemotherapy?

S. Kundu,[1] J. Kell,[1] U. Mohite,[1] M. Haj[1] & A. K. Burnett[2]

[1]*Department of Haematology, University Hospital of Wales;* [2]*Department of Haematology, University of Wales College of Medicine, Cardiff, UK*

Spontaneous remission (SR) in adult acute leukaemia is rare. The incidence of SR cannot be assessed as patients receive chemotherapy immediately after diagnosis. Infections, blood transfusions and G-CSF have been hypothesized to induce SR. However, SR lasts on an average between 7 and 9 months and outcome is worse after relapse. We report two cases in 2 years from our institution. Patient 1 was a 74-year-old woman admitted with pancytopenia in July 2001. BM examination confirmed biphenotypic leukaemia. Cytogenetic analysis was unsuccessful, molecular screening detected bcr-abl and IgH rearrangements. She had sepsis requiring antifungals and was considered unfit for chemotherapy. Repeat BM examination in September 2001 showed 2% blasts and no molecular and clonal rearrangements indicating SR. She remained well, without treatment until Feb. 2002 but relapsed with biphenotypic leukaemia. IgH was clonally rearranged, bcr-abl negative and cytogenetics showed trisomy 8. She received hydroxyurea and low dose Cytarabine. However, she died in August 2002. The SR lasted 6 months. Patient 2 was a 35-year-old man admitted in August 2003 with atraumatic ruptured spleen and high WBC. BM showed FAB AML

Samsung Bioepis Exhibit No. 1002
Page 1413

M5b with normal cytogenetics. He underwent splenectomy and needed blood transfusions and prolonged antibiotics as he had infected aneurysm of right femoral artery. His blood parameters gradually normalized and repeat BM confirmed CR. He is receiving consolidation treatment according to MRC AML 15 schedule but omitted induction, being in SR and remains in CR at 5 months. There is no uniformity in treatment approach to patients who enter SR. We could not find any literature of patients being treated after attaining SR. All of the reports have deferred therapy till relapse resulting in a poor outcome. There is no literature on the outcome of such patients having chemotherapy once SR has been achieved. A review of available literature with respect to mechanisms and treatment approach together with a patient update will be presented.

62

Clarithromycin, dexamethasone and low dose thalidomide (CDT) is effective therapy in relapsed/refractory myeloma and useful in patients with cytopenias

T. C. M. Morris,[1] D. R. Hull,[2] C. Boyd,[2] F. C. G. Jones,[1] P. J. Kettle,[1] M. Drake,[1] R. McLoughlin[3] & J. Quinn[3]

[1]Department of Haematology, Belfast City Hospital; [2]Craigavon Area Hospital; [3]Clinical Trials Unit, Belfast City Hospital, Department of Haematology, UK

We have shown that Clarithromycin in a previous study has a modest anti myeloma effect (Morris *et al, J Med Oncology* 2001;18:79–84) and more recently a combination of Clarithromycin, Dexamethasone and Thalidomide has been shown to have significant activity in patients with myeloma (Coleman *et al, Leukemia & Lymphoma*, 2002;43:1777–1782). We report a very high response rate in a phase II trial of this combination in which the object was to minimize the doses of Thalidomide and Dexamethasone. Sixteen patients (10M, 6F) with relapsed (12) or refractory (four) myeloma, age range 52–83, from whom informed consent was obtained, were given Clarithromycin 250 mg bd continuously. Dexamethasone 10 mg daily for 4 d, once every 4 weeks and Thalidomide 50 mg nocte. All patients received IV biophosphonates once every 4 weeks. Three patients achieved complete remission, two very good partial response, eight partial response and two minimal response and there was one non-responder. The median response duration is 25 weeks. The median overall survival has not been reached but is greater than 9 months. Most patients had one or more cytopenia at the onset of therapy. Eight patients had thrombocytopenia (median 89×10^9/l, range 14–134); all had significant rises in the platelet count, seven to normal levels. Seven of 11 patients with anaemia had haemoglobin increments of >1 g/dl, five reaching normal levels; of seven patients with neutropenia $<1.9 \times 10^9$/l, five had increases to normal values. These results suggest CDT is an effective therapy, which can be given to patients with significant cytopenias.

63

Clinical and experimental study on cardiac effects of general dosage arsenic trioxide on APL patients

J. Zhou, R. Meng, Y. Liu, W. Wang, B. Li & B. Yang

The first Clinical Medical College of Harbin Medical University, Harbin, China 150001

Objective: Study on cardiac effects of arsenic trioxide (ATO) infusion in routine dosage on acute promyelocyte leukaemia (APL) patients. Methods: We monitored the basal cardiac rates, electrocardiograph, arsenic concentrations in blood circulation of APL patients dynamically, investigated the action potential duration and current of ICa-L in guinea pig cardic ventricular myocytes by patch clamp technique, and assayed the elevated cytosolic calcium of guinea pig ventricular myocytes induced by ATO with laser confocal microscopy. Results: Approximately 52.5% of 40 cases of APL patients manifested cardic side-effects in different degree when ATO intravenous infused in routine dosage at 1 or 2 weeks of the initial therapeutic duration, expressing rapid heart rate or prolonged QT interval. ATO with 1, 2, 5 μmol/l in the medium *in vitro* could prolong the action potential duration of guinea pig cardic ventricular myocytes and increased the ICa-L, meanwhile, elevated cytosolic calcium. Calcium channel blocking agent can cut out the effects. Conclusion ATO intravenous infusion in routine dosage could cause tachycardia and prolong QT interval. The probable mechanisms of these side-effects may be that ATO affected the ion channels and cytosolic calcium.

64

The use of imatinib in the treatment of CML in patients over 65 years in Northern Ireland

M. F. McMullin,[1] M. Humphreys[2] & R. J. G. Cuthbert[3]

[1]Department of Haematology, Queen's University; [2]Department of Cytogenetics, Belfast City Hospital; [3]Department of Haematology, Belfast City Hospital, Belfast, UK

Imatinib was licensed in the UK for treatment of CML in November 2001. Physicians in Northern Ireland have been given access to prescribe the drug at their discretion. We have elected to treat newly diagnosed patients with imatinib and convert patients who were not interferon responders. By December 2003, 17 patients aged over 65 years have been treated with imatinib. We present data on their response. There were 12 patients with CML in chronic phase (CP), two in accelerated phase (AP) and three in blast crisis (BC), (nine males and eight females). The mean age at diagnosis was 74.7 years (range 67–86). Of the 12 patients with CML-CP, nine patients were treated with imatinib as first line therapy. Ten obtained a haematological response (HR) and two were not evaluable (N/E). Three patients had a complete cytogenetic response (CtgR), and one a major CtgR. Six were N/E as they did not continue the drug long enough or are too early to assess. There were two early deaths, one from BC and one from old age. Two patients had interrupted treatment due to grade 3/4 haematological toxicity and one interrupted treatment due to non-haematological toxicity. The two patients with CML-AP had HR but no CtgR was seen. One patient died after 16 months and one is still alive after 24 months treatment. The three patients with CML-BC all had a HR, one had a major CtgR and two no CtgR. Two died after 11 and 15 months and one is still alive and well after 34 months of therapy (this patient was in the expanded access programme). Imatinib is well tolerated in the majority of our elderly patients. It provides good palliative care in many and produces cytogenetic responses in some. This may translate into increased survival even in older patients.

65

Effect of Gemtuzumab Ozogamicin on the proliferation of acute myeloid leukaemia blast cells *in vitro*

K. L. Morris, J. A. Adams, M. Blackburn & J. A. Liu Yin

University Department of Clinical Haematology, Manchester Royal Infirmary, Manchester, UK

Although current treatment strategies induce remission in most AML patients, 70% subsequently relapse, engendering interest in novel therapies. Gemtuzumab Ozogamycin (GO or Mylotarg) comprises humanized anti-CD33 antibody conjugated to the antibiotic calicheamicin. CD33 is expressed on the cell surface of leukaemic blasts in 90% of AML patients. On binding to CD33, GO is internalized, the caliche-

Samsung Bioepis Exhibit No. 1002
Page 1414

amicin portion is released and binds to DNA. We investigated effects of GO on AML blast proliferation, viability and apoptosis *in vitro* and related this to absolute amounts of CD33 expressed on the cell surface. Presentation blasts from 21 AML patients isolated from BM or PB were cultured in 96-well plates at 1×10^6 cells/ml in McCoy's medium supplemented with 15% FCS, GM-CSF (100 ng/ml), SCF and IL3 (10 ng/ml). GO, an irrelevant control antibody (HcT-MO1) and unconjugated calicheamicin (CAL) were added at concentrations of 1–1000 ng/ml. Cultures were incubated at 37°C in 5% CO_2, 5% O_2 and 90% N_2. Apoptosis assays were performed between 18 and 96 h of incubation using an annexinV-PE apoptosis detection kit. Tritiated thymidine uptake to measure cell proliferation and WST-1 assays to measure cell viability were performed on day 5 of incubation. CD33 expression was quantified at presentation by flow cytometry using anti-CD33-conjugated 1:1 with PE. Incubation with GO resulted in dose dependent inhibition of proliferation but no decrease in cell viability was detected, control antibody HcT inhibited cell proliferation but to a far lesser extent than GO. Apoptosis was observed in only two of 21 patients. A wide range of CD33 expression was observed on AML blasts (range 2186–25 476 molecules/cell). No correlation between FAB subclass and CD33 level or between CD33 expression and sensitivity to GO was observed, however further data is being accrued. This study is also being expanded to include determination of MDR status, and possible synergy between GO and other commonly used chemotherapeutic agents.

66

Sensitivity and As_2O_3 concentration in cell

J. Zhou, R. Meng, X. Guan, L. Li & B. Yang

Harbin Medical University, Harbin, 150001, China

Arsenic trioxide (As_2O_3) is an effective drug for treatment of acute promyelocytic leukaemia (APL) and other malignant tumours are being reported. However, it is not clear whether sensitivity has been associated with As_2O_3 concentration in target cells. In this study, As_2O_3 concentration and susceptibility in subtype leukaemia and solid carcinoma cells were studied and compared using atomic fluorescence to detect arsenic concentrations and FITC-annexin V and propidium iodide (PI) double staining to measure cell death. The results showed that after maintenance of cells in the presence of As_2O_3, intracellular concentrations of arsenic in different cell lines differ. Our results indicate that the cell lines have concentrations of As_2O_3 NB4 cell > APL primary cell > K562 cell > CML primary cell > HL-60 cell > AML-M2 primary cell > Hela cell > H-22 cell > SGC-7901 cell > cancer of larynx Hep-2 cell. In changing culture medium with low As_2O_3 concentration and long incubation time, higher intracellular As_2O_3 concentrations in NB4, K562 and APL cell strains can be obtained. These results indicate that different leukaemia and solid carcinoma cell lines have different intracellular arsenic concentrations suggesting that leukaemia and solid carcinoma cells may have different sensitivities to As_2O_3 in clinical treatment. The process of As_2O_3 uptake appears to be via an active transport mechanism. Proper administration of As_2O_3, i.e. maintaining continuous low but effective arsenic levels in blood, may increase the efficiency of anti-carcinoma therapy and decrease the side-effects of As_2O_3 treatment.

67

Random and contrast study on two arsenic trioxide administration methods in APL therapy

J. Zhou, R. Meng & B. Yang

Department of Haematology, Harbin Medical University, Harbin, China 150001

Objective: To assess the effectiveness and security of two arsenic trioxide administration methods in acute promyelocytic leukaemia treatment.

Methods: Ninety-six cases of APL treated by arsenic trioxide with two administration methods have been studied randomly; 48 cases were administrated arsenic trioxide with routine method as control, the other 48 cases were given arsenic trioxide with a new method. First, calculated the total quantity of As_2O_3 needed every day of every patient according to the criteria of 0.16 mg/kg, then gave half of the total dosage in the morning and the other half in the afternoon, diluted each half of total amount of As_2O_3 with 5% glucose 250 ml and infused at the speed of 8 drops/min, collected the blood serums of the patients treated with the two methods respectively in different time points, assay arsenic concentration in blood serums.

Results: Most of the APL treated by As_2O_3 gained complete remission at the time of 4th week with the two administrated methods. The total effective rate in new method group was 93.75%, in control group was 83.3%. The effective arsenic level in blood circulation in new method group was lasted at least 18.38 h, while in control group was lasted to 9.38 h, the average hospitalization duration in new method group was 26.4 d, in control group was 35.7 d.

Conclusions: The new method can relieve the side-effects of As_2O_3 and increase complete remission (CR) rate of APL, and accelerate to CR.

68

Arsenic trioxide inhibits proliferation and down-regulates myc-associated zinc finger protein (MAZ) in primary acute myeloid leukaemia (AML) cells

N. Gebril, M. Sweeney, A. Tonks & A. K. Burnett

Department of Haematology, University of Wales College of Medicine, Cardiff, UK

Recently, arsenic trioxide (ATO) was found to be an effective treatment of many haematological malignancies such as refractory/relapsing acute promyelocytic leukaemia (APL) and multiple myeloma (MM). It has been reported that ATO induces apoptosis, inhibits angiogenesis and proliferation of these cells, however, the mechanism of action has not yet been elucidated. At the level of gene transcription, ATO may alter the expression of important genes, which may explain the observed effects. Therefore, the aims of this work were to determine the effects of ATO on both proliferation and MAZ expression in cells from patients with AML. Mononuclear blast cells from different subtypes of AML were treated with ATO (0.5–10 μmol/l). The MTS assay (bio-reduction of tetrazolium) was used to assess the effect of ATO on proliferation. MAZ expression was detected by real-time (RT) PCR using the lightcycler. ATO significantly reduced the proliferation of AML cells when used at 4, 2 and 1 μmol/l of ATO after 24, 48 and 72 h respectively when compared to the control ($P < 0.001$). RT-PCR analysis showed that there was a down-regulation of MAZ after treatment of the cells with 2 μmol/l ATO for 24 h (approximately 50% reduction). Together, these data indicate that ATO inhibits proliferation of primary AML cells in dose and time dependent manner. In addition, the effect of ATO on MAZ might be closely related to its therapeutic effect, as MAZ is important for regulatory function of c-myc. Overall, this might suggest that ATO is also effective in the treatment of the different types of AML other than refractory APL.

69

Audit of febrile neutropenia in patients with acute leukaemia in a district general hospital

S. Mathai,[1] H. Edwards,[2] K. Hunter,[3] P. J. Mounter[1] & Z. T. Maung[1]

[1]*Department of Haematology, University Hospital of North Tees, Stockton on Tees;* [2]*University of Glasgow;* [3]*Department of Microbiology, University Hospital of North Tees, Stockton on Tees, UK*

The pattern of infecting organisms in febrile neutropenia has changed over the last few years. The pattern may also be different in tertiary

Samsung Bioepis Exhibit No. 1002
Page 1415

referral centres and district general hospitals. An audit of febrile neutropenia in acute leukaemia patients, over 2 years in the University Hospital of North Tees which is a district general hospital was analysed. Twenty-three and 10 patients with acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL), respectively, developed 344 episodes of febrile neutropenia between 1 January 2001 and 31 December 2002. Blood cultures were taken at each episode, and positive results were yielded in 72 episodes (20.9%). Gram-negative bacilli were isolated in 51 episodes, Gram-positive cocci were isolated in 41 and *Candia albicans* in one. Most Gram-negative bacilli were sensitive to tazocin and gentamicin *in vitro*. There were eight episodes of blood cultures positive for *Steanotrophomonas maltophilia*, and the organism was resistant to almost every antibacterial *in vitro* and *in vivo*. This organism was always associated with an indwelling central venous catheter and pyrexia settled only by catheter removal. Gram-positive infections were less than expected, and coagulase-negative staphylococcal infections ($n = 28$) were the commonest with no serious clinical sequelae. There were three fatal cases with positive isolates, and two had mixed infections. *Pseudomonas aeruginosa* and group G β-haemolytic streptococcus were isolated in two of fatal cases. In summary, empirical treatment of febrile neutropenia remains to be directed against Gram-negative infections particularly *Pseudomonas*. *S maltophilia* infection must be suspected if there is persistent pyrexia despite treatment in patients with Hickman lines and catheter removal is the only effective treatment. First-line therapy does not warrant a Gram-positive cover, but once an isolate is yielded, the cover should include agents against group G β-haemolytic streptococci.

Poster Presentation

Paediatric and Red Cell Disorders

70

Intermediate purity factor VIII concentrate BPL 8Y as prophylaxis and treatment in congenital thrombotic thrombocytopenic purpura

M. Gattens,[1] K. Khair,[1] S. J. Machin[2] & R. Liesner[1]

[1]*Department of Haematology, Great Ormond Street Hospital;*
[2]*Department of Haematology, University College Hospital, London, UK*

Congenital thrombotic thrombocytopenic purpura (TTP) is characterized by recurrent episodes of microangiopathic haemolytic anaemia and thrombocytopenia, and usually presents in the first 5 years of life, due to a deficiency of von Willebrand factor (VWF)-cleaving protease. Fresh frozen plasma (FFP) has been used to treat acute episodes and to prevent recurrent haemolysis. The intermediate purity factor FVIII concentrate BPL 8Y has been shown to have VWF-cleaving protease activity of at least 100% compared with pooled normal plasma, and its use has been reported in one patient with congenital TTP. We report the successful prophylactic and therapeutic use of BPL 8Y in a further three children with congenital TTP. All three children had evidence of low-grade chronic haemolysis prior to starting treatment and had previously been treated with FFP. They were also shown to have normal coagulation screening tests and factor VIII levels, and VWF-cleaving protease activity between 3% and 22%. One child was treated with one dose of 250 IU of BPL 8Y (30 IU/kg) with each acute episode, with rapid improvement in her haemoglobin and platelet count, sustained for 2–3 weeks. Two other children, both siblings with identified mutations in the ADAMTS13 gene, were treated initially with single doses of BPL 8Y 250 IU with each haemolytic episode. Both brothers had frequent recurrences of symptoms with viral infections and immunizations, and were changed to a prophylactic regimen of BPL 8Y 20 IU/kg every 2–4 weeks. This resulted in a marked reduction in frequency and severity of haemolytic episodes and hospital admissions. Factor VIII levels were raised transiently to a maximum of 220 IU/l following treatment with BPL 8Y during acute episodes, but with no adverse consequences. BPL 8Y provides an effective treatment for congenital TTP, and has the advantage of being suitable for home therapy.

71

Improved engraftment and survival rates in unrelated donor stem cell transplantation in MPS 1H (Hurler's syndrome): a review of three UK centres

E. Tholouli,[1] J. Grainger,[1] A. S. Hassan,[2] J. Motwani,[3] A. M. Will,[1] P. A. Veys,[2] P. J. Darbyshire[3] & R. F. Wynn[1]

[1]*Paediatric Bone Marrow Transplant Unit, Royal Manchester Children's Hospital, Manchester;* [2]*Paediatric Bone Marrow Transplant Unit, Great Ormond Street Hospital for Children, London;* [3]*Paediatric Bone Marrow Transplant Unit, Birmingham Children's Hospital, Birmingham, UK*

Unrelated donor stem cell transplantation (SCT) in patients with severe MPS 1H (Hurler's syndrome) has been reported to have poor outcome. Registry data shows a long term, engrafted survival of 35%. This data have influenced parent's adversely about unrelated donor SCT when no HLA-matched sibling was available. We report the results of unrelated donor SCT in MPS 1H from three UK centres: Manchester, Great Ormond Street and Birmingham. Methods: between January 2000 and December 2003 seven patients with MPS 1H underwent SCT from an unrelated donor. Median age at time of transplantation was 17 months (range 14–22 months). Three patients received conditioning with Fludarabine 30 mg/m^2/d × 5, Busulphan 5 mg/kg/d × 4, Cyclophosphamide 50 mg/kg/d × 4 and rabbit Anti-Thymocyte Globulin (ATG) 2.5 mg/kg/d × 3. Two patients received Fludarabine 30 mg/m^2/d × 5, Busulphan 5 mg/kg/d × 4, Cyclophosphamide 50 mg/kg/d × 4 and Alemtuzumab 0.2 mg/kg/d × 5. One patient received Melphalan 125 mg/m^2 × 1, Cyclophosphamide 50 mg/kg/d × 4 and ATG 2.5 mg/kg/d × 3. One patient received Fludarabine 30 mg/m^2/d × 5, Busulphan 5 mg/kg/d × 4, Cyclophosphamide 50 mg/kg/d × 4. All patients were given Cyclosporin A (CsA) as graft-versus-host disease (GvHD) prophylaxis.
Results: All conditioning regimens were well tolerated. Six of seven patients (86%) survived with a median follow up of 25 months (range 7–45 months). All long-term survivors show full donor engraftment. One of seven patients died 44 d post-SCT with grade 4 GvHD of skin, liver, gastrointestinal tract and lungs. Conclusions: We show improved engraftment and long-term survival in our patients with MPS 1H undergoing unrelated donor SCT when compared with historical data. We believe this is due to more intensive conditioning regimens used. Improving alternate donor transplant results in MPS 1H should be communicated to families to add decision making prior to SCT.

72

A possible role for recombination activating gene-1 (*RAG-1*) allotypes in the aetiology of childhood T-cell acute lymphoblastic leukaemia

H. P. O'Brien,[1] O. B. Eden,[2] J. M. Birch[3] & G. M. Taylor[1]

[1]*Department of Cancer Immunogenetics, University of Manchester;* [2]*Academic Unit of Paediatric Oncology, Christie and Royal Manchester Childrens Hospital (RMCH);* [3]*CRUK Paediatric and Familial Cancer Group, RMCH, Manchester, UK*

The recombination activating genes (*RAG-1*, *RAG-2*) are required for the targeted recombination of the T-cell receptor (TCR) and immunoglobulin (Ig) genes, necessary to produce antigen receptor diversity in the developing immune system. RAG1/2 proteins introduce DNA

Samsung Bioepis Exhibit No. 1002
Page 1416

double strand breaks at recombination signal sequences (RSS) flanking the TCR and Ig coding segments prior to recombination. Evidence that certain types of acquired chimaeric oncoproteins in childhood leukaemia have rearranged TCR genes suggest that they may arise from illegitimate RAG-mediated DSB. About 10% of childhood acute lymphoblastic leukaemias arise in T-cell progenitors, and carry activated oncogenes arising from the fusion of one of the TCR genes to one of several transcription factor genes. We have investigated the possibility that variation in the RAG proteins might influence their recombinogenic function and lead to childhood leukaemia. We screened constitutional DNA from 96 children with ALL and 190 normal infant controls for *RAG-1/2* polymorphic variants by SSCP and sequencing. We found a lineage-specific bias in the frequency of the *RAG-1* nt.858G/A (RAG-1 R249H) bi-allelic polymorphism in T-ALL ($n = 22$) compared with c-ALL ($n = 54$) and pro-B ALL ($n = 18$). The frequency of HH homozygotes was lower in T-ALL (31.8%) than in c-ALL (51.9%) or pro-B ALL (66.7%), whilst the frequency of RR homozygotes was higher in T-ALL (18.2%) than in c-ALL (5.6%) or pro-B ALL (0.0%). There was a similar but less pronounced bias when 73 T-ALL were compared with 144 c-ALL and 28 pro-B ALL. Although HH homozygotes were less frequent in T-ALL, and RR homozygotes more frequent, the differences were not statistically significant, possibly because of the molecular heterogeneity of the T-ALLs. However, these data suggest that lineage-related differences in the function of RAG allotypes increase the risk of leukaemia possibly by predisposing to aberrant TCR recombination.

73

Assessment of liver disease in transfusion dependent thalassaemia major

R. Thiagarajan,[1] S. Mahadevan,[2] R. Brown,[1] P. McKiernan,[1] P. Darbyshire[1] & S. Lawson[1]

[1]Birmingham Children's Hospital; [2]University Hospital of North Staffordshire, UK

Thalassaemia major (TM) is the commonest hereditary form of transfusion dependent anaemia in the United Kingdom. Although with advanced treatment, the life expectancy has improved, complications of iron overload are still common.

Aims: To study the prevalence and severity of liver disease in transfusion dependent TM. To correlate histological changes and liver iron content with non-invasive markers of iron status. To evaluate the relationship between annual red cell consumption (ARC), therapeutic index (TI) and liver iron content (LIC).

Method: Retrospective case notes study of patients undergoing liver biopsy to assess hepatic iron. Mean serum ferritin, ALT level, ARC and TI were calculated for the year/year before the biopsy was done. The continuous variables were expressed as mean ± standard deviation and Student's *t*-test was used for statistical analysis.

Results: Fifty patients (males 24, females 26) were included; 33 patients underwent allogenic BMT. The median age at biopsy was 7.61 ± 4.58 years, at diagnosis 6 months (range 0–42) at starting transfusion 10 months (4–136) and at chelation 41 months (18–177). Liver biopsies were analysed using Ishak scoring system.40% had raised ALT, 62% showed haemosiderosis Grade >3 and 40% had hepatic fibrosis. All patients were seronegative for hepatitis C. The table attached shows the histological findings and associated factors.

Conclusion: Age and serum ferritin level could not predict hepatic fibrosis or LIC. ALT was, however, predictive of the presence of fibrosis. There was no direct relationship between ARC,TI and hepatic fibrosis or LIC, although there was a trend for those with increased ARC to have hepatic fibrosis. Liver biopsy remains necessary to quantify iron overload and provide reliable information on hepatic fibrosis. Serum ALT and ARC may be non-invasive techniques that can help predict fibrosis, although more studies are needed.

Fibrosis	*n*	Age at biopsy (years)	Ferritin	ALT (IU/l)	LIC (mg/g)	Red cell Tx (ml/kg)	TI
Present	20 (40%)	7.93 ± 4.58	2295 ± 1069	75 ± 86.4	1.48 ± 1.04	177.7 ± 42.4	0.02 ± 0.01
Absent	30 (60%)	7.37 ± 4.34	1954 ± 1015	44 ± 34	0.78 ± 1. 02	155.8 ± 37.6	0.019 ± 0.01
P-value		NS	0.14	0.04	0.02	0.05	0.46

74

Enzyme replacement therapy prehaematopoietic stem cell transplantation (HSCT) in Hurler's disease

J. Motwani,[1] A. Chakrapani,[1] P. J. Darbyshire,[1] J. Wright,[1] R. G. F. Gray,[1] A. Cooper,[2] J. E. Wraith[2] & S. E. Lawson[1]

[1]Birmingham Children's Hospital, Birmingham; [2]Royal Manchester Children's Hospital, Manchester, UK

Deficiency of α-L-iduronidase results in the accumulation of glycosaminoglycans (GAGs) in many tissues and a chronic progressive disorder known as mucopolysaccharidosis I (MPS I). The clinical features include organomegaly, dysostosis multiplex, facial dysmorphia, hearing, vision, pulmonary, and cardiovascular dysfunction. GAGs are typically excreted in large quantities in urine and often detected in the initial diagnostic screening tests. Patients with MPS I are classified into three clinical syndromes namely Hurler, Hurler-Scheie and Scheie. Progressive neurodegeneration and death in early childhood are prominent features of Hurler Syndrome (MPS IH). HSCT represents the only effective long-term therapy for selected MPS 1H that prevents neurodegeneration. HSCT is high risk; complications of GvHD are exacerbated by airway disease and cardiomyopathy. Trials with recombinant enzyme replacement therapy (ERT) are ongoing in patients without suitable donors, or those not eligible for HSCT. We report the first patient to receive pre-HSCT ERT. Our patient was diagnosed to have Hurler's disease at 15 months age, at which time he had hepatosplenomegaly, cardiomyopathy and developmental delay. He commenced ERT at 18 months age, while awaiting HSCT. The schedule used was 100 IU/kg as weekly infusions. There were no adverse reactions. The urinary GAGs (quantitated by dimethylbenzadine) were nearly normal after 4 weeks of ERT, and continue to remain normal 2 months post-HSCT. At the time of HSCT his cardiomyopathy and hepatosplenomegaly had completely resolved. He received a successful MUD HSCT 14 weeks after commencing ERT, after being conditioned with busulphan, cyclophosphamide, fludarabine. He engrafted 2 weeks post-HSCT and is fully donor chimaeric. His transplant course was uncomplicated, enzyme levels have been normal post-HSCT. Based on these very promising results, we recommend pre-HSCT ERT, as it significantly improves clinical symptoms. A predicted benefit should be a reduction in transplant-related morbidity and mortality.

75

Outcome of sagittal sinus thrombosis associated with asparaginase therapy in childhood acute lymphoblastic leukaemia (ALL)

R. D. Faulkner, J. C. Welch & A. J. Vora

Department of Paediatric Haematology, Sheffield Children's Hospital, Sheffield, UK

We report two children with acute lymphoblastic leukaemia (ALL) and asparaginase-related sagital sinus thrombosis who had an excellent

Samsung Bioepis Exhibit No. 1002
Page 1417

neurological outcome and were able to resume asparaginase under anti-coagulant cover without recurrence of thrombosis. Patient 1 was a 6-year-old girl with low white cell count common ALL. At D15 of MRC ALL 97/01 induction, following three doses of *E. coli* asparaginase (Elspar, 6000 units/m^2), she developed generalized seizures and head CT scan revealed sagittal sinus thrombosis. AT III level was slightly subnormal at 0.78 units (0.84–1.16), Protein S and C levels normal. She made a full neurological recovery from mild postictal right hemiparesis over 48 h. Although, the remaining induction doses were omitted, she was re-exposed to asparaginase during two intensification courses (Elspar and Oncaspar with DI I and II respectively) under cover of Enoxaparin 20 mg s.c. daily for 21 d without recurrence of thrombosis. Patient 2 was a 2-year-old girl with low white cell count common ALL. She achieved rapid early response to MRC ALL 97/01 regimen A. At D11 of induction, following four doses of Elspar, she developed focal seizures. Head CT confirmed thrombosis of the superior sagittal and right transverse sinuses with associated parafalcine haemorrhages. Her AT III level was normal at 0.99 units, Protein S and C levels were within reference range. She later developed dense left-sided weakness. Repeat scan revealed progressive haemorrhagic infarction in the right front-temporal region with surrounding oedema. However, she made a full neurological recovery over 2 weeks and went on to receive Elspar under cover of Enoxaparin 20 mg s.c. daily for 21 d during two intensification courses without recurrence of thrombosis. Our experience demonstrates that asparaginase related SST has an excellent neurological outcome in children with ALL and it is feasible to recommence asparaginase treatment in such patients. The use of low dose Enoxaparin in this setting was not associated with adverse effects.

76

Toxicity associated with ELSPAR asparaginase in the induction–remission phase of treatment for childhood ALL

C. A. MaCartney, S. I. Dempsey, B. Jackson & A. McCarthy
Department of Paediatric Haematology and Oncology, Royal Belfast Hospital for Sick Children, Falls Road, Belfast BT12 6BA, UK

L-asparaginase reduces L-asparagine required for protein synthesis in leukaemic lymphoblast cells but also normal cells of the liver resulting in hepatotoxicity and coagulopathy. In our centre children are treated according to the MRC ALL 97 (revised 99) protocol. In April 2001 the use of ERWINIA was changed to the *E. coli* derived form ELSPAR following studies demonstrating a better overall and event-free survival. The aim of this study was to look at the toxicity of ELSPAR in all children treated from April 2001 as we had noted a higher incidence of toxic side-effects than had occurred previously. A retrospective analysis was performed of 19 children treated for ALL and T-cell non-Hodgkins lymphoma over a 2-year period. ELSPAR 6000 IU/m^2 was commenced on day 3 of induction therapy with a total of nine doses administered on alternate days. Eight of 19 children had symptoms including vomiting, abdominal pain and diarrhoea. Sixteen patients had an albumin level below 30 g/l, four were below 20 g/l in a symptomatic child requiring intravenous albumin infusion. Nine children had a significant rise in serum transaminases and those under 5 years had the most marked toxicity. No elevated serum amylase was recorded. One child had an axillary vein thrombosis. The antithrombin III level and fibrinogen levels were low (0.6 g/l and 0.73 IU/ml respectively). Toxicity is thus demonstrated in children who have received at least six doses of asparaginase. Studies have shown differing pharmacokinetic properties in preparations of asparaginase although no dose modifications have been made since changing the preparation. Routine coagulation screens have not been recommended during induction chemotherapy or guidelines suggested for management of coagulopathy or thrombotic events. In conclusion, children treated with ELSPAR have a better overall and event free survival but an apparent increase in toxic side-effects.

77

Role of serum methylmalonic acid, homocysteine and transcobalamin II in the diagnosis of vitamin B12 deficiency

A. P. Goringe,[1] R. Ellis,[1] I. F. W. McDowell,[2] C. C. Butler[3] & M. Worwood[1]
[1]Department of Haematology, University Hospital of Wales and University of Wales College of Medicine; [2]Department of Medical Biochemistry, University Hospital of Wales; [3]Department of General Practice, University of Wales College of Medicine, Cardiff, UK

Patients in the UK who are diagnosed as B12 deficient currently receive lifetime treatment with intramuscular B12. However, diagnosis of B12 based on measurement of total B12 concentrations may be unreliable and therefore patients may be receiving unnecessary treatment. We aimed to identify additional factors that may allow clinicians to effectively target B12 treatment to only those who would benefit. Patients ($n = 50$) were recruited from those who had samples submitted to the laboratory with serum B12 < 170 μg/l. Exclusions were age >75, liver disease, hypothyroidism, alcohol abuse, folate deficiency, paraproteinaemia. Following clinical assessment further blood samples were taken and treatment was administered with intramuscular B12 (1 mg/week for 4 weeks, then 1 mg/month) and evaluated after 3 months. At the first consultation transcobalamin II (TCII) concentration was low (<38 pmol/l) in 96%, MCV was >99 fl in 43%, methylmalonic acid (MMA) >0.56 μmol/l in 40% and homocysteine >15 μmol/l in 54% of patients; 12% were anaemic, including three iron deficient women. Following 3 months B12, 52% of patients showed no response, 20% showed a biochemical response (abnormal MMA falling to less than 0.56 μmol/l), 9% a haematological response (abnormal MCV falling to <100 fl) and 18% a combined response. All raised MMA concentrations reverted to normal after therapy but for those with raised homocysteine levels 54% remained high. There was little correlation between biochemical and haematological responses and patients' assessment of their overall well-being after treatment. Six patients described a major, short-lived improvement in well-being. These data suggest that approximately half of patients with 'low' total B12 patients may not be B12 deficient and that further evaluation with haematological indices, MMA, intrinsic antibody status and a therapeutic trial may be helpful before advocating life-long B12 therapy.

78

Role of hydroxyurea (HU) in children with sickle cell disease (SCD)

J. Motwani, S. E. Lawson & P. J. Darbyshire
Department of Haematology, Birmingham Children's Hospital, Birmingham, UK

The short-term beneficial effects of hydroxyurea (HU) in sickle cell disease (SCD) has been shown in several studies in children and adults.. However, doubts remain both about its efficacy and safety in the long term. We report a single centre experience of treating 16 patients (15) with homozygous SCD and (1) with Hb SC disease with HU. Complete follow-up data is presently available for nine patients as seven patients were transferred to adult service (four) or other centres (three). These patients have received HU for a median of 2 years (0.5–3.5 years). They were commenced on HU for recurrent painful crisis (seven), recurrent chest crisis (two), poor growth and failure to thrive in conjunction with recurrent crisis (two) causing a lot of morbidity, prolonged and recurrent hospital admissions and school absenteeism, etc. Four of the patients had received a trial of

Samsung Bioepis Exhibit No. 1002
Page 1418

blood transfusions prior to trial of HU. Clinically, eight children had significant improvement in crisis, hospital admissions and general well being. One patient did not observe any improvement, hence HU was stopped after trial of 6 months. In another child HU was stopped after 6 months as he had a further chest crisis on HU. Laboratory parameters were assessed, comparing mean cell volume (MCV), haemoglobin (Hb) and foetal haemoglobin (HbF) before starting HU and 6 months after commencing HU. Improvement was observed in all these parameters and the difference was statistically significant (P-value = 0.001, 0.005 and 0.0002 respectively for MCV, Hb and HbF respectively). We did not observe any side-effects due to HU in any of the children. We conclude that HU leads to improvement of Hb, HbF and MCV in most patients with SCD and clinical improvement is also observed in children with recurrent painful crisis, and chest syndromes. Further multicentre studies are needed to study these parameters in detail.

79

The C598T *VHL* mutation arose in an ancestor common to Asian and Caucasian populations

M. J. Percy,[1] E. Liu,[2] C. Amos,[2] S. O. Ang,[2] N. Cole,[3] V. Gordeuk,[4] Y. Guan,[2] K. Jedlickova,[2] S. N. Jowitt,[5] M. F. McMullin,[6] Y. D. Pastore,[2] M. Potter,[7] S. Shete,[2] D. W. Stockton,[2] S. L. Thein,[8] M. Treacy,[9] W. H. Watson,[10] T. R. J. Lappin[6] & J. T. Prchal[2]

[1]Department of Haematology, Belfast City Hospital, Belfast, UK; [2]Baylor College of Medicine and VA Hospital, Houston, TX, USA; [3]Department of Haematology, Royal Hospital for Sick Children, Yorkhill, Glasgow; [4]Howard University, Washington DC, USA; [5]Department of Haematology, Stepping Hill Hospital, Stockport, Cheshire; [6]Department of Haematology, Queen's University, Belfast; [7]Department of Haematology, Royal Free Hospital, London; [8]Department of Haematology, King's College, London; [9]Department of Haematology, Chase Farm Hospital, Enfield, Middlesex; [10]Department of Haematology, Monklands Hospital, Airdrie, Lanarkshire, UK

The hypoxia-inducible factor (HIF) transcription complex controls a diverse range of cellular processes including erythropoiesis and angiogenesis, which are regulated by hypoxia. The HIF dimeric complex is composed of two subunits, an α subunit and a β subunit (also known as ARNT). In normoxia HIF-1α is maintained at a low level by association with the von Hippel–Lindau protein (pVHL), followed by ubiquitin-induced degradation by the proteasome. Recently, we identified a C598T base change in the VHL gene from individuals with Chuvash polycythaemia and this was the first familial defect of hypoxia sensing. Subsequently, a further five Asian and seven Caucasian families were also found to possess the C598T *VHL* mutation. The detection of this mutation in a diverse range of ethnic groups suggested that its incidence and geographical distribution were greater than initially suspected. To investigate if there had been recurrent mutational events in human evolution eight single-nucleotide polymorphisms (SNPs) covering 340 Kb spanning the *VHL* gene were utilized to define haplotypes for 101 subjects bearing the C598T *VHL* mutation. In addition, haplotypes for 447 normal unrelated individuals from Chuvash, South-East Asian, Caucasian, Hispanic and African-American ethnic groups were defined. Analysis of the observed haplotypes indicated the presence of one core haplotype of six SNPs and two extended haplotypes. It was found that 99.2% of Chuvash and non-Chuvash individuals homozygous for C598T *VHL* mutation shared the core haplotype, whilst 96.5% shared the two extended haplotypes. Using the frequency of divergence from the core haplotype in those individuals bearing C598T *VHL* mutation allowed us to estimate that the C598T *VHL* mutation originated at least 14 000 years ago. Thus we conclude that the C598T *VHL* mutation, the most common genetic defect of congenital polycythaemia and familial erythrocytosis, has arisen only once in an ancestor common to Asian and Caucasian populations.

80

Free circulating DNA as a biomarker for severity and prognosis in sickle cell disease

S. M. Menzel,[1] S. Kondaveeti,[1] K. Ramasamy,[2] T. Sugai,[2] G. Cheung,[2] M. Awogbadi,[2] J. Cunningham,[1] D. C. Rees[1,2] & S. L. Thein[1,2]

[1]Department of Haematological Medicine, GKT School of Medicine, Kings College London, London; [2]Department of Haematological Medicine, Kings College Hospital, Denmark Hill, London, UK

Free circulating DNA has been shown to be increased in various conditions characterized by increased cell death through necrosis and apoptosis such as cancer and trauma. In sickle cell disease (SCD), an increased cell turnover can be expected through haemolysis and the recurrent episodes of vaso-occlusion causing inflammation, and eventually cell death involving various organs. We propose that the level of free plasma DNA would be elevated in patients with SCD and that these elevations fluctuate with acute crises, and that the extent of increase can serve as a marker of severity of progression of disease. Plasma samples were collected from 28 normal Afro-Caribbean and West African individuals (AA), 62 sickle cell patients (ages 13–67 years), of which 54 were observed in steady state and 12 during an acute crisis. Four patients were studied during steady state and crises. Plasma DNA concentration was measured by real-time quantitative PCR using a probe specific for the β globin gene. The mean plasma DNA levels in genome equivalent (GE)/ml was 1029 (range 236–2980) in AA individuals, 4943 (range 340–38 988) in SCD during steady state, and 7833 (range 1142–36 284) in SCD during crises. The difference in the mean plasma DNA values between AA and SCD patients in steady state and in crises was highly significant, with P-values of <0.008 and <0.0003 respectively. Mean plasma DNA values for sickle patients during steady state vs. crisis were also significant at $P < 0.03$. Plasma DNA values showed no correlation with total white cell counts, haemoglobin, reticulocyte or CRP levels. Our preliminary studies show that circulating DNA concentration may be a reliable biomarker in SCD. We are currently exploring the value of serial plasma DNA levels in longitudinal studies and their association with severity of organ damage in SCD.

81

Surgery, preoperative transfusion and postoperative complications in patients with sickle cell disease: a survey in England

J. Buck,[1] A. Casbard,[2] S. C. Davies[3] & L. Williamson[4]

[1]NBS/MRC Clinical Studies Unit, Cambridge; [2]MRC Clinical Trials Unit, London; [3]Imperial College/Central Middlesex Hospital, London; [4]University of Cambridge, National Blood Service, Cambridge, UK

It has previously been shown that exchange transfusion (ET) offers no advantage over top-up in preoperative preparation of patients with sickle cell disease (SCD) (Vichinsky *et al*, *New England Journal of Medicine*, 1995;333:206–214). To assess the feasibility of an RCT to compare transfusion with no transfusion, we examined surgical procedures, transfusion practice and outcomes in patients with SCD. All hospitals with SCD populations in England were invited to submit data prospectively on patients with SCD having elective surgery between May 2002 and May 2003. A standard data collection form was used to capture history of sickle complications, genotype, surgical procedure, preoperative transfusion, sickle related and other postoperative complications. Data were returned on 114 patients from 21 hospitals. SC/Sβ$^+$thal accounted for 47% untransfused patients, but only 3% of patients who received top-up, and 12% of patients who received ET.

Samsung Bioepis Exhibit No. 1002
Page 1419

Patients with history of sickle complications were more likely to receive a preoperative transfusion. ET was mainly used for moderate risk procedures such as joint replacement, but for low risk procedures no transfusion and top-up were equally prescribed. Untransfused patients underwent mainly obstetric and gynaecological procedures while the top-up group included largely paediatric ENT procedures. Patients having cholecystectomy were as likely to receive a transfusion as not. Postoperative sickle complications were seen in both the top-up and untransfused groups. The rate of 'other' complications (surgical/infections/transfusion) was highest in the top-up group. We conclude that for patients with SS/Sβ^0thal, transfusion practice and outcomes are sufficiently variable to suggest that an RCT including no transfusion continues to be justified.

Table. Patient characteristics, surgery risk and postoperative complications

	None SS/Sβ^0thal ($n = 26$)	None SC/Sβ^+thal ($n = 23$)	Top-up ($n = 39$)	Exchange ($n = 26$)
Age (median and range)	28 (2–45)	33 (5–58)	11 (1–64)	26 (2–40)
Females	19 (73)	15 (65)	20 (51)	15 (58)
History of sickle complications	16 (62)	15 (65)	29 (74)	20 (77)
Surgical risk: low	11 (42)	10 (43)	18 (46)	6 (23)
Surgical risk: medium/high	15 (58)	13 (57)	21 (54)	20 (77)
Postoperative complications	5 (19)	4 (17)	10 (26)	4 (17)*
Sickle	4 (15)	2 (9)	3 (8)	0*
Other	5 (19)	3 (13)	12 (31)	5 (22)*

*Incomplete data ($n = 23$). Values are given as n (%).

82

The Pro582Ser polymorphism in the VHL binding site of HIF-1α is present in patients with idiopathic erythrocytosis but does not diminish binding to pVHL

M. J. Percy,[1] S. M. Mooney,[2] A. Flores,[2] M. F. McMullin,[3] T. R. J. Lappin[3] & F. S. Lee[2]

[1]*Department of Haematology, Belfast City Hospital, Belfast, UK;* [2]*Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, USA;* [3]*Department of Haematology, Queen's University, Belfast, UK*

Many cellular processes including, erythropoiesis, are regulated by hypoxia and controlled by the hypoxia-inducible factor transcription complex (HIF). HIF-1 is composed of two constitutively expressed subunits, α, of which there are three isoforms, and beta (also known as ARNT). In normoxia-conserved prolines 402 and 564 in the oxygen-dependent degradation (ODD) domain of HIF-1α are hydroxylated, allowing association with the von Hippel–Lindau protein (pVHL) and targeting of HIF-1α for degradation via the ubiquitin-proteasome pathway. However, hydroxylation is prevented in the absence of oxygen causing HIF-1α to remain unbound to pVHL. Consequently, HIF-1α is able to escape proteasome degradation and up-regulates target genes such as erythropoietin (Epo). Inappropriately normal or elevated Epo levels are commonly associated with idiopathic erythrocytosis (IE) suggesting a defect in the regulation of Epo. Since Epo gene expression is controlled by HIF-1 and mutations in *VHL* cause a subtle increase in Epo expression, we examined the ODD domain of *HIF-1α* for base changes in a group of IE patients. Sequencing revealed a C to T base change at 1744 causing exchange of proline to serine at codon 582 in nine patients. Screening a group of anonymized healthy controls detected the same change. Loss of Pro582 may diminish VHL and HIF-1α association especially if Pro582 is hydroxylated. Although we did not detect any hydroxylation of Pro582 it may influence hydroxylation of Pro564. Further *in vitro* studies indicated that loss of Pro582 did not influence hydroxylation of Pro564 or association of HIF-1α with either wild type or Arg200Trp VHL. In addition we were unable to show that Pro582Ser could cause up-regulation of a luciferase HRE reporter construct. Therefore, we conclude that the common Pro582Ser polymorphism located in the ODD domain of HIF-1α probably does not contribute to the pathogenesis of IE.

83

A novel mutation in the *ALAS2* gene in a family with X-linked sideroblastic anaemia

M. J. Percy,[1] A. May,[2] M. F. McMullin[3] & R. J. G. Cuthbert[1]

[1]*Department of Haematology, Belfast City Hospital, Belfast;* [2]*Department of Haematology, University of Wales College of Medicine, Cardiff;* [3]*Department of Haematology, Queen's University, Belfast, UK*

Inherited sideroblastic anaemia (SA), caused by a deficiency delta-aminolaevulinate synthase (ALAS), is characterized by a ring of iron-containing mitochrondia ring around the nucleus of erythroid cells. ALAS catalyses the conversion of glycine and succinyl-CoA to delta-aminolaevulinate and is the first rate-limiting step of the haem biosynthesis pathway. The erythroid-specific ALAS2 enzyme is encoded by the X-chromosome gene *ALAS2* and more than 20 different mutations have been reported since the first affected family was described in 1945. A 40-year-old male with a family history of mild chronic anaemia, presented with a microcytic hypochromic anaemia (Hb 7.9 g/dl), elevated ferritin (456 μg/l), and elevated transferrin saturation (88%). The blood film showed microcytic poikilocytes with numerous Pappenheimer bodies while the marrow indicated dyserythropoiesis with >40% ringed sideroblasts. Liver transaminases were elevated and liver biopsy showed mild hepatitis and heavy parenchymal iron deposition with no fibrosis or cirrhosis. He was treated with oral pyridoxine and folic acid. The Hb rose to 10.6 g/dl at 4 weeks and 12.4 g/dl at 10 weeks and is maintained in the range 11.5–12.5 g/dl. Sequencing *ALAS2* revealed a T to C change at base 918 in exon 7, resulting in an amino acid exchange of isoleucine to threonine at codon 289. His brother, who has a similar mild chronic anaemia, also possesses the Ile289Thr mutation. The novel Ile289Thr mutation has not been detected in 50 alleles from healthy controls nor in 20 alleles from other SA patients and appears to be the cause of the disease in this family. Replacement of isoleucine at 289 with threonine, a smaller molecule with a polar side chain, would be expected to cause a minor distortion of the internal α helix of ALAS2 and consequently perturb enzyme function in these individuals.

84

HFE (haemochromatosis) mutations and myocardial infarction

J. Goodfellow,[1] C. Jones,[1] A. Crane,[1] K. Carter,[2] R. R. West[3] & M. Worwood[2]

[1]*Department of Cardiology, Princess of Wales Hospital, Bridgend;* [2]*Department of Haematology, University of Wales College of Medicine, Cardiff;* [3]*Department of Cardiology, University of Wales College of Medicine, Cardiff, UK*

Two prospective surveys have suggested that heterozygosity for the C282Y mutation of the HFE (haemochromatosis) gene is associated with an increased risk of myocardial infarction. In South Wales, the

Samsung Bioepis Exhibit No. 1002
Page 1420

population has an increased incidence of coronary heart disease (CHD) and the frequency of the HFE C282Y mutation is among the highest in Europe with over 15% of the population carrying this mutation. We sought to test whether mutations in the HFE gene may be associated with an increased risk of MI by comparing the frequencies of HFE mutations in blood donors from South Wales with a cohort of patients presenting with MI in the Princess of Wales Hospital, Bridgend. All patients were fully characterized with respect to their past medical history, smoking status and all other major risk factors for CHD. HFE genotypes are shown in the table. Eighteen patients carried S65C. There was a similar frequency in blood donors. There were no significant differences in genotype frequency in the patients with MI when compared with 10 500 blood donors from South Wales confirming a recent report from Scotland (Campbell *et al*, *Heart*, 89:1023). There were no significant differences in frequency for C282Y or H63D between men and women or for smokers and non-smokers. Roest *et al* (*Circulation* 100;1268:1999) found a greater incidence of cardiovascular death from myocardial infarction among women with C282Y who were smokers and had hypertension. We did not find a significant increase in C282Y frequency for patients who smoked and had hypertension.

HFE genotype	Blood donors, male and female (n = 10 556)	Patients, male and female (n = 297)
wt/wt	6152 (58.3%)	164 (55.2%)
wt/H63D	2488 (23.6%)	78 (26.3%)
H63D/H63D	249 (2.4%)	6 (2.0%)
wt/C282Y	1342 (12.7%)	37 (12.5%)
H63D/C282Y	253 (2.4%)	10 (3.4%)
C282Y/C282Y	72 (0.68%)	2 (0.67%)

85

Cannabis use in sickle cell disease: results of an anonymous patient survey

J. Howard,[1] K. Anie,[1] A. Holdcroft,[2] S. Korn[1] & S. C. Davies[1]
[1]*Department of Haematology, Central Middlesex Hospital;* [2]*Magill Department of Anaesthesia, Imperial College, London, UK*

Cannabis use is common in patients with chronic pain. Frequency of use in diseases such as multiple sclerosis and HIV ranges from 27% to 37%. Randomized controlled trials in acute pain have shown dose-related analgesic effects of cannabis extracts. We have anecdotal evidence of cannabis use for pain relief in sickle cell disease (SCD) but the prevalence of this has not previously been surveyed. The aims of this study were to measure the patterns and prevalence of cannabis use in patients with SCD, to evaluate the beneficial or detrimental effect of cannabis on symptom control and to define interest in participation in clinical trials of cannabis extracts. Following LREC approval and verbal consent, adults with SCD were recruited into an anonymous structured questionnaire survey. This took place in the outpatient clinic, day unit and wards at the Central Middlesex Hospital. Questions included demographics, severity of SCD, current or previous cannabis use and its perceived benefits and side-effects. Eighty-six completed surveys were received over 6 months; 34.5% of men and 33% of women (n = 30) were current or previous users of cannabis. 'Medicinal use', defined as usage to control acute or chronic pain, decrease frequency of crises, or to reduce consumption of other analgesics, was reported in 50% of users (n = 15). Cannabis was well tolerated with few side-effects reported (10% reported anxiety and 5% reported blurred vision). Many patients (67%) stated they would be interested in taking part in clinical trials to investigate the efficacy of cannabis. In view of the high prevalence of use documented in this study and the perceived benefits reported, further clinical trials investigating the role of cannabis as an analgesic agent in SCD are indicated.

86

Tosoh-G7 HPLC system HbA2, HbF and HbS levels correlate significantly with in-house Kontron HPLC

S. Marwah,[1] A. Cobley[2] & D. Bareford[3]
[1]*Department of Haematology, City Hospital;* [2]*Department of Haematology, City Hospital;* [3]*Department of Haematology, City Hospital, Birmingham, UK*

The Tosoh-G7 with β-thalassaemia mode is an automated high performance liquid chromatography (HPLC) system that utilizes the principle of high-resolution non-porous cation-exchange chromatography column system for the fractionation and quantification of normal haemoglobin and haemoglobinpathy. In this study 250 EDTA patient samples requested for haemoglobinopathy were analysed both on the in-house gold standard Kontron HPLC system (Central Quality Assurance Scheme 2002 Gold Star award winner for HbA2) and on Tosoh-G7 with β-thalassaemia mode. The association of HbA2, HbF and HbS levels between the two HPLC systems were assessed using the Mann–Whitney U-test and Spearman's rank correlation method. Data is presented as median and inter-quartile range. This study demonstrated that HbA2 levels for Tosoh-G7 [2.50% (2.20–2.70)] and Kontron [2.40% (2.2–2.61)] correlated significantly (r = 0.979, P < 0.001) and also HbF for Tosoh-G7 [0.75 (0.60–3.4)] and Kontron [0.80% (0.60–1.00)] correlated significantly (r = 0.650, P < 0.001). Compared with Kontron HPLC a significant correlation was also found for HbS (n = 27) level (r = 0.994, P < 0.001). Intra-assay CV was 2.5% for HbA2 of 2.60%, 2.9% for HbA2 of 6.1% and 2.3% for HbS of 40.0%. The number of haemoglobinopathies detected by Tosoh-G7 paralleled with the Kontron HPLC. This data demonstrate that Tosoh-G7 with β-thalassaemia mode is reliable and can be used as an effective HPLC system for screening haemoglobinpathies. Tosoh-G7 with β-thalassaemia mode is easy to use without the need of any sample pretreatment and user-friendly HPLC system. This study demonstrates that the Tosoh-G7 HPLC system haemoglobinopathy results correlates with the in house Kontron HPLC system suggesting that Tosoh-G7 HPLC with β-thalassaemia mode could be utilized in a diagnostic haematology laboratory for screening of haemoglobinopathies and reporting of HbS level.

87

Severe sideroblastic anaemia with raised erythrocyte protoporphyrins – a variant of erythropoietic protoporphyria (EPP)

R. Morrell,[1] A. O'Marcaigh,[2] A. May,[3] K. Connolly[4] & G. Crotty[1]
[1]*Department of Haematology, Midland Regional Hospital, Tullamore, Ireland;* [2]*Our Ladys Hospital for Sick Children, Crumlin, Dublin, Ireland;* [3]*University of Wales, College of Medicine, Cardiff, UK;* [4]*Department of Paediatric, Portiuncula Hospital, Ballinasloe, Galway, Ireland*

A 13-year-old male presented 3 years ago with symptomatic hypochromic microcytic anaemia: Hb 6.4 g/dl, MCV 55.6 fl, ferritin 165 ng/ml. Pappenheimer bodies were visible on blood film and marrow aspirate showed large numbers of ringed sideroblasts. Currently our patient is transfusion dependent and has developed secondary iron overload and is on s/c Desferrioxamine. Genetic analyses for ALAS-E and ABC7 transporter protein were normal. However, free erythrocyte protoporphyrin concentration was extremely high

Samsung Bioepis Exhibit No. 1002
Page 1421

(22.9 μmol/l). This finding, unusual in sideroblastic anaemia, being associated more with erythropoietic protoporphyria (EPP), prompted analysis of his ferrochelatase (FECH) gene. He is doubly heterozygous for FECH mutations, bearing (a) a missense mutation C185->G (Pro62->Arg) transversion in exon 2 (previously identified in a case of EPP), and (b) a polymorphism, known to be associated with low FECH expression, in Intron 3. The current view is that EPP results from a severe FECH defect on one allele and a low expression variant on the other. EPP is a rare inherited disorder characterized by partial deficiency of FECH, the last enzyme in the haeme biosynthetic pathway. This leads to overproduction of protoporphyrins with painful skin photosensitivity in the majority of cases. Less frequently it is associated with anaemia and liver disease. Recently our patient developed an erythematous, blistering, painful rash suggestive of photosensitivity (not typical of rash of EPP). In addition he developed symptomatic primary hypoparathyroidism and insulin dependent Diabetes Mellitus, which may be related to iron overload, and has elevated liver enzymes, which could be attributed to iron overload or protoporphyrin accumulation. Lastly, he has bilateral ptosis the cause of which is not clear. Our case is unusual in that the anaemia, which accompanies EPP is usually mild. In less than five cases has a severe sideroblastic anaemia been described in association with EPP. The reasons for this are not wholly understood. Perhaps this particular mutation is associated with a more dramatically reduced enzyme activity.

88

Windy weather and acute pain in sickle cell disease

S. Jones,[1] E. Duncan,[2] N. Thomas,[2] J. Walters,[3] M. C. Dick,[3] S. E. Height,[3] A. D. Stephens,[2] S. L. Thein[2] & D. C. Rees[3]
[1]School of Mathematics, Kingston University, Kingston-upon-Thames; [2]Department of Haematology, King's College Hospital; [3]Department of Paediatric Haematology, King's College Hospital, UK

Sickle cell disease (SCD) is characterized by intermittent episodes of acute, severe pain. Environmental factors are thought to play an important role in triggering these crises and in tropical countries, cold and rainy seasons are associated with increased episodes of acute pain. These findings have not been reliably reproduced in temperate climates. We have retrospectively analysed numbers of daily admission with acute pain and SCD to King's College Hospital, London, together with daily meteorological records collected locally from 1 January 1998 to 31 October 2001. During the 1400 d of the study, there were 1047 admissions of adults and children with acute pain and SCD (78% total admissions of SCD patients). These data were analysed using a graph of the cross-correlation function. There was no relationship between admissions and maximum or minimum temperature, grass temperature, rainfall, humidity and barometric pressure. However, admissions with pain were significantly increased on windy days. This relationship was relatively small, but statistically significant and robust, being present for both the mean wind and maximum gust speed. During the period of the study, there were 280 admissions on days with mean wind speed in the top quartile (>7.95 knots) and 240 on days in the bottom quartile of wind speeds (<3.8 knots). There was no association between calculated wind chill factors and admissions. There was a trend towards fewer admissions in winter, but this did not reach statistical significance. This is the largest and most detailed study analysing climatic effects and acute pain in SCD. High wind speeds are likely to be associated with rapid changes in skin temperature, which has been shown to cause vaso-occlusion and pain. This effect may be equally marked in hot weather, on exposed and sweating skin, and cold weather, when thick clothing reduces any wind chill effect.

89

Vascular endothelial growth factor and proliferative retinopathy in sickle cell disease

J. Mohan, P. L. Lip, D. Bareford, G. Y. Lip & A. D. Blann
Department of Medicine, City Hospital, Birmingham, UK

Patients with sickle cell disease (SCD) are at risk of proliferative retinopathy (PR), which may lead to blindness. PR is characterized by excessive ocular angiogenesis, possibly driven by angiogenic growth factors such as vascular endothelial growth factor (VEGF), which acts on endothelial cells via a specific receptor, Flt-1. We hypothesized raised VEGF, its receptor Flt-1 and endothelial damage marker von Willebrand factor (vWf) in the PR of SCD compared to SCD non-PR eye disease and SCD normal eyes. All markers were measured in citrated venous plasma by ELISA, and also in healthy age, race and sex matched controls. We found raised vWf (median 114 IU/dl vs. 89 IU/dl, $P = 0.002$ Mann–Whitney) and VEGF (median 117 pg/ml vs. 11 pg/ml, $P = 0.001$) but no difference in Flt-1 in 50 patients with SCD compared with 21 controls. Of the 50 SCD patients, 16 had normal eyes, 12 had non-PR eye disease, and 22 had frank PR. However, there was no difference in VEGF, vWf or Flt-1 between the groups (analysis of variance). We conclude that raised vWf and VEGF, reflecting abnormal endothelial function and angiogenesis respectively, does not reflect differences in ocular proliferation in SCD.

Poster Presentation

Transplantation

90

Umbilical cord blood transplantation for paediatric haematological malignancies: single UK institution experience

R. E. Hough, R. D. Faulkner, J. C. Welch & A. J. Vora
Department of Paediatric Haematology, Sheffield Children's Hospital, Western Bank, Sheffield, S10 2TH, UK

Despite the substantial advances made in umbilical cord blood transplantation (UCBT) over the last decade, the utilization of UCB as an alternative stem cell source has been limited in the UK. We report the experience of UCBT thus far at Sheffield Childrens Hospital, UK. 15 patients with a median age of 5.1 years (range 0.7–15.3) were transplanted between June 1998 and February 2003 for AML ($n = 8$), ALL ($n = 2$), JMML ($n = 2$), infantile myelofibrosis ($n = 1$), anaplastic NHL ($n = 1$) and Fanconi anaemia (FA) ($n = 1$). The conditioning regimen was myeloablative in 11 patients, reduced intensity in 3 and non-myeloablative in 1. Graft-versus-host disease (GvHD) prophylaxis consisted of cyclosporin A (CyA) and methylprednisolone. All patients received CMV negative, unrelated donor UCB grafts, which were 6/6 HLA matched with the recipient in 3, 5/6 matched in 7 and 4/6 matched in 5. The median infused cell dose was 5.0×10^7 TNC/kg (range 2.0–8.0), 1.2×10^5 CD34+/kg (range 0.5–4.9) and 4.3×10^4 CFU-GM/kg (range 1.5–52.3). Myeloid engraftment was achieved in 93% after a median of 26 d (range 13–50) with platelet recovery observed at a median of 38 d (range16–64) in 85%. Grade II–IV GvHD was documented in two patients and chronic GvHD was not observed. Five patients have relapsed at a median of 74 d (range 19–94); two of these attained a further complete remission (CR) on withdrawal of CyA. Despite 14 of the 15 patients being at high risk of transplant-related mortality or relapse, nine remain alive and in CR at a median of 21.4 months (range 8.6–60.9). Six patients have died at a median of 2.3 months (range 0.3–5.8) of progressive disease in three and transplant-related

Samsung Bioepis Exhibit No. 1002
Page 1422

complications in three. These data represent the largest single UK institution experience of UCBT to date and confirm the therapeutic value of this approach in high-risk paediatric patients with haematological malignancies.

91

Long-term follow-up of an alemtuzumab (CAMPATH-1H)-containing reduced intensity allogeneic transplant (RIT) regimen for relapsed and refractory non-Hodgkin's lymphoma (NHL)

E. Morris, K. Thomson, C. Craddock, D. Milligan, G. Smith, A. Parker, S. Schey, D. Winfield, R. Chopra, T. Littlewood, J. Tighe, A. Hunter, C. Kyriakou, P. Kottaridis, D. Linch, A. Goldstone, K. Peggs & S. Mackinnon

UK Collaborative Group, UK

Ninety-three patients underwent RIT for NHL with a median follow-up of 22 months (range: 0.5–54). Median age was 48 years. Forty-seven patients had intermediate/HG-NHL and 46 patients had LG-NHL. Thirty-seven patients had received prior autografts. At transplant, 23 patients were in CR, 60 were in chemosensitive PR and 10 had refractory or progressive disease. Conditioning was with alemtuzumab 20 mg/d on days −8 to −4, fludarabine 30 mg/m^2 on days −7 to −3 and melphalan 140 mg/m^2 on day −2. Seventy patients received PBSC from HLA-identical siblings and 23 received BM from matched unrelated donors. GvHD prophylaxis was with cyclosporin A. Acute GvHD developed in 28 patients (30%) [grade I ($n = 14$), grade II–IV ($n = 14$) and grade III–IV ($n = 4$)] and chronic GvHD in seven patients. With a median follow-up of 22 months (range 0.5–54), the estimated overall survival (OS) 4.5 years for all patients is 55% (41% for Intermediate/HG-NHL and 70% for LG-NHL, respectively, $P = 0.001$). Disease status at time of transplant (CR, PR or progressive disease) significantly affected outcome ($P = 0.01$). Overall survival at 4 years was 76% for LG-NHL patients with matched family donors and 50%for those with unrelated donors ($P = 0.02$). The 100-d and 3-year transplant-related mortality (TRM) for patients with LG-NHL were 2% and 9.6% respectively, and were better ($P = 0.02$) than for patients with Intermediate/HG-NHL (26% and 34% at 100 d and 3 years). The estimated current progression free survival (PFS) at 4.5 years, including patients who achieved remission following DLI for progression or persistent disease, was significantly superior for those with LG-NHL (65%) than for HG-NHL (19%) ($P < 0.001$). Again, disease status at transplant was significant ($P = 0.004$). Patients with relapsed LG-NHL and CLL achieve excellent PFS with extremely low TRM and GvHD, even when matched family donors are unavailable. RIT is possibly the treatment of choice for such patients progressing postchemotherapy.

92

Late reactivation of Epstein–Barr Virus (EBV) in recipients of non-myeloablative allogeneic stem cell transplantation conditioned with alemtuzumab

Q. A. Hill,[1] T. A. Collyns,[2] A. D. Hale[3] T. Fawcett,[3] A. Hancox[2] & G. Cook[1]

[1]*BMT Unit, St James' University Hospital;* [2]*Department of Microbiology, St James' University Hospital;* [3]*Health Protection Agency Laboratory, Leeds, UK*

Elevated EBV DNA copy numbers have been shown to be predictive for EBV-induced lympho-proliferative disease (EBV-LPD). EBV-LPD commonly develops within the first 6 months after non-myeloablative allogeneic stem cell transplants (NSCT) using ATG as GvHD prophylaxis. Alemtuzumab in NSCT protocols has been associated with delayed lymphocyte recovery, but the additional effect of B-lymphocyte depletion may reduce the levels of EBV infected B cells resulting in reduced reactivation. The incidence and timing of EBV reactivation in recipients of NSCT protocols incorporating alemtuzumab has not previously been reported. We report a series of 18 patients undergoing NSCT who received alemtuzumab. Pretransplant recipient EBV serology (IgG) was positive in 17 cases and negative in one. Seventeen patients received conditioning with fludarabine (150 mg/m^2) and melphalan (140 mg/m^2), and 1 with cyclophosphamide (120 mg/kg) and TBI (200cCy single fraction). EBV serology was positive in 14 donors (one IgM positive), unavailable for testing in four, and negative in one (EBV negative recipient). Post-transplant, cyclosporin A alone was used as GvHD prophylaxis. Median time to engraftment was 16 d (range 12–29). EBV DNA copy numbers were determined by real-time PCR. EBV reactivation was detected in 6/18 patients (33%). EBV copy numbers ranged from 3×10^2 to greater than 3×10^5/ml plasma. No reactivation was detected before 8 months post-transplant. Therefore, 6/10 (60%) patients surviving more than 8 months post-transplant, had EBV reactivation. No EBV-LPDs were diagnosed. The recipient of the EBV IgM positive stem cells did not reactivate. The delay in EBV reactivation compared to published studies in ATG-containing protocols may relate to the depletion of the B-cell pool caused by alemtuzumab. The risk of EBV reactivation is therefore significant in this setting. With increased surveillance we can effectively monitor this complication and potentially reduce the incidence of EBV-LPD with preventative therapies.

93

Very low dose aciclovir prophylaxis postallogeneic stem cell transplantation prevents varicella zoster infection without development of clinically relevant resistance

D. P. Hart, K. J. Thomson, L. Bannerjee, E. C. Morris, D. C. Linch, A. H. Goldstone, K. S. Peggs & S. Mackinnon

Department of Haematology, University College London Hospital, London, UK

Varicella zoster virus (VZV) establishes a latent state following primary infection. Host T lymphocytes play a crucial role in maintaining latency. Haematopoietic stem cell tranplantation (HSCT) recipients are at particular risk of reactivation. Aciclovir prophylaxis suppresses viral proliferation and has been used following transplantation in differing doses (generally 400–800 mg tds). We performed a retrospective study of 214 patients who underwent allogeneic HSCT between 1998 and 2002. The aim was to identify the frequency and timing of reactivation relative to cessation of aciclovir prophylaxis. The appearance of herpes zoster is sufficiently distinctive that a clinical diagnosis is accurate. All patients received identical antiviral prophylaxis: 250 mg/m^2 aciclovir tds intravenously from D-1, converted to 200 mg bd orally on engraftment, continued until cessation of all immunosuppressive therapy and CD4 > 0.2×10^9/l. VZV infection occurred in 25/214 (11.7%) patients. Twenty-two isolated dermatomal involvement, three disseminated zoster. Only 1/214 patient reactivated zoster whilst on aciclovir prophylaxis. There were no deaths attributable to VZV infection; 62/214 (29%) stopped aciclovir prophylaxis a mean of 489 d (range 116–986) post-transplant. The remaining 152 patients either continue aciclovir prophylaxis having not met the stopping criteria ($n = 57$) or died whilst taking it with no recorded VZV episode ($n = 95$). The mean time to VZV infection after stopping prophylaxis was 244 d (range 16–585). In conclusion, very low dose oral aciclovir provides effective prophylaxis comparable to higher doses used in other series. Emergence of viral resistance does not appear to be a clinical problem. However, 38.7% (24/62)of patients who met the stopping criteria had zoster infection after discontinuation of aciclovir suggesting that CD4 count alone is not a reliable

Samsung Bioepis Exhibit No. 1002
Page 1423

indicator to guide cessation of prophylaxis. If presentation of viral antigens is necessary for re-stimulation of memory responses it may be possible to identify a group in whom vaccination approaches could be targeted prior to cessation of aciclovir.

94

Changes in serum-free light chain concentrations as a marker of disease response in myeloma patients after autologous stem cell transplantation

G. Pratt,[1,2] G. Mead,[3] R. Lovell[2] & A. R. Bradwell[4]

[1]Institute for Cancer Studies, University of Birmingham, Birmingham, B15 2TT; [2]Birmingham Heartlands and Solihull NHS Trust; [3]The Binding Site Ltd, Birmingham, UK B29 6AT; [4]Departemt of Immunology, Medical School, University of Birmingham, Birmingham, B15 2TT, UK

Measurement of serum-free immunoglobulin light chains (FLC) is useful in AL amyloidosis, Bence Jones and non-secretory myeloma. Also, FLC concentrations indicate response to treatment more rapidly than intact immunoglobulin, due to their shorter serum half-life (FLC: 2–6 h, IgG: 20 d). The aim of this study was to follow the changes in serum FLC after peripheral blood stem cell transplantation (PBSCT). Serum samples were collected daily from 14 patients with multiple myeloma (10 IgG, two IgA and two light chain only) during PBSCT with standard melphalan conditioning and assayed for FLC, total immunoglobulins and monoclonal immunoglobulin. Before transplant, 9/14 patients had elevated levels of tumour FLC, 2/14 had normal tumour FLC but abnormal κ/λ ratios due to suppression of the non-tumour FLC and 3/14 patients had normal FLC concentrations and ratios. Within 24 h of high-dose melphalan, all 14 patients showed a fall in their tumour FLC and 11/14 showed falls in their non-tumour FLC. In the three patients whose non-tumour FLC did not fall after transplant, the concentrations were already significantly suppressed before transplantation. In all 12 patients with intact immunoglobulin paraproteins, the FLC fell more rapidly than the immunoglobulin. Two patients were sampled during engraftment and showed that both FLC concentrations rose rapidly, to normal, over 5–6 d. In the majority of patients (10/14) neutrophil engraftment preceded plasma cell engraftment by 1–10 d. All five patients followed to their 3-month assessment had normal or modestly elevated FLC, corresponding with oligoclonal reconstitution of the marrow. FLC assays provided a sensitive monitor of changes in the numbers of tumour and non-tumour plasma cells after PBSCT and predicted the intact immunoglobulin response. This is a preliminary, observational study and further follow up is required to ascertain whether the rate or degree of FLC responses have prognostic value.

95

High response rate to DLI based strategies for the treatment of refractory disease/relapse following allogeneic HSCT for lymphoproliferative disease

N. H. Russell, J. L. Byrne, E. P. Das-Gupta, M. Gilyead & A. P. Haynes

Department of Haematology, Nottingham City Hospital, Nottingham, UK

We have treated 17 patients (median age 51 years; 30–62 years) with refractory disease ($n = 7$) or disease relapse ($n = 10$) following allogeneic HSCT for lymphoma with a DLI-based strategy. Patients received DLI alone ($n = 6$) or following initial chemo/radiotherapy ($n = 11$). The diagnosis was CLL ($n = 5$); mantle cell lymphoma ($n = 4$); high grade NHL ($n = 4$) and follicular NHL ($n = 4$). RIC was either Beam/Campath ($n = 11$), or fludarabine/mel/Campath ($n = 4$). Two patients received TBI/Cyclo conditioning. The median time to DLI was 0.96 years (0.23–4.58 years) and in 15/17 the donor was a matched sibling ($n = 13$) or 1 antigen mismatched sibling ($n = 2$) and in two cases was an unrelated donor. At DLI six patients had mixed chimaerism (20–95%) and eight had full donor chimaerism. The chimaerism status was unknown in five cases. The median first dose of $CD3^+$ cells infused was 20×10^6/kg ($5–60 \times 10^6$/kg); eight patients received a second infusion (median dose 50×10^6/kg; two patients received three doses. Overall 8/17 patients achieved a CR including 2/5 patients with CLL, 3/4 with MCL; 3/4 with follicular NHL and 0/4 with HG.NHL. One patient with CLL is in PR with an ongoing response after the first DLI infusion. No patient achieving a CR has yet relapsed. GvHD developed in 10 patients and was grade II in 8/10 and grade IV in two cases. One patient died in CR of GvHD. One patient with CLL who failed to respond to DLI had a second transplant and is in CR at 14 m. The overall survival at a median of 30 months post-DLI is 53% and 67% for patients with low-grade disease. We conclude that lymphoma patients, particularly those with low grade NHL relapsing following an allogeneic transplant have a high response rate to DLI based strategies comparable with that seen in CML.

96

CD34 and/or CD3 add-back infusions improve donor chimaerism but do not impact lymphocyte subset recovery in children undergoing haplo-identical transplantation

D. Chandra,[1] J. Motwani,[1] P. Davies,[2] D. McDonald,[3] P. Taylor,[3] S. Kirk,[1] P. J. Darbyshire[1] & S. E. Lawson[1]

[1]Bone Marrow Transplant Unit, Birmingham Children's Hospital, Birmingham, UK; [2]Department of Biostatistics, University of Birmingham, Birmingham, UK; [3]National Blood Service, Birmingham, UK

We analysed the factors affecting lymphocyte subset recovery in 35 children undergoing haploidentical transplantation for a variety of malignant (25) and non-malignant (10) diseases. All patients engrafted after receiving CD34 selected PBSC with a mean CD34 dose of 12.4×10^6/kg and mean CD3 dose of 7.9×10^4/kg. Cyclosporine was used as GvHD prophylaxis for the first 6 weeks. Eleven patients received add-back infusions of CD34 and CD3 cells (mean CD34 = 8.5×10^6/kg, mean CD3 = 39×10^6/kg) and four patients received only escalating donor lymphocyte infusions (mean CD3 = 92.5×10^4/kg), 90–150 d post-transplant to improve immune reconstitution (eight) or to reverse progressing mixed chimerism (seven). There was no impact of recipient or donor age, sex, CMV at risk status, graft CD34 or CD3 dose or acute GvHD on the time to recovery of absolute lymphocyte count (ALC) and subsets. In patients who received add-back infusions the median time (in days) to normalize ALC, CD3, CD4, CD8, CD56, and CD19 lymphocyte subsets was 120, 285, 285, 105, 47 and 75 d respectively, compared with 140, 210, 240, 120, 45 and 85 d respectively in those not receiving add back infusions ($P > 0.05$). All patients who received add back infusions for progressive mixed chimaerism improved and stabilized their chimaerism. In the group who received CD34 and/or CD3 add-back infusions, two patients developed acute GvHD greater than grade 2 and 6/15 patients developed opportunistic infections of which four succumbed to these infections. There were a total of 10 deaths (66%) in this group; six due to disease relapse. In the cohort not receiving add-back infusions, 7/20 patients developed opportunistic infections; four died of relapse or toxicity. *Conclusion:* Add-back of CD34/CD3 cells following haploidentical transplantation might stabilize or improve the donor-chimerism, however, there was no positive influence on time to lymphocyte recovery of such add-back in this cohort and needs further study.

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Samsung Bioepis Exhibit No. 1002
Page 1424

97

A single centre experience of reduced intensity conditioned HSCT using BEAM-campath for low-grade lymphoid malignancies

M. Lissack, W. Ingram, M. Lamacchia, A. Ho, N. Greenwood, G. J. Mufti, S. Devereux & A. Pagliuca

Department of Haematological Medicine, Kings College Hospital, London, UK

Low-grade lymphoid malignancies are considered incurable with conventional chemotherapy however allogeneic haemopoietic stem cell transplantation (allo-HSCT) with reduced intensity conditioning (RIC) may offer a curative option. We report 24 patients who underwent RIC allo-HSCT using BEAM-Campath (BCNU 300 mg/m^2, Cytarabine 200 mg/m^2 ×4, Etoposide 200 mg/m^2 ×4, Melphelan 140 mg/m^2, Alemtuzumab 20 mg ×5). Fifteen had a diagnosis of follicular lymphoma, six CLL, two T-prolymphocytic lymphoma and one mantle cell lymphoma. Eighteen were male and six female. Median age was 50 years (range 34–56 years). Eleven underwent sibling allo-HSCT, 13 volunteer unrelated donor HSCT (1DQB1 mismatch). Thirteen patients had received more than two lines of therapy prior to HSCT. Three were in morphological and radiological complete remission (CR) prior to HSCT and 21 were in partial remission (PR). Median duration from diagnosis to HSCT was 134 weeks (range 29–624). Three received bone marrow with a median CD34 count of 3.46×10^6/kg, 21 received PBSC with a median CD34 count of 5.82×10^6/kg. Median time to regeneration of neutrophils greater than 0.5 for BM versus PBSC was 19 vs. 12 d and platelets greater than 20 was 29 vs. 11 d respectively. Nine patients had no evidence of graft-versus-host disease (GvHD), 11 had grade I–II GvHD, four grade III–IV GvHD. Chimerism was monitored in 20 patients. Six showed 100% donor throughout,14 showed mixed chimerism with one resulting in graft failure and eight requiring donor lymphocyte infusions commencing 5×10^5/kg CD3 cells. CMV reactivation was present in eight patients. Transplant related mortality at 100 d was 12%. 71% of patients are alive at follow up. Median duration of follow up was 285 d (range 43–980 d). All survivors are currently in complete clinical and radiological remission. Longer follow up is required, however RIC allo-HSCT may be effective therapy even in heavily pretreated patients with low-grade lymphoid malignancies.

98

Incidence and outcome of herpes simplex virus (HSV) and varicella zoster infection (VZV) following stem cell transplantation in the era of T-cell depletion and the use of non-myeloablative conditioning – implications for prophylactic strategies

S. Narayanan,[1] A. Hodgetts,[1] H. Osman,[2] C. D. Fegan,[1] D. W. Milligan[1] & J. C. Ewing[1]

[1]*Department of Haematology, Birmingham Heartlands Hospital, Bordesley Green East;* [2]*Public Health Laboratory Service, Birmingham Heartlands Hospital, Birmingham, B9 5SS, UK*

Herpes viruses establish a latent phase following primary infection. There is risk of reactivation following haemopoietic stem cell transplant (HSCT) due to escape from immune surveillance during immune reconstitution. Changes in HSCT techniques including stem cell source, T-depletion and non-myeloablative regimen (NMAT) impact on immune reconstitution and may influence incidence, timing and outcome of herpes infection. Improved understanding of tempo of immune reconstitution allows targeted prophylaxis to those at highest risk. Our aim was to retrospectively evaluate incidence and outcome of HSV/VZV in autologous (A-PBSCT) and allogeneic transplant recipients (alloBMT/PBSCT) to define logical, cost-effective parameters for prophylactic strategy. A retrospective study was undertaken in 81 patients undergoing transplant; 60 A-PBSCT and 21 alloBMT/PBSCT (13 sibling, eight MUD). Of these, nine underwent NMAT while the remainder received T-depleted infusions. The prophylactic regimen was aciclovir 400 mg tid from conditioning-neutrophil recovery (median duration = 21 d, range 10–61). HSV reactivation occurred in three A-PBSCT (5%) and 13 alloBMT/PBSCT (63%)and VZV reactivation in six A-PBSCT (10%) and 12 alloBMT/PBSCT (57%). Infection occurred median 137 d (range 3–591 d). Five alloBMT/PBSCT patients had recurrence. Late reactivation (>365 d) occurred in six alloBMT/PBSCT patients. Particularly high incidence was seen in ALL (100%), CLL (83%). Morbidity included disseminated zoster ($n = 3$) and postherpetic neuralgia. Allograft recipients were monitored for lymphoid reconstitution by CD4/8 counts. All 12 episodes of VZV occurred at CD4 count of $<0.3 \times 10^9$/l (nine episodes with CD4 $<0.2 \times 10^9$/l). This may represent a parameter for safe discontinuation of acyclovir prophylaxis. In conclusion, in the era of T-cell depletion and non-myeloablative transplant there is a high risk of potentially life-threatening herpes virus reactivation. Aciclovir prophylaxis may be targeted to those at risk. CD4 count is one clinically applicable indicator of immune reconstitution and our analysis suggests that risk falls at CD4 0.3×10^9/l. It may be safe to stop prophylaxis once this is achieved. A-PBSCT patients are at relatively low risk of viral reactivation 200 d following transplant and may derive limited benefit from prolonged prophylaxis.

99

Estimations of haematopoetic progenitor cells in peripheral blood by the Advia 120 and B-D Vantage flow cytometer: a direct comparison for the prediction of adequate collections

H. M. Greenfield,[1] R. K. Newton,[2] D. A. Sweeney,[2] A. Leather,[1] J. Murray,[1] J. Cavet[1] & J. Chang[1]

[1]*Department of Haematology, Christie Hospital;* [2]*Transplant Laboratory, Christie Hospital, Manchester, UK*

Peripheral blood stem cells are increasingly used to ensure rapid haematological engraftment after myeloablative chemotherapy. CD34 positive cells are used as a surrogate marker for peripheral blood stem cells with 2×10^6 CD34 cells/kg body weight accepted as the minimum requirement. After mobilization, progenitor cells in blood can be enumerated to predict adequate collection by leukapheresis. Recent reports have suggested that the immature cell channel on the Sysmex 95000 automated counter correlates with CD34 peripheral blood cell counts. The Advia 120 has a gating area for immature lymphocytes, which theoretically could also be of similar clinical importance. We have assessed this retrospectively on all patients referred to our centre for stem cell collection prior to autologous transplantation from August 2002 to November 2003 ($n = 91$). A total of 102 apheresis sessions were performed. The minimum data set collected in each case was total white cell count at time of harvest, large undifferentiated cells (LUCs), peripheral blood CD34 count and harvest CD34 count. Our results for the group as a whole show a significant correlation between peripheral blood CD34 and harvest CD34 count estimated by flow cytometry with regression analysis coefficient of $r = 0.75$. We performed sub-group analysis on patients with myeloma ($n = 39$) and NHL ($n = 17$) and found similar strong correlations ($r = 0.63$ and $r = 0.93$ respectively). In contrast we found no relationship between LUC count and CD34 count in peripheral blood and harvest ($r = 0.02$). We conclude that the Advia 120 LUC count cannot reliably be used to predict timing of a successful stem cell harvest.

Samsung Bioepis Exhibit No. 1002
Page 1425

100

Long-term treatment of chronic graft versus host disease with extracorporeal photopheresis does not reduce the number of mononuclear cells that secrete TNF-α and interferon-γ

J. Bladon & P. C. Taylor

Department of Haematology, Rotherham General Hospital, S60 2UD, UK

Graft-versus-host disease (GvHD) remains a major cause of morbidity or mortality following allogeneic bone marrow or stem cell transplantation. The pathology of GvHD involves the stimulation of Th1 pro-inflammatory cytokines following pretransplant conditioning and donor T-cell activation. Initially used for treatment of cutaneous T-cell lymphoma (CTCL), extracorporeal photopheresis (ECP) has more recently been shown to be clinically effective in the treatment of both acute and chronic GvHD. ECP therapy involves the exposure of cells, separated by leucopheresis, to 8 methoxypsoralen (8-MOP) and UVA light. The treated cells are subsequently re-infused. Immediately following ECP, we have detected a fall in the number of mononuclear cells that express the pro-inflammatory cytokines TNF alpha (TNF-α) and interferon gamma (IFN-γ). Our aim was to establish if ECPs down-regulation of these cytokines constituted a mechanism for the long-term treatment of GvHD. Samples were taken from six chronic GvHD patients prior to commencing ECP and after 3 and 6 months of ECP therapy. Following separation, the mononuclear cells were added to a cell culture medium and stimulated with PMA, Ionomycin and Brefeldin A for 5 h. Using flow cytometry, the number of monocytes expressing TNF-α and the number of T cells expressing TNF-α and IFN-γ was determined. No significant reduction in the expression of either TNF-α or IFN-γ, in either mononuclear cell population, was observed. Although a reversal of the Th1/Th2 imbalance has been observed in CTCL patients treated by ECP, a restoration of the Th1/Th2 ratio was not observed for GvHD. Gorgun *et al* have demonstrated that ECP reduces dendritic cell (DC) numbers and speculated that the subsequent down-regulation in ability to process alloantigens reduces GvHD. However, recent criticism of their experimental data means the mechanistic effect of ECP on GvHD is far from resolved. Graft-versus-host disease is a major problem following allogeneic bone marrow or stem cell transplantation. The pathology of GvHD involves upregulation of proinflammatory cytokines. Extracorporeal photopheresis is a clinically effective treatment for GvHD, which has recently been demonstrated to cause the immediate down-regulation of proinflammatory cytokines. This study attempts to establish if a reduction of proinflammatory cytokines is observed in GvHD patients following long term ECP therapy and thus constitutes a mechanistic effect for ECP in GvHD.

101

In vitro generation of T cells cytotoxic to allogeneic AML blasts

P. Baines,[1] J. Fisher,[1] T. Hoy,[2] K. Wilson[1] & A. K. Burnett[2]

[1]*Department of Haematology, University Hospital of Wales;*

[2]*Department of Haematology, University of Wales College of Medicine, Cardiff, UK*

Allogeneic marrow transplantation for leukaemia is associated with improved outcome due to the graft-versus leukaemia (GvL) effect. Clearly, there is a role for alloreactive lymphocytes in leukaemia treatment. We have begun to characterize the *in-vitro* allogenicity of matched sibling donor lymphocytes and recipient acute myeloid leukaemia (AML) blasts. In the first of our matched pairs, donor lymphocytes were primed thrice with either irradiated AML blasts or with dendritic cells (DCs) derived from these AML blasts. Control unprimed donor lymphocytes were cultured for the same period of time (upwards of 4 weeks) in the same conditions (IL2 + IL7). Proliferation of donor lymphocytes was marked only following priming with uncultured AML blasts. $CD4^+$ cells dominated these cultures, which is a characteristic of mixed lymphocyte reactions. Cytotoxicity to priming blasts, to K562 or to residual $CD3^+$ cells sorted from the blast preparation, was measured by chromium release. Cytotoxicity was seen in both primed and unprimed donor lymphocytes to recipient AML blasts and particularly to K562, which suggests high natural killer (NK) activity. Blast-primed donor lymphocytes were fractionated by cell sorting. The ratios of anti-blast over anti-K562 activity were 0.77, 2.7, 0.85, and 0.32, respectively, for unfractionated donor lymphocytes, $CD4^+$ cells, $CD8^+$ 56^- and $CD8^+$ 56^+ cells. In other words, the $CD4^+$ and $CD8^+$ 56^- fractions showed heightened anti blast activity and reduced NK activity. The comparative susceptibility of recipient's blasts to killing by $CD4^+$ cells was reflected in the efficacy of anti-MHCII, but not anti-MHCI antibodies, in blocking killing of AML blasts, but not K562, by unfractionated, blast-primed donor lymphocytes. However, preferential killing of recipient blasts was also seen in $CD4^+$ fractions prepared from cultured but unprimed donor lymphocytes. In summary, both anti-AML and NK activity have been resolved to separate fractions of donor lymphocytes.

102

Nucleated cell doses collected from unrelated donor bone marrow harvests

R. Bailey-Wood, A. Ager & K. M. O. Wilson

Department of Haematology, University Hospital of Wales, Wales, UK

Between December 1996 and December 2003, 248 unrelated donor bone marrow harvests were performed at Cardiff for transplant centres mainly in Europe and North America. Cells were harvested using routine procedures by various haematologists. Prescription requests were for nucleated cell doses generally of $3–4 \times 10^8$ nucleated cells/kg with anticoagulation generally with ACD (1:7) or heparin (10 units/ml) or a combination. Patient body weights ranged from 5 to 169 kg. Those patients (48) with body weights <30 kg were excluded from the analysis as were two patients weighing 123 and 169 kg. Harvest volumes were limited to 25% of donor blood volume and harvests were stopped when the prescription requirement was reached. There were 118 male and 80 female donors. Donor age ranged from 20 to 53 years and weight from 42 to 120 kg. There was a weak significant relationship between total nucleated cells obtained and donor weight ($r = 0.43$, $P < 0.05$) but not to donor age or sex although the median female donor weight (67.7 kg) was significantly less than the median male weight (85.5 kg). As might be expected, there was a significant inverse relationship between the nucleated cell dose/kg and recipient's weight. For recipients of 30–50 kg the median nucleated cell dose was 3.7×10^8/kg for recipients of 50–70 kg the median was 3.1×10^8/kg for recipients 70–90 kg the median was 2.5×10^8/kg and recipients >90 kg the median was 1.8×10^8/kg. This data is relevant to the selection of nucleated cell doses requested by transplant centres.

Samsung Bioepis Exhibit No. 1002
Page 1426

Oral Communication

General Haematology and Transfusion

103

Liver colonization from acetyl LDL incorporating endothelial cells in the mouse embryo

D. Sugiyama,[1,2] K. Arai[2] & K. Tsuji[2]

[1]Developmental Biology Laboratory, UMR CNRS 7622, University Pierre and Marie Curie, Paris, France; [2]IMSUT, Tokyo, Japan

In the early mouse embryo, some special endothelial cells (ECs) have a potential to differentiate into definitive haematopoietic cells (HCs) including haematopoietic stem cells (HSCs). Recently, these haemogenic ECs tagged with DiI-conjugated acetylated low-density lipoproteins (Ac-LDL-DiI) were shown to release the definitive erythroid cells into circulation *in vivo*. However, it remains unclear whether these haemogenic ECs can release the other definitive HCs into circulation and these circulating EC-borne HCs can colonize the foetal liver (FL), the main haematopoietic organ at midgestation. Although it is hypothesized that the FL is colonized by the HCs at extrinsic origins, no route taken by HCs to colonize the FL has been shown. In this study, ECs at 10 d postcoitum (dpc) were tagged with Ac-LDL-DiI and were shown to release DiI+ HCs in circulation after 12 h of whole embryo culture. Flow cytometry analysis revealed that the circulating DiI+ cells expressed c-Kit, and these cells displayed a blastic morphology. *In vitro* haematopoietic culture and *in vivo* haematopoietic reconstitution experiments showed that the circulating DiI+ cells contained definitive multi-potent HCs including HSCs. Furthermore, the sorted DiI+ cells were able to colonize the FL when introduced back into the blood stream of a secondary recipient embryo. We hereby provide the first direct evidence in mammals that ECs can give rise to definitive HCs including HSCs and EC-borne HCs can colonize the FL via circulation *in vivo*, and furthermore that this system would be novel means to investigate the mechanisms of FL colonization.

104

Automated analysis of reticulated platelets and its potential clinical value

C. Briggs, S. Kunka, D. Hart & S. J. Machin

Department of Haematology, University College London Hospitals, London, UK

A new automated method to reliably quantitate reticulated platelets, expressed as the immature platelet fraction (IPF), has been developed utilizing the XE-2100 (Sysmex). The IPF is identified by flow cytometry using a polymethine dye in the reticulocyte channel. The clinical utility of this parameter has been established in the differential diagnosis of thrombocytopenia due to bone marrow failure and increased peripheral consumption, particularly idiopathic thrombocytopenia purpura (ITP) and thrombotic thrombocytopenic purpura (TTP). Reproducibility and stability results over 48 h were acceptable. The IPF normal range was established in adults as 1.1–5.6%, mean 3.1% and 2 standard deviation (SD) 2.3%. A total of 151 patients from several different clinical groups, where platelet production or destruction might be abnormal, were studied and some patients followed serially during treatment. The IPF% is raised in several disease states. The most significant increases are found in patients with untreated ITP, $n = 12$, mean 22.3%, range 9.2–33.1% and active TTP, $n = 5$, mean 17.2%, range 11.2–30.9%. Following these patients during treatment demonstrated that as the platelet count recovered the IPF% fell. Raised levels were also found in third trimester antenatal patients ($n = 12$, mean = 8.4%, range 3.2–14.6%), the IPF being higher in patients with pre-eclampsia. The recovery phase of thrombocytopenia in most chemotherapy and stem cell/bone marrow transplant patients was preceded by a rise in IPF% several days prior to platelet recovery. For the peripheral blood stem cell transplant patients the rise in IPF% occurs 1–3 d before platelet recovery (longer for bone marrow transplant patients, 2–7 d). Had the IPF% results been used as a predictor of platelet recovery, prophylactic platelet transfusions in the patients undergoing stem cell transplants may have been avoided or reduced. These results show the IPF% should become a standard parameter in the clinical evaluation of the thrombocytopenic patient.

105

Clinical and laboratory features of seven British kindreds with hereditary hyperferritinaemia cataract syndrome

S. Siddiq,[1] K. Bower,[2] K. Temple[2] & A. D. Mumford[1]

[1]Department of Haematology, Bristol Royal Infirmary, Bristol; [2]Wessex Regional Genetics Service, Princess Anne Hospital, Coxford Rd, Southampton

Hereditary hyperferritinaemia cataract syndrome (HHCS) is a rare autosomal dominant disorder characterized by early onset bilateral cataracts and elevated serum L-ferritin. Affected individuals have point mutations in a regulatory sequence in the L-ferritin gene 5′ untranslated region (5′-UTR) resulting in increased L-ferritin synthesis that is independent of body iron load. Although the molecular pathogenesis of HHCS is understood, the clinical features of this disorder are less well described. We therefore report the clinical and laboratory features seven British kindreds with HHCS who were referred for molecular investigation. HHCS was diagnosed after the incidental finding of hyperferritinaemia in 7/7 probands (six females, median age 37 years, range 23–45) during investigation of anaemia or antenatal screening. The median serum ferritin concentration was 1420 μg/l (range 740–1960). However, despite hyperferritinaemia, none of the probands had evidence of iron overload. Two of seven probands had previously received erroneous diagnoses of hereditary haemochromatosis. The median age at cataract diagnosis was 6 years (range 4–9). In all cases the cataracts were bilateral and comprised characteristic radiating cortical opacities. Cataract extraction had been performed in 6/7 probands before diagnosis of HHCS, at a median age of 25 years (range 22–42). Molecular analysis showed that affected individuals were heterozygous for point mutations in the L-ferritin gene 5′-UTR [G32C (two kindreds), C39T, G32T, C33T, C36A and A40G]. There was complete segregation within the seven probands and 41 additional affected family members between hyperferritinaemia or premature cataracts and a point mutation within the L-ferritin gene. This case series confirms that the phenotypic manifestations of HHCS include premature cataract hyperferritinaemia but not iron overload and that this disorder is associated with point mutations in the L-ferritin gene 5′-UTR. HHCS should be considered in all individuals with elevated L-ferritin and should be distinguished from other causes of familial hyperferritinaemia, including hereditary haemochromatosis.

106

A large multi-centre evaluation of the methodologies for the accurate platelet counting of severe thrombocytopenia

H. C. Segal,[1] C. Briggs,[2] P. Harrison,[1] S. Kunka,[2] S. J. Machin[2] & M. F. Murphy[3]

[1]Oxford Haemophilia Centre & Thrombosis Unit, Churchill Hospital, Oxford; [2]Department of Haematology, University College London; [3]National Blood Service Oxford Centre, John Radcliffe Hospital, Oxford, UK

The optimal threshold platelet count for prophylactic platelet transfusions in patients with haematological malignancy remains

Samsung Bioepis Exhibit No. 1002
Page 1427

uncertain. A concern in lowering the threshold from the recommended level of 10×10^9/l, is the potential inaccuracy of standard haematology platelet counting by a range of technologies in thrombocytopenia. The aim of this multi-centre study was to evaluate the accuracy of haematology analysers in UK clinical use at platelet counts below 20×10^9/l in patients following cytotoxic chemotherapy. Platelet counts from the XE-2100 ($n = 761$, two sites), Cell-Dyn 4000 ($n = 346$, two sites), LH750 ($n = 501$, one site) and the Advia 120 ($n = 282$, two sites) were compared with the flow cytometric International Reference Method (IRM). Regression analysis of each method compared with the IRM gave Correlation Coefficients (R^2 and y-axis intercept values; see table). Results demonstrate that the Cell Dyn CD61 method is more accurate than other technologies and that optical methods are not superior to the impedance methods with differences in accuracy and calibration between centres. Bland Altman analysis showed methods were biased towards overestimating platelet counts, implying that there is undertransfusion of platelets at the decision thresholds for prophylactic platelet transfusions of 10×10^9/l. This study highlights inaccuracies of platelet counting by available technologies (apart from the CD61 method) emphasizing the need for external quality control of platelet counting in severe thrombocytopenia. Furthermore, the use of prophylactic platelet transfusions at current decision thresholds needs to be reinvestigated.

		Site 1		Site 2	
Instrument	Technology	R^2	Intercept	R^2	Intercept
XE-2100	Impedance	0.77	1.73	0.76	2.63
XE-2100	Optical (fluorescence)	0.77	3.04	0.57	5.62
XE-2100	Reported	0.76	2.1	0.55	5.0
Cell-Dyn 4000	Optical (scatter)	0.81	2.11	0.72	3.73
Cell-Dyn 4000	CD61	0.92	1.2	0.86	1.41
Advia 120	Optical (scatter)	0.78	1.74	0.65	6.47
LH 750	Impedance	0.75	3.42	–	–

107

Transfusion related acute lung injury: a major cause of transfusion associated mortality and morbidity

D. Stainsby, H. Cohen, H. Jones, C. Beatty & L. Williamson

SHOT Steering Group, SHOT Office, Manchester Blood Centre, Manchester, UK

Transfusion-related acute lung injury (TRALI) is a major cause of transfusion-associated mortality and morbidity in the UK. This complication, with 103 cases of varying degrees of probability reported to SHOT over 6 years, has caused 25 deaths (seven definitely, four probably and 14 possibly attributed to transfusion) and 67 cases of major morbidity. Thirty-three suspected cases were reported to SHOT in 2001–2002, contributing to the deaths of seven patients and major morbidity in 18. The majority of patients reported to SHOT subsequently made a full recovery. Eleven patients died: in one death was thought definitely to be transfusion related, in two probably and in four possibly due to transfusion and in four unrelated. Eighteen patients suffered major morbidity. The component most commonly associated with the development of TRALI was FFP (12 cases) with a combination of components in 11 cases, platelets alone in five cases and red cells in five cases. The analysis was unsatisfactory as many cases were not fully investigated and clinical details were sketchy. It is recommended that there is early evaluation of cases by the consultant(s) involved. A team approach including the haematologist and chest physician and/or ICU consultant may be helpful. The blood services are refining the algorithm for investigation of TRALI so the laboratory investigation of cases should be more consistent and complete. Hospitals should continue to be aware of TRALI and to investigate and report possible cases. Continued education of all staff about this condition is encouraged so that cases may be investigated appropriately and implicated donors withdrawn. The blood services are currently evaluating and implementing strategies for reducing the risk of TRALI, including the provision of FFP exclusively from male donors. Meanwhile avoidance of unnecessary use of plasma and a high index of suspicion of TRALI are recommended.

108

Amotosalen hydrochloride (S-59) and UVA light treatment of cryosupernatant has no clinically significant effect on haemostatic proteins and appears suitable for plasma exchange in thrombotic thrombocytopenic purpura

H. Yarranton,[1] A. S. Lawrie,[1] I. J. Mackie,[1] L. Pinkoski,[2] L. Corash[2] & S. J. Machin[1]

[1]*Department of Haematology, University College London, London, UK;* [2]*Cereus Corporation, Concord, CA, USA*

The standard treatment for thrombotic thrombocytopenic purpura (TTP) is plasma exchange (PEX) with fresh frozen plasma (FFP). Cryosupernatant (CSP) may be better, as it lacks high and intermediate molecular weight VWF multimers implicated in the pathogenesis of TTP. A viral inactivated product is preferable. However, recent reports suggest that solvent detergent treated plasma may be associated with an increased risk of venous thromboembolism related to reduced protein S levels. Psoralen amotosalen HCl (S-59) and UVA light has been developed to inactivate pathogens in single plasma units. We studied the influence of S-59 treatment on haemostatic parameters of CSP. Forty single CSP units were prepared from North American blood group A donors. Twenty-one of the plasma units were individually treated with S-59 (test) and 19 units remained untreated (control). Units were frozen and stored between 7 and 9 months before being thawed for CSP preparation and analysis. The CSP process resulted in an expected increase in the APTT and TT, associated with a fall in VWF/FVIII parameters. VWF cleaving protease activity (VWF:CP) determined by electrophoresis [reference range (RR) 80–125%] and collagen binding assays (RR 66–126%) showed mean results of 99% and 81%, respectively, for test CSP and 105% and 95% for control CSP. Protein S levels using total, free antigen and activity assays showed no significant difference between test and control units and remained within the RR. The only statistically significant differences between test and control were for α-2-antiplasmin (102v111 U/dl), antithrombin (98v110 U/dl), protein C (93v103 U/dl) and VWF:CP, but all levels were within the RR. In conclusion, CSP prepared from S-59 treated plasma retained normal VWF:CP activity. We would therefore expect it to be a valuable replacement fluid for PEX in acute TTP. Compared with other viral inactivation methods, protein S activity and other physiological inhibitors of coagulation were maintained within the RR.

Samsung Bioepis Exhibit No. 1002
Page 1428

Oral Communication

Haemostasis & Thrombosis and Anticoagulation

109

Fibrin D-dimer is an independent predictor of future stroke in patients with atrial fibrillation

R. C. Tait,[1] D. L. Murdoch,[2] K. F. O'Neill,[3] A. McMahon,[4] I. W. Wallace,[3] A. Rumley,[4] J. Jackson[4] & G. D. O. Lowe[4]

[1]Department of Haematology, Royal Infirmary, Glasgow; [2]Department of Cardiology, Southern General Hospital, Glasgow; [3]Primary Care Trust, Glasgow; [4]Glasgow University, Glasgow, UK

Anticoagulation with warfarin can significantly reduce stroke risk in patients with atrial fibrillation (AF) but at a cost of increased bleeding. Fibrin D-dimer (Dd), a measure of fibrin turnover, is elevated in some AF patients and may help identify those at high stroke risk, allowing targeted warfarin therapy. We undertook a prospective observational study to assess the predictive value of Dd, other coagulation activation markers and clinical risk factors for stroke in unselected AF patients. A search of general practice records (population base 180 000) identified 1466 confirmed cases of AF, of which 1055 (mean age 72 years; 48% male) consented to participate. Levels of Dd, prothrombin F1 + 2 (F1 + 2), CRP, fibrinogen, thrombin-antithrombin complex (TAT), tPA and von Willebrand factor were measured at recruitment. The majority of recruits (75%) were, by recognized clinical criteria, defined as high or very high risk for stroke, with 21% at moderate risk and 4% at low risk (aged <65 years with lone AF). Two-thirds were receiving warfarin. During follow-up (total 2385 patient-years), there were 71 confirmed thrombotic events (stroke, TIA or other arterial embolism), 13 of which were fatal. Warfarin-treated patients had a lower thrombosis rate [2.1/100 years vs. 4.9/100 years; HR 0.42 (CI 0.26–0.67)] but a higher rate of major bleeds (2.5/100 years vs. 1.7/100 years). Univariate analysis revealed only Dd, F1 + 2, TAT, age and previous stroke to have positive associations with future stroke risk. Analysis of laboratary variables by quintiles showed Dd had the highest hazard ratio (HR) for future stroke (HR 7.87; 95% CI 2.8–22.4). On multivariate analysis, including adjustment for warfarin treatment, Dd remained the strongest predictor of stroke (HR 5.3; 95% CI 1.7–16.4), the only additional predictor being previous stroke (HR 2.17; 95% CI 1.3–3.5). This is the first large prospective study to demonstrate the independent predictive value of Fibrin D-dimer for stroke in AF.

110

Gene defects in patients with factor XI deficiency

J. van Veen,[1] M. Ravara de Matos Pereira,[2] A. C. Goodeve,[2] A. Bowyer,[1] S. Kitchen[1] & M. Makris[1,2]

[1]Sheffield Haemophilia and Thrombosis Centre, Royal Hallamshire Hospital; [2]Division of Genomic Medicine, University of Sheffield, Sheffield, UK

Factor XI (FIX) deficiency is a rare autosomal inherited bleeding disorder. It has variable bleeding tendency with clotting factor levels poorly correlating with bleeding symptoms. The diagnosis is complicated by failure to use a locally derived normal range, by interference from the presence of lupus anticoagulant and by the variable clinical expression. Here, we re-examined the diagnosis in patients with FXI deficiency registered at our centre. We prospectively recruited 23 patients from 23 unrelated families into this study. In addition, we recruited 54 normal healthy controls. All patients had tests for prothrombin time, APTT, FXI assay with synthasil and Actin FS (lupus insensitive) thromboplastins, lupus anticoagulant and FXI mutation analysis using conformation sensitive gel electrophoresis. Control subjects had their FXI measured with both thromboplastins and their DNA was used in the determination of polymorphisms/mutations. The FXI:C normal range was 0.61–1.49 U/ml. The mean FXI level of the patients was 0.38 U/ml. The deficiency was confirmed in 21 of 23 patients. Two patients had normal FXI at 0.61 and 0.71 U/ml, respectively, and had no FXI mutations. Mutations in the FXI gene were found in 16 of the 21 (76%) deficient patients. Four of the mutations have not been previously reported. One mutation Cys128Term was found in six (21%) of the unrelated patients as well as in one of 54 normal subjects suggesting that this may be common in the normal population. The normal subject with the Cys128Term mutation had the lowest FXI among the normals at 0.61 U/ml. Five patients had the lupus anticoagulant but all had mutations in their FXI gene. Eight polymorphisms in the FXI gene were identified of which four are novel. In conclusion, most cases of phenotypically defined mild FXI deficiency have mutations in the FXI gene. A common mutation (Cys128Term) may be responsible for many cases.

111

Heparin cofactor II, new insights into heparin binding

D. J. Okeeffe,[1] T. B. Baglin[2] & J. Huntington[3]

[1]Department of Haematology, Cambridge Institute of Medical Research; [2]Department of Haematology, CIMR, Cambridge; [3]Department of Haematology, University of Cambridge, Cambridge, UK

Unfractionated heparin and low molecular weight heparins have proved ineffective in preventing clot redevelopment in patients postinfarct. The heparin–antithrombin complex appears unable to inhibit clot bound thrombin whereas activated cofactor II (HCII) may be more effective. Using size fractionated heparins, we set out to assess heparin binding to HCII. The affinity of heparins for HCII, ranging in size from 8 to 26 saccharide units, were determined by following change in fluorescence of the extrinsic probe TNS. Dissociation constants were calculated by non-linear least square analysis of the plots of fluorescence change *versus* total heparin concentration. The number of ionic interactions and the strength of the non-ionic contribution were determined from the double log plot of Kd *versus* ionic strength. The affinity of HCII for heparin was size dependent but not linearly dependent as expected. Kds were determined for 8–26mer heparins. Significant improvement in affinity is noted to the 10mer with a fluorescence enhancement. This seems to define the first binding site, as the 12mer binds more weakly than the 10mer, and is the smallest size to produce a fluorescence quench. Disaccharide additions to the 12mer, however, continue the trend towards improvement in affinity with increasing heparin length. A second interaction site seems to be defined by the 16mer, as only a twofold change in Kd is observed between 16 and 26 unit heparins. The ionic strength dependency of heparin affinity for HCII is dramatic. The slope of the double log plot show four ionic interactions and a very weak non-ionic contribution to binding. In summary, we hypothesize that HCII has two contiguous binding sites for heparin, with the principal site accounting for the four ionic interactions and additional improvement in affinity provided by non-ionic interactions.

112

Thrombophilia in pregnancy: a systematic review

L. Robertson,[1] O. Wu,[1] P. Langhorne,[1] S. Twaddle,[2] P. Clark,[3] G. D. Lowe[1,2], I. Walker,[1] I. Brenkel,[4] M. Greaves,[5] L. Regan[6] & I. A. Greer[1]

[1]University of Glasgow, Glasgow; [2]SIGN; [3]University of Dundee, Dundee; [4]Fife Acute Hospitals NHS Trust; [5]University of Aberdeen, Aberdeen; [6]Imperial College School of Medicine, UK

Thrombophilia has been associated with venous thromboembolism (VTE) and adverse pregnancy outcomes. We conducted a systematic

Samsung Bioepis Exhibit No. 1002
Page 1429

review to determine the level of risk of VTE and adverse obstetric complications associated with heritable and acquired thrombophilia in pregnant women. We also assessed the effectiveness of prophylaxis in preventing adverse outcomes. All major electronic databases were searched. Data on VTE, adverse pregnancy outcomes and thrombophilia were extracted into prepiloted forms, and the methodological quality of the studies was assessed. Odds ratios (OR) stratified by thrombophilia type for each VTE and adverse pregnancy outcome were calculated. Where appropriate, meta-analysis was conducted based on the random effects model. Testing of heterogeneity was carried out by standard chi-squared test. Eighty-two studies were included in the review. The magnitude of risk of adverse clinical outcomes was defined. An OR as high as 34.03 was estimated for VTE in pregnant women homozygous for FVL. Significant risks for individual thrombophilic defects were also established for early pregnancy loss (OR 1.40–6.25); recurrent first trimester loss (OR 0.86–2.70); non-recurrent second trimester loss (OR 4.12–8.60); late pregnancy loss (OR 1.31–20.09); pre-eclampsia (OR 1.32–3.49); placental abruption (OR 1.42–7.71) and IUGR (OR 1.36–15.20). There was insufficient data on the effectiveness of thromboprophylaxis in preventing adverse outcomes. This is the first review that measures the level of risk of both VTE and all adverse obstetric outcomes with heritable and acquired thrombophilia in women who are pregnant or postpartum. However, further studies are required to establish the effectiveness and cost effectiveness of thromboprophylaxis in pregnancy.

113

Homology modelling of mutations causing factor XI deficiency using related crystal structures and bioinformatics

N. M. O'Connell,[1] R. E. Saunders,[2] C. A. Lee,[1] D. J. Perry[1] & S. J. Perkins[2]

[1]The Katharine Dormandy Haemophilia Centre and Haemostasis Unit, The Royal Free and University College Medical School; [2]Department of Biochemistry and Molecular Biology, University College London, London, UK

Analysis of the effect of residue mutations in factor XI (FXI) causing FXI deficiency has been restricted by the lack of a three-dimensional structure for FXI. Here, we describe the first full homology model for FXI based on closely related crystal structures. CLUSTALW and phylogenetic tree searches of serine protease domains in the Protein Data Bank identified β-acrosin to be the closest related structure to that in FXI, requiring only three insertions and deletions to create the model. The four apple domains of FXI were modelled using the homologous crystal structure of the N-terminal domain of hepatocyte growth factor, a member of the PAN superfamily. Modelling required only three insertions and deletions. Models were constructed using INSIGHT II and O software, then energy refined and validated using PROCHECK. The resulting models enabled the effect of 39 missense mutations in FXI to be examined. Protein misfolding was implicated as the likely mechanism of disease in eight of 17 mutations in the serine protease domain, and likewise 10 of 22 mutations in the four apple domains (a type I disorder). Unlike other coagulation factor deficiencies, clinical reports of a catalytically dysfunctional protein in FXI deficiency (a type II disorder) have been uncommon. From the structural models, four mutations in the serine protease domain are predicted to disrupt the active site, and six mutations in the apple domains are predicted to cause functional defects in substrate or co-factor binding. Phenotypic data are available for six of these mutations, and are consistent with the predictions for four of them. This first model of the FXI protein is considered to be sufficiently reliable to assess FXI mutations. This will enable new mutations to be characterized by analysis of their effects on protein structure to complement other phenotypic and expression data.

114

A comparison of bleeding and rethrombosis rate in primary antiphospholipid syndrome and inherited thrombophilia at a target INR of 2.0–3.0

P. R. J. Ames,[1] A. Ciampa,[2] M. Margaglione,[3] G. Scenna,[4] L. Iannaccone[4] & V. Brancaccio[4]

[1]Academic Department of Rheumatology, Leeds General Infirmary, Leeds, UK; [2]Ospedale G. Moscati, Avellino; [3]Department of Genetics, University of Foggia, Foggia; [4]Coagulation Unit, Ospedale A. Cardarelli, Naples, Italy

Oral anticoagulation (OA) is the mainstay of re-thrombosis prevention in antiphospholipid syndrome (APS). The relative risk of re-thrombosis and bleeding of primary PAPS (PAPS) in comparison with other conditions requiring oral anticoagulation is not known. Relative risk of re-thrombosis and bleeding was prospectively evaluated in 67 patients with PAPS (38 females, 29 males, mean age 40 ± 16 years), 89 with inherited thrombophilia (IT) (38 females, 51 males, mean age 46 ± 16 years) and 24 with aortic valve replacement (AVR) (13 females, 11 males, mean age 45 ± 17 years). The rate of minor bleeding was 5.01 event/patient/year ($n = 19$, two patients bled more than once) in the IT group, 13.26 event/patient/year in the APS group ($n = 46$, nine patients bled more than once) and 5.35 event/patient/year in the V group ($n = 29$; seven patients bled more than once). The rate of major bleeding was 0.53 event/patient/year ($n = 2$, one patients bled twice) in the IT group, 0.87 event/patient/year in the APS group ($n = 3$, all three with minor events as well) and 0.19 event/patient/year in the V group ($n = 1$). The hazard ratio between APS and AVR groups was not significant. Re-thrombosis occurred in 1.1% ($n = 1$) of the IT group and 12% ($n = 9$) of the PAPS group. The rate of re-thrombosis was 0.26 event/patient/year ($n = 1$) in the IT group and 2.88 event/patient/year in the APS group ($n = 10$, one patient with two recurrences). The hazard ratio for APS compared with the IT group was 13 (95% IC 1.6–102.2; $P = 0.015$). In the PAPS group, amongst baseline IgG aCL titre, lupus anticoagulant pattern, inherited thrombophilic defects, sex and duration of anticoagulation, a multiple regression analysis identified IgG aCL titre >80 GPL as an independent predictor of re-thrombosis. PAPS is more thrombogenic than IT with a 13-fold higher relative risk of recurrence. A baseline IgG aCL higher than 80 GPL predicts re-thrombosis in primary APS.

Poster Presentation

Cellular & Molecular Biology

115

Ras promotes monocyte lineage selection

R. L. Darley, L. Pearn, J. Fisher, T. Hoy & A. K. Burnett

Department of Haematology, UWCM, Cardiff, UK

Deregulation of Ras is particularly associated with disease with monocytic features suggesting that Ras may differentially influence the development of this lineage. In order to test this, we expressed mutant Ras in human $CD34^+$ cells and found it doubled the proportion of monocyte (CD14hi, CD15lo) colonies formed (66 ± 21% of colonies vs. 31 ± 4% in controls; $P < 0.05$) and also increased overall colony forming efficiency (84 ± 0.21% vs. 56 ± 18%; $P < 0.05$). We also found that immediately following retroviral transduction with Ras (day 3) the frequency of monocytic progenitors ($CD34^+$ and $CSF\text{-}1R^+$) cells was more than twice that of control cultures (17 ± 7% vs. 8 ± 3%; $P < 0.01$) suggesting this oncogene might be influencing monocyte commitment. We therefore examined its effect on lineage negative myeloid ($CD34^+$ and $CD38^+$) subpopulations as recently defined by

Samsung Bioepis Exhibit No. 1002
Page 1430

Manz *et al* who have defined the common myeloid progenitor cell (CMP) as CD123lo, CD45RA-. Bipotential populations with CFU-G/M potential (GMP) and erythromegakaryocytic potential (MEP) have also been defined as CD123lo, $CD45RA^+$ and $CD123^-$ $CD45RA^-$ respectively. Using four-colour cytometry, we isolated these uncommitted myeloid subpopulations expressing mutant Ras and examined its effect on colony formation. We found that Ras caused significant distortion to the pattern of colony formation. Within the GMP (bipotential) population, expression of Ras resulted in almost complete abrogation of CFU-GM formation compared with controls (2 ± 3% vs. 24 ± 2%; $P < 0.05$). Instead nearly all colonies formed were CFU-M (91 ± 9% vs. 56 ± 7%; $P < 0.05$). A similar distortion was observed in the CMP (multipotent) population: 32 ± 5% of colonies were CFU-M (vs. 3 ± 5% in control; $P < 0.05$). However, Ras also significantly reduced the colony forming efficiency of both CMP and MEP cells. These data indicate that expression of Ras can grossly perturb lineage selection of granulocyte/monocyte bipotential cells in favour of monocyte colony formation and provides a likely explanation for the association of deregulated Ras with monocytic leukaemias such as CMML.

116

Profiling proteasome activity in multiple myeloma and lymphoma

L. Crawford,[1] T. C. M. Morris,[2] D. Chauhan,[3] K. C. Anderson,[3] B. Walker[4] & A. E. Irvine[1]

[1]Department of Haematology, Queen's University; [2]Department of Haematology, Belfast City Hospital; [3]Medical Oncology, Dana-Farber Institute, Boston; [4]School of Pharmacy, Queen's University, Belfast, UK

The proteasome is a multicatalytic proteinase with three distinct catalytic activities – chymotrypsin-like (CT-L), trypsin-like (T-L) and peptidylglutamyl peptide-hydrolyzing (PGPH). Proteasome inhibition is an emerging therapy for many cancers and a novel treatment for multiple myeloma (MM). Most proteasome inhibitors target the CT-L activity, believed to be rate limiting in protein degradation; however, the precise role of individual catalytic activities remains unclear. We have developed a method to extract and measure all three activities of the proteasome. Cells were obtained from five MM cell lines (U266, OPM-2, KMS-11, KMS-18, IM-9), bone marrow (BM) from 10 MM patients, and PS-341 resistant and sensitive lymphoma cell lines (DHL-4, DHL-6). Cells were lysed with an extraction buffer containing 5 mmol/l ATP and 0.1 mmol/l DTT. Individual enzyme activities were measured as rate of turnover of specific fluorogenic substrates (CT-L:Succ-LLVY-AMC, T-L:Z-ARR-AMC, PGPH:Z-LLE-AMC) and recorded in Arbitrary Fluorescence Units (AFUs). Addition of BZLLL, a proteasome inhibitor that targets all active subunits with comparable affinity, caused 85% inhibition in cell lines and 91% in primary cells confirming the specificity of our measurements. T-L activity was highest and CT-L lowest in all MM cell lines and eight of 10 BM samples. In OPM-2 cells T-L activity was 4.6-fold greater than CT-L and 3.5-fold greater than PGPH. Primary cells from MM patients showed a similar profile. In the PS-341 resistant lymphoma cell line, DHL-4, CT-L activity was similar to MM lines but was significantly higher than T-L (1.3-fold) and PGPH (twofold). DHL-6 (PS-341 sensitive) displayed on average 11-fold less activity than DHL-4. The T-L and PGPH activities account for a large proportion of total proteolytic activity in MM cells. These profiles suggest the balance of proteasome activity is cell-type specific and imply that targeting individual activities with specific inhibitors could enhance therapeutic efficacy.

117

Inhibitors of lysophosphatidic acid acyltransferase-β (LPAAT-β) are anti-proliferative, induce apoptosis and down-regulate PA-requiring signalling pathways in ATRA sensitive and resistant myeloid cell lines

H. Al-Bakistani,[1] A. F. Gilkes,[1] D. Hollenback,[2] L. Bonham,[2] J. Singer[2] & K. I. Mills[1]

[1]Department of Haematology, Cardiff, Wales, UK; [2]Cell Therapeutics Inc., Seattle, WA, USA

Lysophosphatidic acid acyltransferase-β (LPAAT-β), an intrinsic membrane protein that catalyses the synthesis of phosphatidic acid (PA) from lysophosphatidic acid (LPA), has been implicated in cellular transformation. Recently, the identification of enzymes that mediate the production and degradation of LPA have suggested mechanisms by which the production or action of LPA could be modified. We have used specific inhibitors of the β-isoform of LPAAT on ATRA sensitive and resistant variants of the NB4 and HL60 cell lines which express LPAAT-β. The inhibitors were potent inducers of anti-proliferation and cytotoxicity of all four cell lines in a dose- and time-dependant manner. These inhibitors, after a 1 and 24 h exposure, also reduced LPAAT-β activity when measured with an intracellular-based assay. An increase in Annexin V expression, slightly increased activated caspase 3 staining and an accumulation of cells in G2/M, was also observed, while no effect of the inactive homologues of the inhibitors was seen. Western blot analysis of P-AKT, p70s6K, ERK and m-TOR showed a profound decrease in the phosphorylation status of the PA-requiring signalling pathways after treatment with the inhibitors. Affymetrix gene expression profiling following a 1 h treatment with the active and inactive compounds has been used to identify genes with altered expression. Several genes with high fold change between untreated, inactive and active treatments were identified, including caspase 5 and CDC2, and have been further analysed using RQ-PCR. These data indicate that LPAAT-β has an important role in AML cell survival and the effect of the inhibitors on ATRA-sensitive and -resistant cell lines suggest that LPAAT-β represents a novel therapeutic target for acute myeloid leukaemia.

118

Defining complete *Hox* signatures in murine haematopoietic cells by Q-PCR

G. J. McGonigle,[1] D. G. Grier,[1] A. C. Kwasniewska,[1] M. C. Yoder,[2] S. J. Collins,[3] T. R. J. Lappin[1] & A. Thompson[1]

[1]Department of Haematology, Queen's University, Belfast, UK; [2]Department of Paediatrics, Indiana University School of Medicine, Indianapolis, IN; [3]Human Biology Division, Fred Hutchinson Cancer Research Centre, Seattle, WA, USA

Developmental processes, such as haematopoiesis, depend on the coordinated expression of regulatory gene and protein networks in a temporal and spatial manner. Members of the *Hox* gene complex play a major role in haematopoiesis, but the contribution of the overall network has not been evaluated. Q-PCR is a rapid and sensitive method for the evaluation of known gene or gene networks. Taqman primer and probe oligonucleotides were designed for measuring the expression of all 39 murine *Hox* genes. Specificity was verified by cloning and sequencing. Plasmids were used for standard curve generation and the correlation coefficients; gradient and intercept values were highly reproducible for all *Hox* genes, indicating the robustness of this technology. The *mHox* Q-PCR system was applied to the EML-MPRO cell model and primary haematopoietic precursor cells. EML and MPRO were obtained by transducing a dominant-negative RAR-α into bone marrow cells. The *Hox* signature for the SCF-dependent EML cells showed higher gene expression than the more differentiated promyelocyte MPRO cells (*Hox b4* and *Hox a6*, 855 and 631 more copies

Samsung Bioepis Exhibit No. 1002
Page 1431

respectively). These data support findings that a global downregulation of *Hox* genes is associated with APL patient samples (Thompson *et al* Blood 2003; 101: 1558–1565) and suggests that *Hox* gene expression decreases with increasing cell maturity. To evaluate the expression of *Hox* genes during maturation c-kit$^+$/Lin$^-$ cells were sorted from fetal liver (e14.5) and adult bone marrow samples. The *Hox* signature for fetal liver derived cells showed global upregulation in expression over adult bone marrow derived cells (*Hox a4*, 30 186 additional copies). This is of particular interest due to the reported preferential engraftment and proliferation capacity of these cells. The application of the *mHox* Q-PCR system to murine models will help define the interrelationships within the *Hox* network in haematopoiesis.

119

Regulation of *Hox* genes by IL-3 in 32D and FDCP-mix cells

G. J. McGonigle,[1] A. Glasow,[2] D. G. Grier,[1] T. Enver,[2] A. Zelent,[2] T. R. J. Lappin[1] & A. Thompson[1]

[1]*Department of Haematology, Queen's University, Belfast;* [2]*Institute of Cancer Research, Chester Beattie Laboratories, London, UK*

Haematopoiesis is a highly organized developmental process that depends on complex patterns of gene expression under the control of haemopoietic growth factors. Interleukin-3 (IL-3) regulates the earliest identifiable stem cell and is thus a critical factor in the developmental process. Members of the clustered class I homeobox (*HOX*) gene complex play a major role in haematopoiesis, but the contribution of the overall network has not been evaluated. Some HOX proteins are leukaemogenic in murine models, while others expand the stem cell pool. In this study, the effect of IL-3 on *Hox* gene expression was investigated in two well-characterized growth factor-dependent murine cell lines (32D cl13 and FDCP-mix). A Q-PCR approach was taken for the simultaneous analysis of the *Hox* gene network. Taqman primer and probe oligonucleotides were designed and validated for all 39 murine *Hox* genes. Withdrawal of WEHI conditioned media (source of mIL-3) from the culture media of the 32D cells, for 16 h, caused the onset of apoptosis and the coincident increases in the expression of several Hox genes. Replenishment of the media with WEHI reduced the level of *Hox a5* and *a9* expression by 50% within 1 h and to control values by 24 h. Withdrawal of recombinant murine IL-3 from 32D cells resulted in the upregulation of 28 out of 38 *Hox* genes (including *Hox a4* and *b4*). Replenishment studies showed the upregulation of expression is transient and suggests a direct effect of IL-3 in regulating these particular *Hox* genes. Differential *Hox* gene expression was also observed in FDCP-Mix cells treated with different growth factors, e.g. high and low dose IL-3 alone and in combination with differentiation agents such as all-trans retinoic acid. These findings position *Hox* genes as potential targets of haematopoietic growth factors in particular IL-3, or the pathways they evoke.

120

Clonal haemopoiesis may occur after conventional chemotherapy and is associated with accelerated telomere shortening and defects in the NQ01 pathway; possible mechanisms leading to the development of t-AML/MDS

L. Fern, M. Pallis, G. I. Carter, C. Seedhouse, N. H. Russell & J. L. Byrne

Department of Haematology, Nottingham City Hospital, Nottingham, UK

The molecular pathogenesis of t-AML/MDS remains uncertain. However, we know that clonal haemopoiesis may develop following SCT and precede the development of t-AML/MDS; that accelerated telomere shortening may be induced by replicative stress or oxidative damage and lead to genomic instability; and that inactivating polymorphisms of the gene encoding NADPH-quinone oxidoreductase (NQO1) are more frequently observed in patients with t-AML. We studied clonal haemopoiesis, telomere length and NQO1 status in 146 patients receiving conventional chemotherapy for non-myeloid malignancies. Clonal haemopoiesis was demonstrated in 8/98 (8%) of female postchemotherapy patients studied. Telomere length was reduced in patients following chemotherapy particularly in those with clonal haemopoiesis ($P < 0.001$). There was a trend towards telomere shortening in control subjects homozygous for NQO1-187Ser which warrants further study since such individuals may be at risk of significant telomere shortening due to background exposure to reactive oxygen species from environmental and dietary agents. In the chemotherapy-exposed patients polymorphic for the NQO1-187Ser allele telomere length was significantly compared with those with wild type NQ01 ($P < 0.001$). Furthermore chemotherapy-treated patients with the NQO1-187Ser polymorphism were more likely to develop clonal haemopoiesis than patients with wild type NQO1 (OR = 8; 95% CI 1.3–45.5). We conclude that a switch to clonal haemopoiesis may occur after conventional chemotherapy and is associated with accelerated telomere shortening. Patients with the NQO1-187Ser polymorphism have an increased risk of developing both clonal haemopoiesis and telomere shortening which may in part explain the predisposition to t-AML in NQO1-187Ser null individuals.

121

Raf, PI3K and RalGDS each contribute to erythroid lineage misprogramming mediated by mutant Ras in primary human erythroid cells

R. L. Darley,[1] L. Pearn,[1] J. Fisher,[1] T. Hoy,[1] A. K. Burnett[1] & D. Wynford-Thomas[2]

[1]*Department of Haematology, UWCM;* [2]*Department of Pathology, UWCM, Cardiff, UK*

Previously, we have shown that mutant Ras is able to subvert the development of normal human erythroid cells by misprogramming early development giving rise to inappropriate expression of megakaryocyte antigens and concurrent reduction in colony-forming capacity; defects which closely parallel erythroid lineage dysplasia in preleukaemia. These defects are dependent on inappropriate activation by Ras of protein kinase C isoforms (PKC), which acts as a lineage-determining signal for megakaryocyte development. To investigate the role of individual downstream effectors of Ras, we employed effector mutants of Ras which contain additional point mutations resulting in selective activation of Raf(S35), PI3-kinase(C40) or RalGDS(G37). We expressed each of these in normal human erythroid progenitor cells by retroviral gene transfer. While mutant Ras itself (RasV12) resulted in a 40% reduction in erythroid colony-forming capacity (compared with control cells), none of the individual effector mutants of RasV12 had any significant effect on colony formation. Only by co-expressing pairs of effector mutants was colony formation inhibited. Each combination was equal in this respect indicating each effector made an equivalent contribution to the phenotype (mean 33% reduction). We also examined the effect on the induction of the megakaryocyte antigen, CD41, which is a PKC-responsive gene strongly induced by RasV12 (73 ± 10% of cells). In this case individual effector mutants did result in significant CD41 induction on erythroid cells (40 ± 15%, 52 ± 12% and 50 ± 15% respectively); data which were corroborated by repeating these experiments using constitutively active forms of Raf, PI3K and RalGDS. Surprisingly, but in agreement with these data, we also found stimulation of each effector of Ras gave rise to chronic activation of conventional and novel isoforms of PKC. These data

Samsung Bioepis Exhibit No. 1002
Page 1432

indicate stimulation of Raf, PI3-kinase and RalGDS each contribute equivalently to the misprogramming of erythroid development mediated by Ras, probably by chronic stimulation of PKC.

122

Influx and efflux of imatinib are mediated by active transport via drug transporter proteins

J. Thomas,[1] L. Wang,[2] R. E. Clark[2] & M. Pirmohamed[1]
[1]*Department of Pharmacology, University of Liverpool;* [2]*Department of Haematology, Royal Liverpool University Hospital, Liverpool, UK*

Imatinib mesylate (Glivec) is an effective drug in chronic myeloid leukaemia (CML) and in gastrointestinal stromal tumours. However, the development of imatinib resistance is becoming an increasing clinical problem. Mutations in BCR-ABL may be important in conferring resistance. However, acquired alterations in drug transport proteins have been associated with resistance in non-haematological settings (e.g. epilepsy), and there is data from CML cell lines suggesting that MDR1 overexpression may confer imatinib resistance. Here, we have investigated the mechanisms of imatinib cellular uptake and efflux. We used the leukaemic cell line CCRF-CEM and its drug resistant subline VBL100 to measure the uptake of ^{14}C-labelled imatinib. Imatinib uptake was temperature dependent (40.4 ± 5.9 ng/million cells at 4°C compared with 420.6 ± 140.5 ng/million cells at 37°C; $P < 0.05$), indicative of an active uptake process. Additionally, incubations with transport inhibitors showed that verapamil, amantadine and procainamide, inhibitors of the human organic cation transporter 1 (hOCT1) significantly decreased imatinib uptake into CEM cells, while inhibitors of hOCT2 or hOCT3 had no effect, indicating influx into the cells is an active process likely to be mediated by hOCT1. Studies on transepithelial drug transport model MDCK cells showed that in MDR1 (ABCB1) transfected MDCK cells there was a greater rate of basolateral to apical (B–A, secretory transport) transport of imatinib than apical to basolateral sides (A–B, absorptive transport) ($P < 0.001$), indicating the presence of an active secretory process from the cell. The B–A transport was inhibited by the specific MDR1 inhibitor PSC-833. Together, these data confirm an active efflux component attributable to MDR1. The results indicate that active transport processes mediate both influx and efflux of imatinib. Differential expression of influx (hOCT1) and efflux (MDR1) transporters may be a critical determinant of intracellular drug levels, and hence of resistance to imatinib.

123

Increased expression of the low affinity IgG receptor FcRγIIA (CD32) in platelet RNA from patients with chronic myeloproliferative disorders demonstrated by representational difference analysis

A. J. R. Allen, M. J. Nash, D. C. Linch & R. E. Gale
Department of Haematology, University College London, London, UK

There are no known disease-specific markers for the chronic myeloproliferative disorder (cMPD) essential thrombocythaemia (ET). The disorder may be heterogeneous, with both polyclonal and clonal myelopoiesis demonstrated in informative females using X-chromosome inactivation patterns. To identify potentially diagnostic markers of ET, we performed representational difference analysis on platelet cDNA from a clonal ET patient and a haematologically normal control (NC) with two rounds of hybridization and PCR in which the cDNAs were mixed in the ratio 4:1 (ET:NC or vice versa), then 16:1. The resulting PCR products were cloned, screened and clones showing apparent differential expression sequenced. Three were encoded by mitochondrial DNA (12S ribosomal RNA, cytochrome c oxidase II, cytochrome b), but no relative difference in expression of these three transcripts was detected using a variable number of amplification cycles in RT-PCR of platelet RNA from three ET patients and three NCs. Three clones showed an apparently greater expression in clonal ET platelets: ferritin light peptide, the low affinity IgG receptor FcRγIIA (CD32) and the chemokine RANTES. Radioactive semi-quantifiable PCR was performed to obtain the relative ratio of CD32 to GAPDH transcripts in platelet cDNA. The median ratio was 1 in 10 NCs (range <1–3) and was significantly higher in 15 clonal ETs (median 25, range 8–60) ($P = 0.005$). It was also significantly raised in 18 polyclonal ETs (median 23, range 2–73), eight with reactive thrombocytosis postsurgery (median 10, range 2–25) and 11 with PV (median 30, range 7–63) ($P < 0.01$ for all). Cross-linking of FcRγIIA has been implicated in a number of platelet activities, including GPVI and GpIb-mediated activation and downstream signalling. These results suggest platelets from patients with cMPDs and acute thrombocytosis may be partially activated, but CD32 is not sufficiently discriminatory to be useful as a diagnostic tool.

124

Improved quantitation of haematopoietic chimaerism by real-time polymerase chain reaction

L. W. Harries,[1,2] C. L. Wickham,[1,2] S. A. Rule,[4] M. V. Joyner[3] & S. Ellard[1,2]
[1]*Department of Molecular Genetics, Royal Devon and Exeter NHS Healthcare Trust;* [2]*Institute of Biomedical and Clinical Science, Peninsula Medical School;* [3]*Department of Haematology, Royal Devon and Exeter NHS Healthcare Trust, Exeter;* [4]*Department of Haematology, Derriford Hospital NHS Trust, Plymouth, UK*

Allogeneic bone marrow transplantation is the treatment of choice for certain haematopoietic malignancies. The use of mini-allografts with reduced conditioning regimes has enhanced the use of this treatment for patients previously ineligible for transplantation because of age or co-existent disease. Reduced conditioning regimes allow the initial persistence of a proportion of recipient cells in blood or bone marrow (mixed chimaerism; MC). Monitoring of MC is required to assess the success of the procedure and predict subsequent rejection or impending relapse. Existing methods of measuring MC include analysis of patient-specific restriction fragment length polymorphisms (RFLPs) and microsatellites. These techniques vary in their accuracy and sensitivity. We present here a quantitative real-time PCR approach for the measurement of MC, which exploits the presence of single nucleotide polymorphisms (SNPs) as a means to distinguish cells of patient or donor origin. A panel of 6 SNPs and an XY-specific marker were chosen for study. All assays were validated by standard curve analysis, which showed that the technique is reliably linear from 1% to 99% patient DNA in artificial mixtures. We examined 12 patient:donor combinations and all were found to be informative. We have performed a comparison between the use of microsatellites and the real-time approach to identify and measure MC. The overall correlation between the methods was good ($r_2 = 0.95$) where MC was present between 25% and 75% patient cells. However, when either patient or donor DNA was present in vast excess or in very small quantities, MC was only reliably and accurately measured by the real-time approach. Hence, patients who may be about to relapse (with low but increasing amounts of MC) may not be accurately identified by microsatellite methods. Improved quantification of the persistence or re-emergence of recipient cells in the blood or bone marrow during the post-transplant period should permit earlier intervention and hence improve clinical outcome.

Samsung Bioepis Exhibit No. 1002
Page 1433

125

Analysis of anagrelide-induced apoptosis in megakaryocytic cell lines

R. C. Boocock & C. N. Harrison

Department of Haematology, Guy's and St Thomas' NHS Trust, London, UK

Anagrelide has recently entered the clinical arena as an effective modality in the management of essential thrombocythaemia. It is a potent inhibitor of blood platelet formation with an IC50 value of *c.* 120 nm (30 ng/ml) in $CD34^+$ maturation assays; plasma drug concentrations rarely exceed 5–10 ng/ml. (Shire data on file). Precisely how anagrelide induces thrombocytopenia is unknown, although it has been postulated to act at the postmitotic phases of megakaryopoiesis. A small anti-proliferative effect within the megakaryocytic lineage has also been observed. The present study evaluated the *in vitro* effect(s) of anagrelide using a human megakaryocytic cell line, HEL. Cells were continuously exposed to anagrelide (2.67 ng/ml–43.75 mcg/ml) and cellular activities (apoptosis, cell cycle changes and viability) assessed over 72 h. Anagrelide induced significant cytotoxicity in the dose range 1–43.75 mcg/ml. Apoptosis was confirmed as the mode of cell death through caspase activation, DNA fragmentation and mitochondrial depolarization. Microscopy failed to detect classical apoptosis (membrane blebbing and/or apoptotic body formation), indicating a 'pseudo' or 'atypical' form of apoptosis. Cell cycle analysis revealed a gradual arrest in 'G_{0G1}' peaking at 24 h, which coincided with the onset of cell death and down modulation of Bcl-2, cMyc and p53. Cell cycle positioning by bivariate analysis ascertained that cells in 'G_{0G1}' were highly sensitive to the cytolytic effects of anagrelide. We show here for the first time that anagrelide, at supraphysiological concentrations, has the ability to induce a genetically defined form of cell death. The discrepancy between anagrelide concentrations required to inhibit megakaryocyte maturation, and the concentrations observed to induce apoptosis in the HEL cell line are unknown; although 'drug processing' and/or the pleiotropic effects of growth factors associated with megakaryopoiesis may be involved. We are currently evaluating the role of apoptosis in mediating the anti-cellular activities of anagrelide using haematopoietic growth factors and $CD34^+$, erythroid and megakaryocytic progenitors.

126

Isolation and properties of non-transduced and HoxB4-transduced bone-derived murine mesenchymal stem cells

J. D. Grainger,[1] M. D. Milsom,[2] L. J. Fairbairn[2] & R. F. Wynn[1]

[1]*Stem Cell Research Group, Royal Manchester Children's Hospital;* [2]*Patterson Institute Cancer Research, Manchester, UK*

The isolation of murine mesenchymal stem cells (muMSC) is complicated by the lack of specific markers and by haemopoietic contamination of the murine CFU-F. We describe the isolation of muMSC from femurs following mechanical depletion of bone marrow. Following enzymatic digestion of bone fragments with Collagenase type II the adherent population is expanded in MesenCult murine media (StemCell Technologies) and subsequently depleted of $CD45^+$ and $CD31^+$ cells. The initial cell count following enzymatic digestion of bone was half that of whole bone marrow cells (11×10^6 vs. 23×10^6 cells/femur). However, after 14 d the adherent population from bone was fivefold higher than that from bone marrow (4×10^6 vs. 0.8×10^6 adherent cells). The $CD45^-/CD31^-$ population in bone was an average of 21.5% vs. 7.6% for bone marrow resulting in a total output of 3.5×10^6 cells/femur compared with 0.05×10^6 from bone marrow. The muMSC derived in this manner can be expanded for over 60 population doublings and following transfer to stimulant media are able to differentiate into both adipocytes and osteocytes. Following staining with LacZ the muMSC derived from the Rosa26 murine strain can be demonstrated to express β-galactosidase. Exposure to HoxB4-GFP producers resulted in a transduction efficiency of 27% and expression was maintained for over 30 population doublings and was not seen in either in untransduced or mock-transduced mock-transduced muMSC. HoxB4-GFP-muMSC exhibited no difference in expansion kinetics or differentiation potential compared with non-transduced muMSC. In conclusion, muMSC are more abundant in bone compared with bone marrow but maintain the properties described for human bone marrow derived MSC. The muMSC isolated in this manner are suitable for preclinical studies in a murine model.

127

Proteolytic activities of the proteasome in chronic myeloid leukaemia

P. Windrum,[1,3] B. Walker,[2] M. F. McMullin[1,3] & A. E. Irvine[3]

[1]*Department of Haematology, Belfast City Hospital;* [2]*School of Pharmacy, Queen's University;* [3]*Department of Haematology, Queen's University, Belfast, UK*

The proteasome is a multicatalyic enzyme complex that plays a key role in cell cycle regulation. It has three distinct peptidase activities: chymotrypsin-like (CT-L), trypsin-like (T-L) and postglutamyl peptide hydrolase (PGPH). The aims of this study were to measure these individual proteolytic activities in a murine model for chronic myeloid leukaemia (CML), human CML cell lines and primary human cells. Proteasome was extracted in a DTT/ATP lysis buffer. Individual proteolytic activities were measured by rate of turnover of specific fluorogenic substrates (Succ-Leu-Leu-Val-Tyr-AMC, Z-Ala-Arg-Arg-AMC and Z-Leu-Leu-Glu-AMC for CT-L, T-L and PGPH respectively) and expressed as arbitrary fluorescence units (AFU)/50 μg protein/min. Addition of the novel proteasome inhibitor Bz-Leu-Leu-Leu was used to confirm reaction specificity. Proteasome activities were measured in an FDCP-Mix murine haematopoietic stem cell line transfected with a temperature sensitive Bcr-Abl gene. All three activities were higher in the Bcr-Abl positive cells compared with Bcr-Abl negative controls (CT-L 316 ± 26 vs. 163 ± 22, T-L 645 ± 97 vs. 393 ± 152 and PGPH 1169 ± 97 vs. 837 ± 29 AFU/50 μg protein/min) with PGPH highest and CT-L lowest ($n = 3$). Addition of Bz-Leu-Leu-Leu caused >95% inhibition of all three activities. Three human CML cell lines were studied: K-562, LAMA-84 and MEG-01. In all lines, T-L activity was highest and CT-L lowest, e.g. for LAMA-84 cells: CT-L 223 ± 6, T-L 1296 ± 134 and PGPH 683 ± 97 AFU/50 μg protein/min ($n = 3$). Addition of Bz-Leu-Leu-Leu caused >95% reduction in T-L and PGPH. Proteasome activity in one newly diagnosed CML patient was similar to Bcr-Abl positive cell lines while levels in three normal subjects were significantly lower and comparable with Bcr-Abl negative cells. First generation proteasome inhibitors such as Bortezomib target the CT-L peptidase of the proteasome. This study indicates future potential for targeting the T-L and PGPH proteases.

128

Are we getting as much information as possible from bone marrow biopsies?

K. M. A. Ramadan, J. Anderson, M. Drake, M. F. McMullin, T. C. M. Morris, P. Kettle & G. M. Markey

Department of Haematology, Queens University, Belfast, UK

Immunocytochemical definition of 'macroscopic' microscopic disease visible in H&E stained sections of bone-marrow biopsies is now a routine process. However, haematologists are frequently looking for minimal evidence of malignant disease or other disease processes to account for cytopenias or other phenomena. In this paper, we provide data from four instructive biopsies in which very small, but clinically significant, populations of cells were shown although H&E/Giemsa

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Samsung Bioepis Exhibit No. 1002
Page 1434

stained sections did not suggest their presence and currently would not routinely have been investigated further. The populations exposed were: (i) a tiny λ light chain-restricted population of IgD positive small lymphocytes in a patient with λ light chain amyloidosis; (ii) a relatively large polyclonal T lymphocytosis in a patient with pure red cell aplasia; (iii) a large population of CD20 positive lymphocytes of which one-third were CD5 positive in a patient with unexplained neutropenia; (iv) a tiny, but significantly increased, population of IgD positive plasma cells in the iliac crest biopsy from a patient with 'plasmacytoma' of cervical vertebrae. We conclude that aggressive investigation of bone marrow biopsies is warranted to determine the presence of 'clones' either possibly responsible for symptomatology (and possibly influencing their treatment) or for determining the presence of minimal neoplastic disease. Only by building a body of knowledge from such investigations will it be possible to determine fully and accurately the exact significance of these populations in individual patients in the future.

129

Detection in serum of tumour-derived antigen receptor DNA in chronic lymphoproliferative disorder patients

M. A. Catherwood, J. P. Wall & H. D. Alexander

Department of Haematology, Belfast City Hospital, Belfast, UK

Detection of minimal residual disease in haematological malignancies is best achieved by PCR-based molecular or flow cytometric techniques. Molecular studies have usually involved isolation of mononuclear cells from blood or bone marrow aspirate samples, followed by extraction of DNA and subsequent molecular analysis. This study was designed to investigate previous reports suggesting that tumour DNA may be detectable in the plasma or serum of patients with active disease. Tumour-derived antigen receptor DNA in serum, and DNA extracted from peripheral blood mononuclear cells, were analysed in 56 patients with B ($n = 54$) or T ($n = 2$) cell chronic lymphoproliferative disorders. DNA was amplified using PCR and analysed using polyacrylamide gel electrophoresis and a fragment analyser with Genescan software. In all 56 patients, clonal antigen receptor gene rearrangements were detected in DNA extracted from peripheral blood mononuclear cells. In 49 of the 56 (87%) serum samples, an antigen receptor gene clone was demonstrated. Twenty-nine of the 49 positive serum clones were identified using polyacrylamide gel electrophoresis, while the remaining 20 were detected using Genescan. Degradation of clonal tumour-derived DNA *in vitro* at room temperature reduced the sensitivity of the assay. Fresh serum gave optimal results. Biochemical and immunophenotypic markers for tumour load and active disease were not found to be statistically significantly different in patients in whom clonal tumour-derived DNA was detected in serum, either by polyacrylamide gel electrophoresis or Genescanning, and those in whom no clonal DNA was detected in serum. The use of Genescan in this study has significantly enhanced sensitivity, and hence accuracy, compared to previous studies. The use of PCR to detect tumour-derived DNA in serum is time conserving and could have benefits in diagnostic screening and for detection of minimal residual disease or prediction of clinical relapse.

130

Effect of calcium on bone marrow-derived stromal cell growth and differentiation

W. R. Almond, G. B. Tennant & A. K. Burnett

Department of Haematology, University of Wales, College of Medicine, Cardiff, UK

Bone marrow-derived stromal cells (BMdSCs) were isolated from the mononuclear cell fraction by plastic adherence and were grown and passaged in α-modified minimal essential medium (α-MEM) *in vitro*. When subconfluent, these cells were induced to differentiate down the osteogenic lineage in α-MEM, which has a relatively high calcium concentration of 200 mg $CaCl_2$/l. Induction involved the widely used differentiation protocol: dexamethasone (0.1 μmol/l), ascorbic acid-2-phosphate (50 μmol/l) and sodium β-glycerophosphate (10 mmol/l). We found that within several days of induction in α-MEM (variable between cultures), scattered mineral deposits began to form over the cell layer. These deposits gradually became so dense that in <21 d of induction (sometimes as little as 7 d) the cells had begun to break up, releasing the cytoplasmic contents. This made it difficult to analyse bone lineage gene expression quantitatively for >21 d as the extractable levels of RNA from differentiating BMdSC decreased as the mineralization in culture increased. We have therefore examined the effects of calcium concentration on stromal cell growth and differentiation with a view to limiting the rate of mineralization by reducing the medium calcium concentration. An alternative strategy is also being investigated by varying the concentration of sodium β-glycerophosphate. The addition of calcium chloride (1.56–400 mg/l) to stromal cell growth in α-MEM resulted in increased growth to 400 mg/l. On average, a twofold reduction in calcium concentration resulted in <20% reduction in stromal density. It was therefore possible to reduce the calcium concentration without seriously compromising stromal cell growth. Reducing the concentration of calcium chloride in α-MEM slowed the rate of mineralization in cultures allowing for RNA extraction for >21 d.

131

Acetyl carnitine stimulates *in vitro* myeloid progenitor growth

G. S. Masters,[1] G. B. Tennant[2] & A. K. Burnett[2]

[1]*Department of Haematology, University Hospital of Wales;*

[2]*Department of Haematology, University of Wales College of Medicine, Cardiff, UK*

It has been reported that erythroid colony formation is stimulated in cultures of fetal mouse liver cells by carnitine derivatives (Matsumura *et al* Am. J. Nephrol. 1998; 18: 355). This prompted us to check the effect of carnitine on our erythroid and myeloid clonal assays. Carnitine is involved in transporting long chain fatty acids across membranes for oxidative catabolism and for incorporation of lipid material into membranes. Carnitine also reduces the *in vitro* toxicity of glutamine-derived ammonia; and has been investigated as a possible treatment for anaemia in patients undergoing haemodialysis. As carnitine is only synthesized in liver, kidney and brain cells it is likely to be deficient in cultured haemopoietic cells, especially under serum-free conditions. Our serum-free assay of burst forming units erythroid (BFU-E) from normal peripheral blood was unaffected by acetyl carnitine levels between 0.3 and 10.0 μmol/l, in combination with optimal (2 U/ml) or suboptimal (0.5 U/ml) concentrations of erythropoietin (carnitine = 0 μmol/l, mean = 171 BFU-E/ml blood, 95% confidence range = 102–287; carnitine = 2.5 μmol/l, mean = 192, 138–267; $n = 15$). The growth of normal peripheral blood myeloid progenitors from the same patients (cultured with 10% FCS) was significantly stimulated by acetyl carnitine at all concentrations between 0.3 and 10.0 μmol/l ($P < 0.001$ at 5.0 μmol/l; $n = 9$). Colony forming unit granulocyte macrophage (CFU-GM) numbers were more than doubled with 2.5 μmol/l acetyl carnitine (mean = 30.2 CFU-GM/ml blood; 95% confidence range = 17.3–52.6; $n = 9$) compared with no addition (mean = 12.7; 4.9–32.9; $P < 0.01$, $n = 9$). The reason for the unexpected difference in the response to acetyl carnitine of myeloid and erythroid progenitors has yet to be resolved. The specific mechanism of myeloid stimulation is being further investigated. In the meantime, we have a useful increase in the myeloid clonal assay sensitivity.

Samsung Bioepis Exhibit No. 1002
Page 1435

132

Improving the accuracy of detection of t(11;14)(q13;q32) and t(14;18)(q32;q21) chromosomal translocations by PCR

C. L. Wickham,[1,2] L. W. Harries,[1,2] P. Sarsfield,[3] M. V. Joyner[4] & S. Ellard[1,2]

[1]Department of Molecular Genetics, Royal Devon and Exeter NHS Healthcare Trust; [2]Institute of Biomedical and Clinical Science, Peninsula Medical School; [3]Department of Histopathology, Royal Devon and Exeter NHS HealthcareTrust; [4]Department of Haematology, Royal Devon and Exeter NHS HealthcareTrust, Exeter, UK

The polymerase chain reaction is commonly used to identify chromosomal translocations for diagnostic purposes. A translocation is generally considered to be present upon amplification of a product of a similar size to the positive control. However, non-specific products may also be generated using PCR, in particular when using nested reactions to amplify from low templates. If these non-specific products are of a similar size to the positive control then it may be difficult to distinguish them from genuine translocation products, using agarose gel detection. It may also be difficult to determine if the product is genuine if it is significantly different in size from the positive control. We report the use internal control primers and sequencing to improve the accuracy of detection of t(14;18)(q32;q21) and t(11;14)(q13;q32) translocations. Samples were analysed from patients with suspected non-Hodgkin's lymphoma over a 5-year period (1997–2003). Internal control primers were introduced into published t(11;14)(q13;q32) and t(14;18)(q32;q21) translocation PCR reactions. Sequencing was employed to distinguish between translocation and non-specific PCR products. The number of non-specific products was decreased but not eliminated using internal control primers. The sizes of t(11;14)(q13;q32) and t(14;18)(q32;q21) PCR products amplified from patient samples were often very different in size from the positive controls, with products varying by up to 700 base pairs (bp). Confirmatory sequencing was required in 65% of cases, of which 70% were found to contain genuine translocation products. Genuine t(11;14) translocation products ranged in size from 234 to 934 bp and 143 to 560 bp for the MTCA and MTCB breakpoints respectively. Similarly two t(14;18) MCR products sequenced were found to be genuine despite the products being only 219 and 237 bp, in contrast to the positive control which spanned 498 bp. This data highlights the importance of using internal control primers and sequencing to accurately identify genuine translocation products.

Poster Presentation

Haemostasis & Thrombosis and Anticoagulation

133

Acute endothelial cell activation in the pathogenesis of severe and cerebral malaria

C. Donkor,[1,2] M. J. Hollestelle,[3] A. Osei Akoto,[1] J. O'Donnell,[4] J. A. van Mourik[3] & J. Bunn[2]

[1]Komfo Anoke Teaching Hospital, Kumasi, Ghana; [2]School of Tropical Medicine, Liverpool, UK; [3]Plasma Proteins, Sanquin, Amsterdam, the Netherlands; [4]Hammersmith Hospital, Imperial College, London, UK

Plasmodium falciparum malaria is a major cause of death in African children. Clinical features of severe and cerebral malaria are well described, but the pathogenesis remains unknown. Sequestration of malaria-infected erythrocytes in the peripheral circulation has been implicated, and *in vitro* studies have shown parasitized erythrocytes adhere to endothelial cells (EC). To investigate EC activation in severe malaria, we studied 87 Ghanaian children with severe ($n = 73$) or cerebral ($n = 14$) malaria, compared with 24 febrile but malaria-negative OPD controls. Plasma VWF:Ag and FVIII:C levels were both increased in severe malaria (medians 3.41 and 2.39 IU/ml), compared with malaria-negative controls (medians 1.21 and 1.5 IU/ml). However, the FVIII:VWF ratio was significantly reduced in malaria (0.7), a finding consistent with EC activation. VWF-propeptide levels provide a more specific measure of acute EC perturbation, and were markedly elevated in cases of severe malaria compared with controls (medians 25.3 and 10.4 nmol/l; $P < 0.001$). The highest VWF-propeptide levels were found in children with cerebral malaria (28.1 nmol/l) and in those children who died. Lactic acidosis (>5 mmol/l) is a reliable marker of malaria severity, and in children ($n = 20$) with lactic acidosis VWF-propeptide levels were significantly higher (medians 31.7 and 21.3 nmol/l; $P < 0.001$). In cerebral malaria ($n = 14$), follow-up samples demonstrated a rapid fall in propeptide (to 18.3 nmol/l at 24 h, and 10.9 mmol at 72 h) concordant with clinical recovery. In contrast, VWF:Ag and FVIII:C levels had increased further by 24 h, but declined by 72 h. Although VWF and FVIII are acute phase reactants, CRP values were normal in 73% of cases, and demonstrated no correlation with VWF-propeptide levels. In conclusion, acute EC activation is a hallmark of severe malaria and correlates with disease severity. Further studies are required to investigate the role of HMW-VWF multimers, VWF-propeptide, P-selectin, or other Weibel-Palade body components, in the pathogenesis of cerebral malaria.

134

Prospective evaluation of a new algorithm for diagnosis of pulmonary embolism

R. K. Patel,[1] C. Bagot[1] M. Buxton-Thomas,[2] S. Desai[3] & R. Arya[1]

[1]Department of Haematological Medicine, King's College Hospital; [2]Department of Nuclear Medicine, King's College Hospital; [3]Department of Radiology, King's College Hospital, London, UK

There is a lack of consensus regarding the optimal diagnostic strategy for pulmonary embolism (PE). We assessed the utility of a new diagnostic algorithm for PE at King's. All subjects with suspected PE were initially assessed using clinical pretest probability (PTP), plasma D-dimer (DD) and CXR. Subjects with low PTP and negative DD (<200 mg/l) were discharged without further investigation. In remaining subjects CXR result determined further imaging. Subjects with normal CXR underwent ventilation-perfusion (VQ) scan, requiring subsequent spiral CT (CTPA) only if results were non-diagnostic. Subjects with abnormal CXR underwent CTPA, requiring further investigation with lower limb venous duplex or pulmonary angiography only if results were equivocal. Over a 12-month period, we prospectively assessed 437 patients (62% female) with suspected PE (mean age 56.8 years, SD 19.5). PTP scores were as follows: 15.3% high, 81.2% moderate and 3.5% low. DD level (range 14–9807 mg/l, median 599 mg/l) was >200 mg/l in 90.8% of subjects and CXR was abnormal in 57.2% subjects. VQ scan was performed in 42.1% (60.9% low probability, 29.3% intermediate probability and 9.8% high probability scans) and CTPA in 59.7% of subjects (72.4% normal, 3.8% equivocal, 23.4% positive and 0.4% failed) with 10.1% having both types of scan. Of subjects selected for CTPA, 75.5% had an abnormal presentation CXR; of those having VQ scan 84.2% had a normal presentation CXR; 18.9% of subjects were diagnosed as having PE (66.3% by CTPA and 22.9% by VQ, 9.6% both). In PE subjects, CXR was abnormal in 57.8%. DD levels of <200 mg/l had a 100% negative predictive value (NPV) for PE (range 201–8265 mg/l, median 1227 mg/l). Low PTP had a 92.3% NPV for PE (1.2% low PTP, 75.9% moderate PTP and 22.9% high PTP). Use of such an

Samsung Bioepis Exhibit No. 1002
Page 1436

algorithm helps streamline PE diagnosis, improves diagnostic accuracy and allows rational and efficient use of imaging resources.

135

Potentiation of fibrinolysis by inhibition of thrombin activatable fibrinolysis inhibitor in whole human blood

N. L. Cruden,[1] L. Edwardson,[1] L. Lawes,[1] S. Robinson,[1] C. A. Ludlam[2] & D. E. Newby[1]

[1]Cardiovascular Research, University of Edinburgh; [2]Department of Haematology, University of Edinburgh, Edinburgh, UK

Exogenous tissue plasminogen activator (t-PA) fails to achieve reperfusion of the infarct related artery in *c.* 30% patients presenting with acute myocardial infarction. Thrombin-activatable fibrinolysis inhibitor (TAFI) is a carboxypeptidase present in human plasma that inhibits t-PA mediated fibrinolysis in animal models of coronary artery occlusion. Investigation of the role of TAFI in man has been limited by the lack of a suitable bioassay. Thromboelastography (TEG) is a potential method for assessing clot dynamics *in vitro* through changes in the viscoelastic properties of whole blood over time. Using TEG of human whole blood from healthy volunteers ($n = 5$ for each study), we investigated the effects of potato carboxypeptidase inhibitor (PCI), a specific inhibitor of TAFI, and tranexamic acid (TA), a competitive inhibitor of plasminogen activation, on t-PA mediated clot lysis. Incremental doses of recombinant t-PA (0–560 ng/ml) were applied with saline placebo, PCI (27 μg/ml) or tranexamic acid (420 ng/ml) during tissue factor initiated TEG and the percentage of clot lysed at 60 min was recorded. In the presence of placebo, t-PA produced a significant dose-dependent increase in clot lysis in all studies ($P < 0.001$). PCI or TA alone did not alter clot lysis. The presence of PCI or TA, caused left and rightward shifts respectively in the dose response curve for t-PA mediated lysis ($P < 0.001$). The mean concentrations of t-PA producing 50% clot lysis in the presence of PCI, t-PA alone or TA were 160 (130–190) ng/ml, 220 (186–268) ng/ml and 372 (125–1111) ng/ml respectively. Thromboelastography is a suitable bioassay in which to assess inhibitors and adjuncts of t-PA mediated lysis in human whole blood. Tissue plasminogen activator-mediated clot lysis is potentiated by PCI, a specific inhibitor of TAFI, and inhibited by TA suggesting functional activity of TAFI in human whole blood. Inhibition of TAFI may be a novel adjuvant to thrombolytic therapy in man.

136

Low incidence of high titre FVIII inhibitors in previously untreated children with severe haemophilia A treated only with intermediate FVIII concentrate (BPL 8Y). Experience from five comprehensive care centres in the UK

S. A. Brown,[1] M. D. Williams,[2] D. M. Keeling,[3] T. T. Yee,[1] P. W. Collins,[4] H. Dasani,[4] R. Liesner,[5] F. G. Hill[2] & C. A. Lee[1]

[1]Katharine Dormandy Haemophilia Centre, Royal Free Hospital, London; [2]Department of Haematology, Birmingham Children's Hospital, Birmingham; [3]Oxford Haemophilia Centre, Churchill Hospital, Oxford; [4]Arthur Bloom Haemophilia Centre, University Hospital of Wales, Cardiff; [5]Department of Haematology, Great Ormond Street Hospital for Children, London, UK

Development of high titre FVIII inhibitors in severe haemophilia A (SHA) remains the most significant complication of replacement therapy. It remains controversial whether the type of FVIII concentrate used influences the development of FVIII inhibitors in SHA. Previous reports have shown a low incidence of inhibitors in children who had only received an intermediate purity FVIII concentrate (BPL 8Y). The aim of the current study is to obtain data on BPL 8Y use in relation to the incidence of high titre inhibitors (>5 BU/ml) and underlying FVIII gene mutations. Seventy-four children with SHA, from five centres, were treated solely with BPL 8Y as the first FVIII concentrate. The median age at first exposure to BPL 8Y was 12 months (range 0.5–70 months) and median duration of treatment was 78 months (range 15–149 months). In this cohort, only two high titre inhibitors developed (an incidence of 2.7%). This compares with an incidence of high titre FVIII inhibitors of between 11.3% and 18% seen in three studies of recombinant FVIII concentrate use in previously untreated children. Although molecular analysis remains incomplete, 26 of 54 (48%) individuals tested possess the intron 22 inversion. In a further 20 individuals, an underlying mutation in the FVIII gene has been detected. A review of the Hamsters FVIII database revealed there was information on inhibitor development for the mutations in 12 individuals, of which the mutations in six individuals had been associated with FVIII inhibitor development. ITI has failed to provide long-term tolerization in the two individuals with FVIII inhibitors. The underlying FVIII gene mutation has been identified in one of the inhibitor patients: this nonsense mutation (C1336T) is associated with inhibitor development. In conclusion, the use of BPL 8Y was associated with a low incidence of high titre FVIII inhibitors.

137

Circulating endothelial cells predict outcomes in patients with acute coronary syndrome: relationship to plasma indices of endothelial damage/dysfunction and interleukin-6

K. W. Lee & G. Y. H. Lip

University Department of Medicine, City Hospital, Birmingham, UK

It is well recognized that markers of inflammation and coagulation as well as endothelial activation are altered in patients with acute coronary syndrome (ACS). The aim of this study was to compare the short-term prognostic value of interleukin-6 (IL-6, an inflammatory marker), von Willebrand factor levels (VWF, an index of endothelial damage/dysfunction) and circulating endothelial cells (CECs, indicating endothelial damage) in patients presenting with ACS. A total of 156 patients (107 males, 64 ± 12 years) consecutively admitted to the coronary care unit with ACS were included. Blood was drawn within 24 h of admission (T1) and 48 h thereafter (T2) for plasma levels of VWF and IL-6 (ELISA). CECs were quantified using epifluorescence microscope after immunomagnetic separation. Baseline values were compared with 35 matched patients with stable coronary heart disease (CHD) and 35 healthy controls. Levels of VWF, IL-6 and CECs were significantly higher in ACS patients compared with stable CHD and controls (all $P < 0.01$). CECs, VWF and IL-6 were highly correlated with each other (all $P < 0.001$). Forty-eight patients had a major adverse clinical event (MACE; 10 deaths, 33 refractory angina, five readmitted with ACS) at 30 d. CECs and IL-6 levels at T1 and T2, VWF levels at T2 and changes of CECs, VWF and IL-6 levels over 48 h of admission were strongly and significantly correlated with 30-d MACE (all $P < 0.002$). CECs at T2 were the strongest independent predictor for 30-d MACE, followed by the changes of IL-6 and VWF levels over 48 h (all $P < 0.05$). CECs level at T2 was the only single independent predictor of death at 30 d ($P = 0.007$). Raised plasma levels of VWF, IL-6 and numbers of CECs during the first 48 h of ACS were strongly associated with MACE at 30 d. CECs levels at 48 h were the only independent predictor of both death and MACE at 30 d, indicating the crucial role of endothelial/vascular damage in the pathogenesis of ACS.

Samsung Bioepis Exhibit No. 1002
Page 1437

138

Effects of hypobaric hypoxia on platelet activation and reactivity

C. I. Jones,[1] I. Ford,[2] R. J. Pearse,[1] V. Chudasama,[1] L. R. Mitchell,[2] S. J. Watt,[2] A. V. Hurley,[3] A. J. Batchelor,[3] D. P. Gradwell,[3] H. G. Watson,[2] M. Greaves,[2] A. H. Goodall[1] & W. D. Toff[1] for the WRIGHT Group (World Health Organisation Research Into Global Hazards of Travel)

[1]Department of Cardiovascular Science, University of Leicester, Leicester; [2]Department of Medicine and Therapeutics, University of Aberdeen, Aberdeen; [3]RAF Centre of Aviation Medicine, Henlow, UK

Links between air travel and deep vein thrombosis (DVT) have been widely reported but it is unclear if specific factors in the aeroplane cabin increase the risk over that of seated immobility at ground level. The role of platelets in the pathogenesis of DVT is also uncertain, although aspirin has been advocated as prophylaxis. We investigated the effects of hypobaric hypoxia, similar to that which might be experienced during a commercial long-haul flight, on platelet activation and reactivity. Thirty-one healthy volunteers sat for 8 h in a hypobaric chamber and were exposed to normobaric normoxia (ground level) or hypobaric hypoxia (equivalent to an altitude of 8000 feet). Subjects were exposed to both conditions, in a double-blind randomized fashion, 1–2 weeks apart. Blood was taken before and within 10 min after exposure. Whole blood flow cytometry was performed to measure platelet response *ex vivo* [percentage of platelets binding FITC-fibrinogen (Dakocytomation)] following activation with ADP (1×10^{-6} mol/l; Sigma) and TRAP (1×10^{-6} mol/l; in-house) and platelet activation *in vivo* [percentage of leukocyte-platelet aggregates (LPA), separating leukocyte populations by forward and side scatter characteristics and the presence of platelets by anti-CD42b antibodies (BD Pharmingen)]. There was no significant change in fibrinogen binding to platelets following either normobaric or hypobaric exposures, in response to ADP (69.3 ± 12.3% vs. 67.3 ± 12.5%, $P = 0.11$, and 69.6 ± 10.6% vs. 70.0 ± 12.1%, $P = 0.93$ respectively) or TRAP (4.36 ± 1.6% vs. 5.33 ± 3.1%, $P = 0.15$, and 4.55 ± 1.7% vs. 4.71 ± 2.07%, $P = 0.41$ respectively). The percentage of LPA increased after both normobaric (monocytes: 9.15 ± 1.3% vs. 10.16 ± 1.4%, $P < 0.0001$; lymphocytes: 8.51 ± 1.3% vs. 9.21 ± 1.4%, $P < 0.0001$; granulocytes: 8.44 ± 1.3% vs. 9.20 ± 1.4%, $P < 0.0001$) and hypobaric (monocytes: 10.1 ± 3.3% vs. 10.43 ± 2.5%, $P = 0.708$; lymphocytes: 8.88 ± 1.6% vs. 9.71 ± 2.0%, $P = 0.004$; granulocytes: 8.59 ± 1.7% vs. 9.49 ± 2.1%, $P = 0.0002$) exposures but there was no significant difference in the change between the two conditions. In conclusion, mild hypobaric hypoxia has no effect on platelet activation or reactivity.

139

Thrombin generation and correlation with bleeding tendency in inherited FXI deficiency

N. M. O'Connell,[1] J. H. McIntosh,[2] A. Riddell,[1] G. Pascoe,[1] A. Griffioen,[1] C. A. Lee[1] & D. J. Perry[1]

[1]The Katharine Dormandy Haemophilia Centre and Haemostasis Unit, The Royal Free and University College Medical School, London, UK; [2]The National Institute for Biological Standards and Control, Potters Bar, Hertfordshire

Factor XI (FXI) deficiency is characterized by a variable bleeding tendency but routine coagulation assays do not readily identify individuals at risk of excessive haemorrhage. We have developed and evaluated a thrombin generation assay in a group of 23 patients with FXI deficiency with well-defined bleeding phenotypes and in a subgroup of patients treated with recombinant factor VIIa (rFVIIa). Defibrinated plasma was incubated with phospholipid and coagulation initiated with a low concentration of recombinant tissue factor (10 pg/ml) and $CaCl_2$. Subsamples of the reaction mix were withdrawn into a stop solution at 30–60 s intervals for 15–30 min and thrombin generation determined using the chromogenic substrate (S2238). Study participants consisted of 12 patients with severe deficiency of FXI and 11 with partial deficiency. Significantly less thrombin generating potential was observed in severely deficient plasmas compared with partially deficient plasmas, with a median *AUC* of 1574.2 U/ml vs. 3199.5 U/ml (ranges 1260.4–3119.4 U/ml and 1402.5–5354.1 U/ml; $P = 0.0062$). The median peak thrombin was also less in severely deficient plasmas at 1.95 U/ml vs. 6.5 U/ml [ranges 1.4–4.3 U/ml and 3.7–12.8 U/ml; $P < 0.0001$]. There was a non-significant trend for higher *AUC* and peak thrombin in non-bleeders *versus* bleeders (median *AUC* 1790.9 U/ml vs. 1357.6 U/ml and 3480.6 U/ml vs. 2322.1 U/ml in severely and partially deficient plasmas). Six patients received rFVIIa to cover elective surgical procedures. A marked increase in the *AUC* and peak thrombin (median *AUC* 2147.55 U/ml vs. 3804.5 U/ml, median peak thrombin 4.9 U/ml vs. 11.5 U/ml) was seen after the first dose of rFVIIa. These preliminary results suggest that the thrombin generation test can differentiate severe and partial FXI deficiency and may be effective in identifying patients with a bleeding tendency. Treatment with rFVIIa normalizes thrombin generation. A continuous thrombin generation assay is currently under development to investigate the relationship between bleeding tendency and thrombin generation in a larger patient group.

140

Successful use of magnetic resonance direct thrombus imaging (MRDTI) and inferior vena cava (IVC) filter insertion in pregnancy and the puerperium

H. Lyall,[1] R. Ganatra,[2] R. Gregson,[2] M. M. Ramsay,[3] D. K. James[3] & J. Strong[1]

[1]Department of Haematology, Queen's Medical Centre University Hospital; [2]Department of Radiology, Queen's Medical Centre University Hospital; [3]Department of Fetomaternal Medicine, Queen's Medical Centre University Hospital, Nottingham, UK

Magnetic resonance direct thrombus imaging (MRDTI) is a new imaging technique. It allows assessment of deep vein thrombosis (DVT) and pulmonary emboli (PE) as a single investigation. Intravenous contrast is unnecessary and there is no radiation exposure. This makes its use appealing, particularly in pregnancy. inferior vena cava (IVC) filters have a limited but important role in the treatment of venous thromboembolism (VTE). Filter usage in pregnancy and the puerperium is not widely reported. We present a series of three women who developed VTE in either the third trimester or puerperium. Standard management with therapeutic heparin failed. Two patients presented with DVT. Despite therapeutic anticoagulation they developed PE. The third patient had bleeding that necessitated minimization of anticoagulation. MRDTI accurately evaluated both DVT and PE. All three patients had IVC filters inserted. In the two patients with recurrent VTE despite anticoagulation MRDTI delineated thrombus in both the pulmonary vessels and the proximal extent of clot in the pelvic veins. In pregnancy visualization of pelvic thrombus with ultrasound is difficult owing to the presence of a gravid uterus. One patient received a permanent filter, the other a temporary one. The third patient presented with chest symptoms. Ventilation/perfusion scanning gave an intermediate probability of PE. Definitive diagnosis was made by MRDTI. She developed a rectus sheath haematoma. A temporary filter was inserted and the baby was delivered safely by caesarean section. The filter was later removed without complication. We believe MRDTI is the gold standard imaging modality for VTE in pregnancy. These cases illustrate a role for IVC filters in pregnancy and the puerperium. Long-term follow-up data in this setting is currently lacking. Given the rarity of these events a national database would be of value.

Samsung Bioepis Exhibit No. 1002
Page 1438

141

Circulating platelet microparticles in patients with antiphospholipid antibodies

B. J. Hunt, A. O'Mahony & K. Parmar

Department of Haematology, St Thomas' Hospital, London, UK

Platelet microparticles (PMPs) are released upon platelet activation that are prothrombotic. The aim of this study was to assess PMPs in the pathogenesis of thrombosis in patients with antiphospholipid antibodies (aPL). Ethical committee approval was obtained. PMP levels were measured using both a functional assay of PMP procoagulant activity, and flow cytometry assay. There were 20 healthy controls (median age 27 years, range 20–62 years), and 17 patients with aPL (median age 42 years, range 29–74 years). All patients with aPL had a positive lupus anticoagulant or anticardiolipin antibody on at least two occasions 8 weeks apart. Fifteen had previous thrombotic events and were using oral anticoagulation. Blood was collected by flawless venepuncture into trisodium citrate. PMPs were detected by flow cytometry (Becton Dickinson) using CD42b-PE (PharMingen) as a platelet marker and annexin V-FITC (PharMingen). Platelet-derived procoagulant activity of the following plasma fractions; PRP (platelet rich plasma), PPP (platelet poor plasma), 0.1-μm filtered PFP (particle free plasma) and 0.2-μm filtered PFP was assayed by Russells Viper Venom Test (RVVT) on the ACL300R (IL, UK Ltd.). Results were compared with RVVT times of doubling dilutions of brain phospholipid (Diagen) in filtered pooled plasma. Statistical analysis was performed using the Mann–Whitney test. All patients receiving anticoagulant therapy had significantly prolonged RVVT times in comparison to normal controls, making this method unsuitable for determining the procoagulant activity of PMP. The PMP levels in patients with aPL were significantly higher (median 1.72, range 0.59–3.72) than normal controls (1.03, 0.19–2.29) ($P = 0.02$). Thus PMPs may play a role in the pathogenesis of thrombosis in antiphospholipid syndrome. Unfortunately, the procoagulant activity of PMP using RVVT is not reliable in patients receiving oral anticoagulation.

142

Defective fibrinolysis in children with severe haemophilia A

M. C. Mathias,[1,2] I. Mackie,[1] K. Khair,[2] S. J. Machin[1] & R. Liesner[1,2]

[1]*Department of Haematology, University College London Medical School;* [2]*Great Ormond Street Children's Hospital, London, UK*

In addition to the coagulation defect in children with haemophilia A, there is increasing evidence that they have abnormal regulation of fibrinolysis compared with non-haemophiliacs, which is thought to result from reduced generation of activated TAFI because of an insufficient thrombin burst at the initiation of coagulation. We present clot lysis data from 19 children (aged 3 months to 15 years) with severe haemophilia A (FVIII <1%), 14 untreated and another five treated 9–48 h previously. Platelet poor plasma, tPA (1.43 nmol/l), Hepes buffer and either BSA or recombinant FVIII (final concentration *c.* 1 IU/ml) were combined and then a thrombin/calcium chloride mixture added before pipetting in triplicate onto a microtitre plate and reading absorbance at 405 nm at 10–15-min intervals until clot lysis was complete. The time to 50% clot lysis was calculated graphically. Children with baseline FVIII levels had shorter lysis times (mean ± SD, 59.9 ± 8.9 min) compared with 10 normal adults (85.4 ± 23.9 min); this was corrected with *in vitro* FVIII concentrate (88.0 ± 15.3 min, $P < 0.000$ post-FVIII). We found no significant correction of the lysis times with *in vitro* addition of rVIIa (Novo Nordisk). Two children given FVIII <12 h previously had lysis times greater than those of normal plasma (mean = 119.5 min vs. 85.4 min for normal plasma) and lysis was only prolonged marginally by further *in vitro* FVIII. Three children who received FVIII 48 h previously had lysis times similar to those with baseline FVIII levels. Severe haemophilia A is associated with shortened clot lysis times which are prolonged by addition of FVIII, an effect which is maintained *in vivo* for up to 12 h, but with this assay more than a trace amount of FVIII is needed to have an impact on the clot lysis time.

143

A second cohort of patients with coronary heart disease and reduced VWF cleaving protease activity

M. Coakley,[1] S. Mallet,[1] G. Purdy,[2] R. Self,[2] S. J. Machin[2] & I. J. Mackie[2]

[1]*Department of Anaesthesia, Royal Free Hospital;* [2]*Haemostasis Research Unit, Department of Haematology, UCL, London, UK*

In a previous study (Br. J. Haematol. 2003; 121(Suppl. 1): 21–22), we found a mild reduction of von Willebrand factor cleaving protease (VWFcp), using a collagen binding assay, in four of 26 acute coronary syndrome patients. We now present a separate cohort of 33 patients (age 44–83 years) with ischaemic heart disease (stable angina), attending cardiology outpatients or having elective angiography. Twelve patients had previous MI, eight PTCA and nine CABG surgery. VWFcp was measured by an electrophoretic method based on the measurement of residual VWF multimers after incubation of patient plasma with pure VWF. Of 33 patients, nine had reduced VWFcp (median ratio 0.26, IQR 0.48 and normal range 0.80–1.25). Three of these showed a gross reduction (ratios: 0.02, 0.06 and 0.07), similar to that commonly seen in acute TTP. Mixing tests suggested the presence of an inhibitor in three of the nine patients with low VWFcp. There was no association between low VWFcp and clinical history or current medication. All patients were receiving 75 mg daily aspirin, but a large proportion showed apparent aspirin resistance as measured by the PFA-100. Six of the nine patients with reduced VWFcp had PFA-100 closure times <200 s using CEPI cartridges, although 13 of 24 patients with normal VWFcp ratios also had closure times <200 s. There was a trend towards shorter closure times and lower VWFcp with higher VWF:Ag values ($rs = -0.38$, $P = 0.03$; $rs = -0.4$, $P = 0.02$ respectively). The reduction in VWFcp confirms our previous findings and is in agreement with Yoo *et al* (2003), where 14 patients with stable coronary heart disease were found (using a method based on residual VWF:RCo activity) to have a mild reduction in VWFcp, as well as increased VWF:Ag. These results suggest that a large study of VWF function and metabolism in coronary syndromes is merited, to investigate the potential contribution of VWFcp in cardiovascular disease.

144

The combined use of D-dimers and diagnostic imaging in the exclusion of deep vein thrombosis in a nurse led outpatient service

P. M. Baker, S. R. Davidson & D. M. Keeling

Department of Haematology, Level 4, John Radcliffe Hospital, Oxford, UK

The use of D-dimers in the exclusion of venous thromboembolism is of maximal clinical benefit when incorporated into an algorithm in combination with diagnostic imaging. This approach has been adopted in our DVT clinic over the past 2 years to screen 3182 patients using the diagnostic scoring categories of Low, Intermediate and High probability as described by Wells *et al* (1997; 191: 3182) (6%) were categorized as low probability of which 100 (52%) were excluded from further testing with a negative (<500 μg/l FEU)

Samsung Bioepis Exhibit No. 1002
Page 1439

D-dimer (Biomerieux, UK). Those that were D-dimer positive (48%) went on to have ultrasonography with all but one being negative. Those in the high probability group (1186/3182, 37%) went straight to have ultrasonography with 333 being positive. Those that were negative (853) had a repeat scan in 1 week. This identified 25 more positive cases. In the intermediate group, 1805 (57%) were identified. Ultrasonagraphy and D-dimers were performed in all cases; 1662 (92%) of the patients were ultrasound negative with approximately 48% of those D-dimer negative. Repeat scanning was withdrawn in this category with <1% positive after 1 week. Of those that were D-dimer positive 23 of 870 (3%) were subsequently found to be positive on repeat so follow-up in this category is currently maintained. In the intermediate group, 138 (8%) were ultrasound positive of which 13 (9%) had a negative D-dimer. These figures give a negative predictive value of >98% for D-dimers in the intermediate probability group, similar to the recent medical devices agency (MDA) recommendations. In conclusion, D-dimers in conjunction with a diagnostic algorithm have saved potentially 1231 of 4147 (30%) ultrasound investigations without adverse clinical outcome. Currently, the goal is to further evolve the categories similar to that of Wells *et al* (2003) limiting still further the numbers of ultrasounds that are performed.

145

Elevated factor VIII:C levels in patients with pulmonary hypertension. A marker of endothelial dysfunction?

J. van Veen,[1] C. A. Elliot,[2] L. Martin,[2] I. Armstrong,[2] D. G. Kiely,[2] A. E. Bowyer[1] & M. Makris[1]

[1]*Department of Haematology, Royal Hallamshire Hospital;* [2]*Pulmonary Vascular Disease Unit, Royal Hallamshire Hospital, Sheffield, UK*

Patients with pulmonary hypertension are known to have endothelial dysfunction and an increased risk of thrombosis. We prospectively performed tests for thrombophilia over a 6-month period in patients referred with suspected pulmonary hypertension, up to 8 weeks prior to cardiac catheterization. Fifty-three patients (37F, 16M) were subsequently confirmed to have pulmonary hypertension; 11 patients had chronic thromboembolic pulmonary hypertension (CTEPH) and 42 had pulmonary arterial hypertension (PAH). Of the patients with PAH, 18 had primary pulmonary hypertension (PPH), 17 in association with connective tissue disease (CTD) and seven associated with other diseases. Standard thrombophilia tests were performed and the abnormalities identified were antithrombin deficiency in 5.6%, protein C deficiency 5.6%, protein S deficiency 13.2%, factor V Leiden 1.8%, prothrombin 20210A 3.8%, lupus anticoagulant 6.9%, IgG anticardiolipin antibodies 15.1%, IgM anticardiolipin antibodies 13.2% and anti-β_2 glycoprotein 1 in 1.8%. Factor VIII, a recently recognized prothrombotic risk factor, was measured using a two-stage assay. We also measured the FVIII:C in 103 healthy individuals. Patients with CTEPH and PAH had mean FVIII:C levels of 2.77 and 1.84 IU/ml, respectively, which were significantly higher than the mean in normal subjects which was 1.18 IU/ml ($P < 0.001$). Patients with CTEPH had significantly higher FVIII:C than the PPH (2.04 IU/ml) and CTD (1.74 IU/ml) groups ($P < 0.001$). There was no significant difference between the PAH subgroups themselves. Our normal range for FVIII:C is 0.52–1.84 and the percentage of patients with a result above the upper limit of normal were CTEPH 91%, PAH 45%, PPH 61% and CTD 41%. We conclude that elevated FVIII:C is the most frequent haemostatic abnormality in patients with pulmonary hypertension and although highest in CTEPH, it is elevated in all the other subgroups of PAH. Further work is required to determine if the high FVIII levels are causal in the pathogenesis of pulmonary hypertension or the result of endothelial dysfunction/damage.

146

Neutral endopeptidase inhibition potentiates bradykinin mediated vasodilatation and tissue plasminogen activator release in patients with heart failure on chronic angiotensin converting enzyme inhibitor therapy

N. L. Cruden,[1] C. A. Ludlam,[2] K. A. A. Fox[1] & D. E. Newby[1]

[1]*Cardiovascular Research, University of Edinburgh;* [2]*Department of Haematology, University of Edinburgh, Edinburgh, UK*

Angiotensin converting enzyme (ACE) and neutral endopeptidase (NEP) degrade kinins. ACE inhibition potentiates bradykinin mediated vasodilatation and endothelial release of the pro-lytic factor, tissue plasminogen activator (t-PA) in patients with chronic heart failure (CHF). We investigated whether additional NEP inhibition with thiorphan further augments the vascular actions of bradykinin in CHF patients on chronic ACE inhibitor therapy. Ten patients with CHF on chronic ACE inhibitor therapy received an intrabrachial infusion of thiorphan (30 nmol/min) or saline placebo for 3 h in a randomized, double blind, cross over trial. Thiorphan or placebo was co-infused with Lys-des-Arg9-bradykinin (B1 kinin receptor agonist; 1–10 nmol/min), bradykinin (B2 kinin receptor agonist; 30–300 pmol/min), atrial natriuretic peptide (10–100 pmol/min) and sodium nitroprusside (2–8 mcg/min) in random order. Bilateral forearm blood flow (FBF) was recorded using strain gauge plethysmography and plasma t-PA antigen determined using ELISA. There were no changes in heart rate, blood pressure or non-infused FBF or t-PA release during thiorphan or placebo infusion. Bradykinin, atrial natriuretic peptide and sodium nitroprusside caused dose dependent increases in infused FBF in all studies ($P < 0.001$). There were no significant changes in FBF during Lys-des-Arg9-bradykinin infusion. Bradykinin but not Lys-des-Arg9-bradykinin or atrial natriuretic peptide caused a dose dependent increase in t-PA antigen and estimated t-PA antigen release in all studies ($P < 0.001$). Compared with placebo, thiorphan augmented the increase in infused FBF, t-PA antigen and estimated t-PA antigen release ($P < 0.001$, $P = 0.076$, $P < 0.005$ respectively) to bradykinin but not atrial natriuretic peptide or sodium nitroprusside. In the presence of chronic systemic ACE inhibition, local NEP inhibition potentiates bradykinin mediated vasodilatation and endothelial t-PA release. Despite the results of recent trials of vasopeptidase inhibition, these findings support the hypothesis that combined ACE and NEP inhibition may afford greater cardiovascular protection than ACE inhibition alone.

147

Rebound elevation of factor VIII:C levels following successful treatment for acquired haemophilia: a risk factor for venous thrombosis?

C. E. Grimley & G. Dolan

Department of Haematology, Queen's Medical Centre, Nottingham, UK

Acquired haemophilia A is a rare, life-threatening immune-mediated haemorrhagic disorder, due to autoantibodies against factor VIII (FVIII). After eradication of the autoantibody with immuno-modulatory treatment, FVIII:C returns to normal levels. Rebound elevation of FVIII:C following treatment has not previously been reported. We reviewed 13 cases of acquired haemophilia treated at our centre over 14 years (seven females, six males; age range 30–88 years). Of these, FVIII:C returned to normal (50–200 IU/dl) in seven patients. In four patients, FVIII:C rebounded to very high levels. Patient characteristics were: three females, one male; age range 35–87 years. All had pre-existing conditions known to be associated with acquired haemophilia: two had rheumatoid arthritis, one systemic lupus erythematosus and one chronic lymphocytic leukaemia. Immuno-modulatory therapy with oral prednisolone was given to all four, and additional agents including cyclophosphamide, vincristine and intravenous

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Samsung Bioepis Exhibit No. 1002
Page 1440

immunoglobulin were used in three cases. FVIII:C levels rebounded to between 307 and 616 IU/dl after 2–12 months of treatment. One patient, a male aged 56 years with rheumatoid arthritis and end stage chronic renal failure, developed a pulmonary embolus when his FVIII:C was 463 IU/dl. Elevated FVIII:C (FVIII:C>150 IU/dl) has been shown to be a risk factor for venous thrombosis. Very high levels may confer a substantially greater risk of thrombosis in a dose dependent fashion. Our review suggests that FVIII:C can rebound to very high levels after recovery from acquired haemophilia in some cases. This phenomenon may be associated with pre-existing autoimmune or iymphoproliferative disorders and could increase thrombotic risk.

148

Audit of the management of thrombotic thrombocytopenic purpura in adult patients in Scotland

D. E. Plews,[1] J. Davis[2] & C. A. Ludlam[3]

[1]Edinburgh & South East Scotland Blood Transfusion Service; [2]Department of Haematology, Western General Hospital; [3]Department of Haematology, Royal Infirmary of Edinburgh, Edinburgh, UK

This audit aimed to review the management of adult patients with TTP in Scotland against guidelines produced by the British Committee for Standards in Haematology (2002). Data were collected by questionnaire and clinical note review as necessary. In Scotland in 2001, 23 patients were treated for a presumed diagnosis of TTP; 3 were subsequently given alternative diagnoses. The overall incidence was 4.1/million with a mean age of presentation of 50.3 years. Six patients died (this included all three with alternative diagnoses), two entered a partial remission and 14 had a complete remission with a mean follow-up of 6 months. There were three relapses. A normal coagulation screen in addition to the defined criteria of thrombocytopenia, red cell fragmentation and a raised LDH was found to be diagnostically important. All three cases of misdiagnosis had evidence of DIC at initial presentation. All patients were treated with plasma exchange, over 50% within 24 h of diagnosis. The median number of exchanges was 10, although there was large variability in exchange regimens, suggesting the need for comparative trials. Treatment complications occurred in 16 patients, which is significantly higher than anticipated by the guidelines. Infusions of fresh frozen plasma were useful as initial treatment pending transfer to a plasmapheresis centre and as maintenance therapy in two patients. The centralization of treatment in specialized centres throughout Scotland did not delay treatment. This audit has demonstrated the true incidence of TTP requiring treatment in Scotland. The sharing of knowledge has led to a review of the way in which TTP is managed and has enabled the introduction of standard apheresis protocols. The work is ongoing and the establishment of a Scottish TTP database will promote further evidence-based improvements in patient care.

149

Thrombophilia in the black population: results of the Camberwell Thrombophilia Study

R. K. Patel, E. Ford, J. Thumpston & R. Arya

Department of Haematological Medicine, King's College Hospital, London, UK

In the Camberwell Thrombophilia Study conventional thrombophilia testing was unrewarding in black subjects with venous thromboembolism (VTE) revealing an abnormality in only 9% black cases as opposed to 30% white cases. We previously identified elevated FVIII:C levels as a major risk factor for VTE in blacks. We now studied levels of fibrinogen, D-dimer and thrombin-antithrombin complexes (TAT) in 61 black patients with DVT (27 males, 34 females, mean age 56.2 years, SD 14.6) and 64 healthy black volunteers (nine males, 55 females, mean age 55 years, SD 14). Cases had completed standard anticoagulant treatment for objectively confirmed DVT at least 3 months previously. Controls had no known history of VTE, malignancy or intercurrent illness. Mean fibrinogen was significantly higher ($P = 0.048$) in cases (mean 3.19 g/l, SD 0.72, range 1.8–4.9 g/l) than controls (mean 2.94 g/l, SD 0.67, range 1.7–4.4 g/l). Fibrinogen level was independent of gender ($P = 0.10$) and age ($P = 0.11$). Odds ratio (OR) for DVT was significantly higher for subjects with fibrinogen in excess of 3 g/l (OR 2.41, 95% CI 1.11–5.30, $P = 0.025$) using levels less than 3 g/l as comparator. Median D-dimer was significantly higher ($P = 0.007$) in cases (median 179 ng/ml, interquartile range 86–432 ng/ml) than controls (median 101 ng/ml, interquartile range 54–203 ng/ml). OR for DVT of D-dimer levels >347 ng/ml (the 90th centile value for controls) was 4.3 (95% CI 1.48–13.5, $P = 0.005$). D-dimer levels were independently associated with FVIII:C ($P = 0.034$) in cases but not in controls. We could not demonstrate any association between fibrinogen and either D-dimer or FVIII:C in either cases or controls. D-dimer levels were associated with elevated TAT levels ($P = 0.03$) in cases but there was no association between TAT and either fibrinogen or FVIII:C level. In blacks elevated levels of coagulation proteins may constitute significant risk factors for thrombosis and should be considered when evaluating such patients for thrombophilia.

150

A UK survey of prophylaxis in paediatric patients with haemophilia

A. E. Thomas

Royal Hospital for Sick Children, Edinburgh, UK

A questionnaire was sent to all haemophilia centres in the UK to determine the use of prophylaxis in patients with severe or moderate haemophilia A or B aged <16 years on 30 September 2002. The ISTH definitions of severity of haemophilia and type of prophylaxis were given. Forty-one centres responded which included 17 of 21 Comprehensive Care Centres treating children. Eleven responders treated no children. A total of 753 children with severe or moderate haemophilia were identified; 82% had haemophilia A, of whom 78% were severe and 18% had haemophilia B, of whom 56% were severe. Seventy-three percentage of patients with haemophilia A were on prophylaxis, both primary and secondary, compared with 52% of patients with haemophilia B. The median frequency of dosing for both haemophilia A and B was ×3/week with a range of twice per week to daily. The median dose range of factor replacement was 25–40 units/kg for factor VIII and 25–50 units/kg for factor IX. Twenty-three centres monitored prophylaxis using trough levels, with fewer using half-life studies or recovery instead or in addition; three centres used clinical criteria alone; 27 centres (90%) used clinical outcome measures to judge efficacy whereas only 20% used the X-ray or orthopaedic scores. Prophylaxis was given via peripheral veins in 86% of children, although 28% had used a central venous access device at some point, usually for poor venous access. Carers administered factor in 71% and the patients themselves in 24%; 31% were not on prophylaxis, the most frequent reason being a mild phenotype. Compliance was the commonest reason for discontinuing prophylaxis. In the UK, 69% of those with moderate or severe haemophilia are on prophylaxis. In 95% of children, the carer or child gives the prophylaxis. Continued support is important to prevent non-compliance with such demanding treatment.

Samsung Bioepis Exhibit No. 1002
Page 1441

151

u-PA expression in normal myeloid differentiation and leukaemia

Y. L. Chee, S. Berry, M. Greaves & B. Bennett
Department of Medicine and Therapeutics, University of Aberdeen, Aberdeen, UK

The urokinase-type plasminogen activator (uPA) plays an important role in extra- and intravascular proteolysis. We have shown that u-PA is present in myeloid leukaemic cells and so can induce primary hyperfibrinolysis that may cause severe bleeding, particularly in APL patients. We have also shown that normal peripheral blood stem cells do not express functional u-PA but acquire this ability during the process of myeloid differentiation, suggesting that u-PA production by myeloid leukaemia cells may be a reflection of their stage of maturation and not aberrant gene expression. However, not all APL express functional u-PA. To study this further, we analysed cells from six APL patients and the HL-60 cell line (human-derived APL cell line). HL-60 cells were stimulated with 1.2% DMSO to induce granulocytic differentiation and examined at serial intervals. uPA antigen and activity were detected using ELISA or APAAP, SDS–PAGE/zymography and clot lysis assay and uPA mRNA using RT-PCR. PAI -1 antigen and activity were detected using ELISA and SDS–PAGE/reverse zymography. Results from the HL-60 cell line showed that uPA antigen and activity was detected in Hl-60 blasts and promyelocytes prior to and in the progeny following differentiation induction with 1.2% DMSO. The pattern of uPA mRNA expression paralleled that of uPA activity. PAI-1 antigen/activity was undetected. Mononuclear cells and plasma were obtained from six APL patients at presentation. u-PA antigen was detected in six of six and u-PA activity in four of six patients. Plasma PAI-1 was found to be within normal limits in all patients. These results suggest that the expression of u-PA antigen in APL is not dependent on the leukaemic process while the expression of u-PA function is more complex and may reflect upregulation of u-PA, high cell load or absence of functional inhibitor.

152

Protein Z concentrations in normal pregnancy

F. Stewart,[1] J. E. Ramsay,[1] H. Friel,[2] I. D. Walker,[3] I. A. Greer[1] & M. D. McColl[1,2]
[1]*University Department of Obstetrics, Royal Infirmary, Glasgow;* [2]*Department of Haematology, Crosshouse Hospital, Kilmarnock, Ayrshire;* [3]*Department of Haematology, Royal Infirmary, Glasgow, UK*

Protein Z (PZ) is a vitamin K dependent glycoprotein that functions as a natural anticoagulant. We have previously shown that PZ concentrations are significantly reduced in subjects with antiphospholipid syndrome and may contribute to the thrombotic phenotype observed in this condition. Data suggest PZ deficiency may also play a role in fetal loss. However, data relating to PZ concentrations during normal pregnancy are lacking. In a prospective study, we assayed PZ concentrations in all three trimesters and 3 months following delivery in 40 healthy women. Mean maternal age was 28.4 (SD 5) years and BMI 28.6 (SD 6.4) kg/m^2. Mean PZ increased from 1.73 mcg/ml (SD 0.7) in the first trimester (T1) to 1.96 mcg/ml (SD 0.7, $P < 0.001$) in T2 and 1.9 mcg/ml (SD 0.8) in T3 ($P = 0.7$ vs. T2). Levels fell at 3 months following delivery to 1.3 mcg/ml (SD 0.5, $P < 0.001$ vs. T3; $P < 0.001$ vs. T1 and T2). There was no significant difference in non-pregnant PZ concentrations between lean (BMI ≤28 kg/m^2) and obese (BMI >28 kg/m^2) individuals. Mean PZ concentration was significantly higher in T2 and T3 in obese compared with lean women. However, mean PZ also increased significantly even in lean women during pregnancy. We conclude that PZ concentrations increase early in pregnancy. This may be a physiological response to control the hypercoagulability of pregnancy. Failure of this mechanism may predispose to a pro-thrombotic state explaining the association with poor pregnancy outcome.

153

An analysis of the β_2-glycoprotein I Cys306Gly polymorphism in patients with thrombotic and obstetric disorders

M. J. Nash, R. S. Camilleri, I. J. Mackie, S. J. Machin & H. Cohen
Haemostasis Research Unit, University College London, London, UK

The Cys306Gly polymorphism of β_2-glycoprotein I (B2GPI) results in the disruption of a disulphide bond between Cys281 and Cys306. These results in a change in the conformation of the fifth domain of B2GPI, a region crucial for the interaction of the molecule with negatively charged phospholipids. We have analysed the frequency of this polymorphism in 284 normal healthy controls and 290 consecutive patients referred to our clinic for investigation of a thrombotic episode or obstetric morbidity. Morbidity among the patients was distributed as follows: thrombosis (arterial and/or venous) 165; late pregnancy morbidity 55; recurrent/repeated miscarriage 86; miscellaneous 11; thrombocytopenia three (some patients had more than one clinical condition). Ninety-seven patients were persistently positive for antiphospholipid antibodies (APA) (anticardiolipin antibodies and/or lupus anticoagulant and/or anti-B2GPI antibodies). No significant differences were found between the APA positive and negative patient groups with regard to the frequency of the allele coding for the Cys306Gly polymorphism. This finding remained after analysis of patient subgroups by clinical presentation. In addition, no significant differences were observed between the normal control group and patients with thrombosis or late pregnancy morbidity. However, the frequency of the allele was increased in APA negative patients with miscarriage (7.35%) compared with female only controls (2.79%, $P = 0.01$, chi-squared test). This difference however failed to reach statistical significance when only Caucasian patients were considered (frequency in miscarriage APA negative group 6.9%, frequency in control group 2.9%; $P = 0.06$, chi-squared test). This effect was not seen in either case with APA positive patients. Our results suggest that the Cys306Gly polymorphism has little influence on the antigenicity of B2GPI with regard to the production of APA or does it affect the incidence of thrombosis. However, the polymorphism may increase the risk of miscarriage via a mechanism independent of the production of APA.

154

An analysis of the antiphospholipid antibody status of 111 women with late pregnancy morbidity

M. J. Nash, R. S. Camilleri, T. Everington & H. Cohen
Haemostasis Research Unit, University College London, London, UK

Late pregnancy complications associated with the antiphospholipid syndrome include pre-eclampsia (PET) and placental insufficiency. We have analysed the clinical presentations and thrombophilia screen results of 111 such consecutive patients referred to our haematology department. Patients were considered positive for antiphospholipid antibodies (APA) only if they were positive on two separate occasions at least 6 weeks apart [tests were performed for anticardiolipin antibodies (aCL) of IgG and IgM class, anti-β_2-glycoprotein I (b2GPI) antibodies of IgG class and for lupus anticoagulant activity]. Clinical presentations were as follows: PET 59; intrauterine growth restriction (IUGR) 40; stillbirth 33; placental abruption 15 (some patients had more than one presentation). The patient's thrombophilia screens showed eight heterozygotes for the factor V Leiden mutation; one homozygote and 39 heterozygotes for the thermolabile variant of the MTHFR gene; two heterozygotes for the G20210A prothrombin gene mutation and two patients deficient for protein S. Sixteen of the

Samsung Bioepis Exhibit No. 1002
Page 1442

patients were found to be persistently APA positive: 11 had persistent IgG aCL (median titre 15 GPLU, range 5–85 GPLU); four persistent IgM aCL; two persistent lupus anticoagulant (LA) activity; four persistent IgG anti-b2GPI antibodies (median 26%, range 13–35%). Both of the LA positive patients were strongly positive for IgG aCL (>50 GPLU). Six of the APA positive patients were heterozygous for the thermolabile variant of the MTHFR gene and one was homozygous; no other additional thrombophilia makers were observed in the APA positive group. Eight of the APA positive patients had PET and one of these had suffered a cerebral infarction previously. Seven of the APA positive patients had IUGR (some overlap with PET cases). The prevalence of APA in women with late obstetric complications was 14.4% and the proportion of APA positive patients with PET in this cohort was 13.5%. The optimal haematological management of these patients requires a larger evidence base.

155

High pulse pressure but not mean arterial blood pressure is associated with abnormal thrombogenesis and endothelial damage/dysfunction in patients with coronary heart disease with and without hypertension

K. W. Lee & G. Y. H. Lip

University Department of Medicine, City Hospital, Birmingham, UK

Both pulse pressure (PP) and mean arterial blood pressure (MAP) have been implicated as independent predictors of cardiovascular (CV) risk. PP measures the pulsatile component, whereas MAP measures the steady component of BP. We hypothesized that high 24-h ambulatory PP in patients with coronary heart disease (CHD) is associated with an increased risk of haemostasis, which may be related to abnormal haemorheology (raised haematocrit, plasma viscosity and fibrinogen levels), endothelial damage/dysfunction (raised plasma von Willebrand factor, VWF), thrombogenesis (raised D-dimer, Dd) and platelet activation (raised soluble P-selectin, sPsel). We studied 72 patients with stable CHD who were in sinus rhythm and with preserved left ventricular systolic function (mean ejection fraction 61 ± 10%). All completed a 24-h ambulatory BP monitoring. Patients were divided into two groups according to high (above median) or low (below median) values of the ambulatory PP (51 mmHg). The two groups were not different in clinical and demographic characteristics. Twenty-four hours PP was positively correlated with age ($r = 0.48$), VWF ($r = 0.41$), Dd ($r = 0.25$), fibrinogen ($r = 0.3$) and sPsel ($r = 0.22$) levels (all $P < 0.05$; see table), but not with plasma viscosity or haematocrit. None of haemostatic indices correlated with 24-h MAP. On multivariate ANCOVA analysis, after controlling for age and MAP, VWF, fibrinogen, Dd and sPsel levels remained significantly elevated in patients with high 24-h PP (all $P < 0.05$; see table). High 24-h PP load in patients with CHD was associated with a prothrombotic state (with higher levels of fibrinogen, Dd and sPsel) and evidence of endothelial damage/dysfunction (VWF), independently of age. These may contribute to higher CV risk associated with increased PP.

	High-PP ($n = 37$)	Low-PP ($n = 35$)	P-value
Age (year)	63 ± 11	55 ± 9	<0.001
VWF (IU/dl)	212 ± 58	172 ± 49	0.002
D-dimer (mg/l)	0.35 (0.23–0.59)	0.24 (0.21–0.35)	0.004
Fibrinogen (g/l)	3.4 ± 0.8	3.0 ± 0.8	0.025
Soluble P-selectin (ng/ml)	87 (77–98)	78 (63–91)	0.034

Mean ± SD or median (IQR).

156

Impaired left ventricular diastolic function is associated with abnormal haemorheology, thrombogenesis and endothelial damage/dysfunction in patients with coronary heart disease

K. W. Lee, K. Jolly & G. Y. H. Lip

University Department of Medicine, City Hospital, Birmingham, UK

Heart failure (HF) is associated with an increased risk of thromboembolic events. About 35% of patients with HF have normal left ventricular (LV) systolic function, with diastolic dysfunction as the underlying cause. We hypothesized that LV diastolic dysfunction is associated with an increased risk of haemostasis, which may be related to abnormal haemorheology [raised plasma fibrinogen (Fg) and plasma viscosity (PV)], endothelial damage/dysfunction (raised von Willebrand factor, VWF) and thrombogenesis (raised D-dimer, Dd). Seventy-two patients with CHD (58 males, 59 ± 11 years) who were in sinus rhythm and with preserved LV systolic function (mean ejection fraction 61 ± 10%) and 35 matched healthy controls (23 males, 57 ± 6 years) were studied. Transmitral pulsed Doppler indices [early/atrial (E/A) velocity ratio, E-wave deceleration time (EDT), isovolumic relaxation time (IVRT)] and pulsed Doppler Tissue imaging indices [peak systolic (S'), early and late diastolic velocities (E', A')] obtained at the septal and lateral mitral annuli were measured and related to plasma haemostatic markers levels. Patients with CHD have significant prolonged EDT (median 240 ms vs. 204 ms) and IVRT (149 ms vs. 125 ms), shortened S' (10 cm/s vs. 12 cm/s), E' (12 cm/s vs. 14 cm/s) and A' (12 cm/s vs. 13 cm/s) velocities with higher E/E' ratio (6.2 vs. 5.4) compared with controls (Mann–Whitney test, all $P < 0.05$). PV (median 1.7 vs. 1.6 mPa/s), Fg (3.3 g/l vs. 2.5 g/l), Dd (0.28 ng/ml vs. 0.18 ng/ml) and VWF (182 IU/dl vs. 120 IU/dl) levels were significantly higher in CHD patients than in controls (all $P < 0.01$). PV and Fg levels were correlated with S', E' and E/E' ratio (Spearman's $r = 0.3$, $P < 0.01$). VWF levels were correlated with E/A ratio ($r = 0.2$), EDT ($r = 0.3$), IVRT ($r = 0.3$), S' ($r = 0.4$) and E' ($r = 0.2$) (all $P < 0.05$). Dd was correlated with EDT ($r = 0.3$). On multivariate analysis, VWF was independently related to S' ($P = 0.03$) and IVRT ($P = 0.02$), whilst PV was independently related to E/E' ratio ($P = 0.03$). Diastolic LV dysfunction in patients with CHD is significantly associated with abnormal haemorheology, thrombogenesis and endothelial damage/dysfunction.

157

Platelet activation, coagulation and angiogenesis in breast and prostate cancer

A. Blann, G. Caine, P. Ryan, P. Stonelake & G. Lip

Department of Medicine and Surgery, City Hospital, Birmingham, UK

In health, haemostasis and angiogenesis are tightly regulated processes, but may become deregulated in cancer. Recent evidence suggests that platelet activation may link these processes as platelets can release angiogenic factors, such as vascular endothelial growth factor (VEGF). Recent data also implicate inflammation in regulating both coagulation and angiogenesis, possibly by activating platelets directly and increasing (e.g. fibrinogen). We hypothesized relationships between these plasma markers of the processes in two common forms of cancer. Plasma levels of VEGF (reflecting angiogenesis), soluble P selectin (sP-sel), marking platelet activation, tissue factor (TF), fibrinogen and D-dimer (coagulation markers), and serum levels of IL-6 (inflammation) were measured by ELISA in 30 patients with biopsy-proven breast cancer, 30 patients with biopsy-proven prostate cancer, and 30 age- and sex-matched controls per group. Prostate specific antigen was also measured in the men. Release of VEGF from IL-6 stimulated platelets was assessed by ELISA. Results were that plasma levels of IL-6 ($P < 0.02$), VEGF, sP-sel, fibrinogen and fibrin D-dimer (all $P < 0.01$)

Samsung Bioepis Exhibit No. 1002
Page 1443

were significantly raised in breast cancer, whereas plasma fibrinogen ($P < 0.05$), VEGF, sP-sel and fibrin D-dimer (all $P < 0.01$) were significantly raised in prostate cancer. Strongly significant correlations were found between IL-6 and VEGF in breast and prostate cancer, and between PSA and sP-sel in prostate cancer (all $P < 0.01$). There was an IL-6 dose-dependent release of VEGF from platelets. We conclude that strong relationships between IL6 and VEGF, but not with coagulation or platelet markers, and release of VEGF from IL-6 stimulated platelets, suggest a role for inflammation and platelets in angiogenesis.

158

Vaccino-associated immune thrombocytopenic purpura in childhood

V. Petrov,[1] V. Vdovin[2] & P. Svirin[2]

[1]Departments of Haematology, [2]Haematology Consulting Department, Izmailovskaya Children's Hospital, Moscow, Russia

We observed 96 children aged 1–15 years with ITP, associated with vaccination, in the period of possible postvaccinal complications. ITP developed: in 25 children after rubella vaccination on the 15th day (in the average); in 17 children after DTP-vaccination on the 14th day; in 16 children after influenza vaccination on the 12th day; in 12 children after measles vaccination on the 10th day; in 10 children after hepatitis B vaccination on the 17th day; in nine children after poliomyelitis vaccination on the 11th day; in five children after mumps vaccination and in two children after BCG revaccination on the 9th day. The ITP in all patients was characterized with skin bleeds. Besides at 60% of the patients, the various bleedings (epistaxis, mouth bleedings, haematuria and menorrhagia) were marked. At 88% of children there was revealed the deep thrombocytopenia (10 × 10 milliards/l). The hardest ITP was marked after hepatitis B and influenza vaccination. At these patients in 90% and 75% of cases, accordingly, there were marked various bleedings. At the majority of the patients (73%) were revealed high level of antiplatelet antibodies. There was investigated a condition of general immunity in all patients, that marked on changes, characterized for infectious and autoimmune mechanisms. There also was researched the specific immunity in the majority of patients, that revealed higher level of antibodies to vaccin-associated infections, which required the exact connection of ITP and vaccination. Fifty-five patients received corticosteroid therapy and 35 received symptomatic therapy. Six children received IVIG. Among children received corticosteroid therapy complete recovery has come at 84% of the patients. Among children received symptomatic therapy the recovery has come at 45% of children. The efficiency of therapy IVIG was 66.7%.

159

Multi-centre survey of the management of antenatal venous thromboembolism in the UK and Ireland 2003–2004

J. M. Voke,[1] A. J. Keidan,[2] J. A. Copplestone,[3] M. D. Creagh,[4] C. R. Shiach,[5] D. S. Thompson,[6] K. Murphy[7] & S. Pavord[8]

[1]Department of Haematology, E&N Herts NHS Trust, AL7 4HQ; [2]Department of Haematology, Kings Lynn & Wisbech NHS Trust, PE 30 4ET; [3]Department of Haematology, Derriford Hospital, Plymouth PL6 8DH; [4]Department of Haematology, Royal Cornwall Hospital, Truro TR1 3LJ; [5]Department of Haematology, Manchester Royal Infirmary, M13 9WL; [6]Department of Haematology, Luton & Dunstable NHS Trust, LU4 0DZ; [7]Department of Haematology, St Vincent's University Hospital, Dublin DR4; [8]Department of Haematology, Leicester Royal Infirmary, LE1 5WW, UK

Antenatal venous thromboembolism (VTE) occurs in <1 in 1000 pregnancies (Simpson *et al* BJOG 2001; 108: 56–60); therefore, many hospitals manage fewer than five such patients per year. Most haematologists and obstetricians follow UK guidelines published by the RCOG and the BSH, some of which are extrapolations from level 1 evidence in non-pregnant patients. The dearth of randomized studies on VTE in pregnancy is partly due to the lack of any multi-centre organization to conduct such research. This multi-centre MREC approved study was set up after requests from many haematologists to share information on current diagnosis and treatment of antenatal VTE. An informal survey by the BSH Obstetric Haematology group confirmed that management varied widely, with different heparins, dosage regimes and monitoring protocols being used (Copplestone *et al* Br. J. Haematol. 2004; 124: 124–125). The primary objective of the study is to record current management of over 200 antenatal VTE patients in the UK and Ireland, from March 2003, using information sheets and data forms designed by participants and obtained from the website http://www.ukivtepsurvey.co.uk/. Forty-eight haematologists have signed up to date and the study is still open. Data are being submitted to a secure database for analysis in 2004 and subsequent publication. A secondary objective is to find out whether an on-line data collection survey of this type will be successful. If it is, this method of multi-centre audit of the management of rare conditions will be a cost-effective tool for future multi-centre work. Having established a national group interested in this clinical area, overcome problems in setting up the study and demonstrated that such a survey can be conducted mainly by e-mail, we will be in a strong position to support, participate in and recruit for randomized research.

160

A novel R1205C candidate type 1 von Willebrand disease mutation in the same codon as type 2 'Vincenza' in two affected siblings of a Scottish family

M. S. Enayat,[1] EA Chalmers,[2] M. D. Williams[1] & F. G. H. Hill[1]

[1]Molecular Haemostasis Laboratory, Department of Haematology, Birmingham Children's Hospital NHS Trust, Birmingham; [2]Department of Haematology, Royal Hospital for Sick Children, Yorkhill, Glasgow, UK

Type 1 von Willebrand disease is characterized by reduced, but qualitatively normal von Willebrand Factor (VWF) and the reported gene mutations are scattered throughout the gene. We have investigated two affected members of a Scottish family with reduced plasma levels of FVIII:C, VWF:Ag and VWF:Ricof, and having normal VWF:Ag multimers. Although these two siblings had minimal bleeding symptoms, their father, paternal grandfather and an aunt had repeated bleeding episodes. Initially, DNA from these two patients was used in targeted direct DNA sequencing of exon 27 and 28 of VWF gene, revealing an heterozygous 3613C>T transition, predicting the substitution of R1205C in exon 27. This mutation was confirmed using *Nae*I restriction enzyme, and found to be absent in 50 normal individuals. The Arginine 1205 codon is the site of other reported changes leading to R1205L and R1205H 'Vincenza' phenotypes, which results in presence of ultra high molecular weight multimers (UHMWM) in plasma. We have previously reported five affected patients from two unrelated families with R1205H 'Vincenza' mutation who similar to present patients did not show UHMWM [Lester *et al* J. Thromb. Haemost. 2003; 1(Suppl. 1): July (ISSN 1740 3340). Abstract no.: P0096]. The findings from this study also refute other reports suggesting that any mutation involving destruction or creations of Cysteine will result in an abnormal multimer smearing effect. It is likely that the Arginine 1205 codon is target of many mutations which occur frequently: this amino acid has a significant position in VWF gene and different base changes are likely to lead to a spectrum of bleeding phenotypes in these patients.

Samsung Bioepis Exhibit No. 1002
Page 1444

161

Changes in plasma haemostatic markers with hospital-based versus home-based cardiac rehabilitation in patients with coronary heart disease: the Birmingham Rehabilitation Uptake Maximization Study (BRUM)

K. W. Lee, K. Jolly & G. Y. H. Lip

University Department of Medicine, City Hospital, Birmingham, UK

Raised levels of plasma viscosity (PV), fibrinogen, D-dimer (Dd, an index of thrombogenesis) and von Willebrand factor (VWF, an index of endothelial damage/dysfunction) are known independent predictors of cardiovascular (CV) risk in patients with coronary heart disease (CHD). The effects of home-based (nurse facilitated, based on the UK Heart Manual) *versus* hospital-based cardiac rehabilitation (CR) programmes on these haemostatic indices in patients with CHD are unknown. Seventy-two patients (58 males, 59 ± 11 years) with CHD (following myocardial infarction or coronary revascularization) were randomized to either a hospital- ($n = 35$) or home-based ($n = 37$) CR programme. Plasma levels of VWF, Dd, fibrinogen and PV were measured at baseline and after 3 months of CR. Baseline values were compared with 35 matched healthy controls (23 males, 57 ± 6 years). Patients with CHD had significantly elevated PV [(mean ± SD) 1.7 ± 0.2 mPa/s vs. 1.6 ± 0.8 mPa/s], fibrinogen (3.3 ± 0.8 g/l vs. 2.6 ± 0.6 g/l), VWF (189 ± 58 IU/dl vs. 127 ± 32 IU/dl) and D-dimer [median (IQR) 0.28 (0.2–0.5) ng/ml vs. 0.18 (0.13–0.3) ng/ml] when compared with healthy controls (all $P < 0.001$). The baseline levels of haemostatic indices were not significantly different between the two CR programmes. After 3 months of CR, PV, fibrinogen, VWF and Dd levels were significantly reduced [to 1.6 ± 0.1 mPa/s, 2.9 ± 0.7 g/l, 172 ± 50 IU/dl and 0.2 (0.12–0.3) ng/ml respectively] in the whole patient cohort when compared with baseline (all $P < 0.001$). The improvements in these haemostatic markers levels were not significantly different between patients of the home- and hospital-based programmes. In conclusions, CR improves haemostatic profiles in patients with CHD, with no significant differences between home- and hospital-based CR programmes. These effects may contribute to the beneficial effects of CR on CV risks.

162

Incremental shuttle walking is associated with activation of haemostatic and haemorheologic markers in patients with coronary heart disease

K. W. Lee, J. Ingram, K. Jolly & G. Y. H. Lip

University Department of Medicine, City Hospital, Birmingham, UK

Activation of platelet reactivity and coagulation cascade after short-term physical exertion has been shown in patients with CHD. However, most previous studies have used either bicycle ergometer or treadmill exercise protocol, which is not widely available to most patients. Most CHD patients choose walking as a mean of exercise and therefore the aim of this study was to assess the effect of an incremental shuttle walk test (ISWT) on plasma haemostatic markers. Plasma viscosity (PV), fibrinogen (Fg), D-dimer (Dd), plasma soluble P-selectin (sPsel) and von Willebrand factor (VWF) levels were measured before and immediately after ISWT in 52 CHD patients (47 males, 58 ± 10 years) who had completed a 12 weeks course of cardiac rehabilitation and in 19 matched healthy controls (15 males, 59 ± 7 years). The ISWT is a standardized incremental field-walking test that provokes maximal performance and has been shown to correlate strongly with maximum oxygen uptake (see table). Baseline Fg, VWF and sPsel levels were significantly higher in CHD patients than in controls (all $P < 0.05$). Fg, vWf, Dd and PV but not sPsel levels were significantly raised immediately after ISWT. Increment of Fg was correlated with total walking distance and peak heart rate ($P = 0.02$). A single bout of shuttle walking in patients with CHD appears to elevate fibrinogen, VWF, Dd and PV compared with healthy individuals with intact endothelium and normal haemostasis.

Table. Pre- and post-ISWT in CHD patients *versus* controls.

	CHD		Control	
	Pre	Post	Pre	Post
VWF(IU/dl)	163 ± 44	179 ± 49*	122 ± 17	126 ± 17
Dd(ng/ml)	0.23 ± 0.1	0.26 ± 0.2*	0.22 ± 0.1	0.23 ± 0.1
Fg(g/l)	2.9 ± 0.7	3.2 ± 0.7*	2.5 ± 0.7	2.7 ± 0.7*
PV(mPa/s)	1.6 ± 0.1	1.7 ± 0.1*	1.6 ± 0.1	1.6 ± 0.1

Values are given as mean ± SD.
*$P = 0.001$.

163

Anti-hypertension treatment reduces platelet derived VEGF and angiopoietin-1 in hypertension

A. Blann, S. Nadar & G. Lip

Department of Medicine, City Hospital, Birmingham, UK

Platelets carry angiogenic growth factors vascular endothelial growth factor (VEGF) and angiopoietin 1 (Ang-1). Although both growth factors are important in the pathogenesis and metastasis of malignancy, their role in the pathogenesis of complications and the response to treatment in hypertension is not known. To test the hypotheses (i) that there are differences in VEGF and Ang-1 in the plasma and within platelets from patients with hypertension, and (ii) that levels change with successful treatment. We recruited 42 previously untreated patients with hypertension (25 males; mean age 53 years) and 30 age- and sex-matched controls. Plasma VEGF, Ang-1 and soluble P-selectin (sPsel, an index of platelet activation), and total platelet [platelet VEGF (pVEGF) and platelet Ang-1 (pAng-1)] were measured by ELISA. The patients were treated for 6 months with amlodipine-base therapy, achieving a blood pressure below 140/80 mmHg. Patients with hypertension had significantly higher levels of plasma sPsel ($P = 0.01$), VEGF ($P < 0.001$) and Ang-1 ($P = 0.01$), as well as pVEGF ($P < 0.001$) and pAng-1 ($P = 0.02$). The levels of plasma and platelet angiogenic growth factors were significantly reduced after antihypertensive treatment (VEGF, $P = 0.01$; pVEGF $P < 0.001$; Ang-1, $P < 0.001$; pAng-1 $P = 0.04$). There were no correlations with blood pressure or the levels of sPsel. Levels of plasma and intra-platelet VEGF and Ang-1 are increased in hypertension and are decreased with treatment. Platelet levels of VEGF and Ang-1 are probably not related to platelet activation and may involve other mechanisms, such as the general vascular and haemodynamic changes that are seen in hypertension.

164

Soluble CD40 ligand, P-selectin, interleukin-6 and tissue factor in diabetes with and without overt cardiovascular disease

H. S. Lim, A. D. Blann & G. Y. H. Lip

Haemostasis, Thrombosis and Vascular Biology Unit, University Department of Medicine, City Hospital, Birmingham, UK

CD40L-CD40 interaction has been linked to vascular inflammation and the hypercoagulability. High levels of soluble CD40 ligand (sCD40L) have been found in diabetes (DM). We hypothesized that sCD40L is related to indices of platelet activation, inflammation and hypercoagulability in patients with DM with and without clinically

Samsung Bioepis Exhibit No. 1002
Page 1445

overt cardiovascular disease (OCVD). We measured plasma sCD40L, soluble P-selectin (sP-sel, a marker of platelet activation), interleukin-6 (IL-6, a pro-inflammatory cytokine) and tissue factor (TF, an initiator of coagulation) by ELISA in 97 patients with DM (41 with and 56 without OCVD) and 39 age- and sex-comparable controls. [All data as mean (SD) or median (IQR) and in the following order: controls, DM without OCVD, DM with OCVD. Differences between patient groups by ANOVA] HbA1c [5.4 (0.3), 7.5 (0.5), 7.8 (1.2), $P < 0.001$] and systolic blood pressure [128 (17), 138 (15), 137 (21), $P = 0.048$] were higher in patients compared to controls, but not between patient groups. Plasma sCD40L [30 (10–175), 520 (183–1475), 700 (228–1425) pg/ml], sP-sel [85 (65–95), 95 (80–120), 100 (85–115) ng/ml], IL-6 [1.0 (1.0–2.0), 1.0 (1.0–5.7), 2.6 (1.0–17.5) pg/ml] and TF [300 (11–925), 560 (250–1400), 975 (400–2574) pg/ml] were higher in patients with DM compared with controls (all $P < 0.01$). IL-6 and TF were highest in patients with OCVD ($P < 0.01$). Plasma sCD40L correlated significantly with IL-6 and TF (all $r > 0.5$, $P < 0.001$) but not with sP-sel and HbA1c in both patient groups. On multivariate regression analysis, patient group was the only significant predictor of plasma sCD40L ($P = 0.04$). Our data suggest a link between the CD40-CD40L system and the inflammatory and hypercoagulable state in DM. Higher levels of plasma IL-6 and TF in patients with OCVD may explain the higher cardiovascular risk in these patients.

165

The effect of intensive multi-factorial intervention on soluble CD40 ligand, interleukin-6 and tissue factor in diabetes with and without overt cardiovascular disease

H. S. Lim, A. D. Blann & G. Y. H. Lip

Haemostasis, Thrombosis and Vascular Biology Unit, University Department of Medicine, City Hospital, Birmingham, UK

CD40L–CD40 interaction has been linked to vascular inflammation, a hypercoagulable state and may be involved in the pathogenesis of atherothrombotic complications. We sought to determine if: (i) intensified multi-factorial intervention could reduce plasma sCD40L, indices of inflammation and the hypercoagulable state in diabetes, and (ii) patients with overt CVD (OCVD) would derive similar benefit when compared with those without. Sixty-eight patients with diabetes (36 patients with and 32 without OCVD) participated in multi-factorial intervention, targeting glycaemia, hyperlipidaemia and blood pressure over 1 year. We measured plasma sCD40L, interleukin-6 (IL-6, a pro-inflammatory cytokine) and tissue factor (TF, an initiator of coagulation) by ELISA at baseline and 1 year. [The significance of changes in clinical and biochemical indices were evaluated with paired t-test or Wilcoxon's signed rank test] HbA1c (by mean of 0.9% and 0.8%) and total cholesterol (by mean of 1.1 and 0.8 mmol/l) were reduced in patients without and with OCVD respectively (all $P < 0.01$). Blood pressure was reduced to 135/77 and 135/74 mmHg at the end of the study, but this was not statistically significant. These changes were associated with reductions in sCD40L in both patient groups (by mean of 195 and 280 pg/ml, $P < 0.001$). TF fell only in patients without OCVD (by mean of 180 pg/ml, $P < 0.001$). There was no significant change in IL-6 in both patient groups. Changes in sCD40L and TF did not correlate with changes in metabolic parameters. Contemporary multi-factorial intervention is associated with reduction in plasma sCD40L but can only be partially correct the underlying vascular inflammation and hypercoagulability in diabetes, particularly in patients with OCVD. This highlights the limitations of contemporary management strategies and suggests the need for earlier, possibly more aggressive intervention in diabetes.

166

Plasma vascular endothelial growth factor, angiopoietin-1, -2 and soluble Tie-2 levels in diabetes: relationship with glycaemia, microalbuminuria, endothelial damage/dysfunction and the effect of multi-factorial intervention

H. S. Lim, A. D. Blann & G. Y. H. Lip

Haemostasis, Thrombosis and Vascular Biology Unit, University Department of Medicine, City Hospital, Birmingham, UK

Vascular endothelial growth factor (VEGF) and angiopoietin/Tie-2 are angiogenic growth factors implicated in the pathogenesis of diabetic vascular disease. Plasma levels of these growth factors in patients with diabetes and their relationship with the presence of overt cardiovascular disease (OCVD), measures of glycaemia, albuminuria and endothelial perturbations are unclear, as are the effects of intensified diabetes and cardiovascular risk management. We measured plasma von Willebrand factor (VWF, a marker of endothelial damage/dysfunction), VEGF, angiopoietins-1&2 (Ang-1, Ang-2) and soluble Tie-2 (all ELISA) in 94 patients (38 with OCVD) with diabetes and 34 normal controls. Thirty-three patients with and 31 without OCVD were subjected to a year's intensified cardiovascular risk management. Plasma VWF ($P = 0.009$), VEGF ($P = 0.001$) and Ang-2 ($P = 0.001$), but not Ang-1 ($P = 0.432$) or soluble Tie-2 ($P = 0.052$), were higher in both groups of diabetics, both with and without OCVD, compared with controls. Ang-2 correlated with urine albumin: creatinine ratio ($r = 0.307$, $P = 0.021$) and VWF ($r = 0.398$, $P = 0.005$) in patients without OCVD but not in patients with OCVD. VEGF correlated with HbA1c in patients with ($r = 0.367$, $P = 0.012$) and without ($r = 0.338$, $P = 0.024$) OCVD. Correlation between Ang-2 and HbA1c was significant only in patients without OCVD ($r = 0.356$, $P = 0.007$). There were significant reductions in HBA1c and total cholesterol after 1-year treatment of 33 patients with and 31 without OCVD associated with reductions in plasma VWF, and VEGF in both groups, but Ang-2 fell only in the patients without OCVD (all $P < 0.001$). Our data indicate that plasma Ang-2, like VEGF and VWF, is elevated in patients with diabetes regardless of overt vascular disease and may be associated with glycaemia, microalbuminuria and endothelial damage/dysfunction. Intensive multi-factorial intervention results in improvements in plasma VEGF and (in patients without overt CVD) Ang-2 levels. Further studies to assess the prognostic value of these pathophysiological changes are warranted.

167

Comparison of multiplex PCR with thirdwave invader DNA analysis for the detection of the factor V Leiden and prothrombin 3'UTR gene mutations

A. Riddell, G. Mellars & D. J. Perry

Haemophilia Centre & Haemostasis Unit, Royal Free Hospital, London, UK

Screening for factor V Leiden and prothrombin 3' UTR mutations is part of the assessment of many patients with a suspected prothrombotic disorder. Analysis is commonly performed by PCR followed by restriction enzyme analysis. This approach is labour intensive, time consuming and involves multiple steps. A novel approach to the detection of the factor V Leiden and prothrombin 3' UTR mutations, developed by thirdwave technologies (Madison, WI, USA) involves using two partly overlapping DNA probes one of which contains the allele specific sequence of interest. Hybridization to a nucleic acid target results in the formation of a structure recognized by a proprietary Cleavase enzyme. In a secondary reaction, the cleaved product releases a detectable fluorescent signal. We have retrospectively evaluated the thirdwave invader DNA technique

Samsung Bioepis Exhibit No. 1002
Page 1446

in 151 patients proven by PCR and restriction analysis to be normal, heterozygous or homozygous for factor V Leiden and/or the prothrombin 3' UTR mutations. Our current multiplex PCR method uses mutant primers that create a *Hin*dIII restriction site if the mutation is present. Results using the 3'UTR prothrombin gene invader assay identified 10 of 151 patients as heterozygous, four of 151 had equivocal results, nine of 151 had no result and the remainder were normal. Results using the factor V Leiden invader assay identified 17 of 151 as heterozygous, two of 151 as homozygous, 11 of 151 had no result, one of 151 had an equivocal result and the remainder were normal. All patients were correctly identified by the invader assay. Failure to generate a result or an equivocal result was due solely to the quality/quantity of DNA used. Overall, the thirdwave invader DNA assay is a simple, accurate rapid and reproducible procedure for correctly identifying Factor V Leiden and the 3' UTR prothrombin gene mutations.

168

Risk factor profile and provisional outcomes findings for confirmed venous thromboembolism: the VEenous thromboembolism RegIsTrY (VERITY)

D. O'Shaughnessy,[1] P. Rose,[2] D. Cummins,[3] N. Scriven[4] P. Walton[5] & N. Amlot[6]

[1]Southampton General Hospital, Southampton; [2]Warwick Hospital, Warwick; [3]Southend Hospital, Essex; [4]The Calderdale Royal Hospital, Halifax; [5]Dendrite Clinical Systems, Henley-on-Thames; [6]Aventis, Kent, UK

VERITY is a UK, prospective, observational registry of patients with suspected venous thromboembolism (VTE). Data on demographics, medical history, symptoms, diagnosis, treatment and outcomes are recorded. Uniquely, data from VTE-negative cases are available for comparison. All confirmed cases are treated with low-molecular-weight heparin, enoxaparin (LMWH). VERITY is expanding rapidly (15 December 2003: 11 276 entries; 34 centres active; 11 new centres confirmed). The 2003 analysis will be reported (February 2004) in five key areas: (i) DVT and PE diagnosis/outcomes, (ii) provision of thromboprophylaxis, (iii) cancer, (iv) pregnancy and (v) practice variations. In a preliminary analysis undertaken for this submission (5 December 2003), there were 10 268 patient entries, 2167 confirmed cases of DVT and 176 confirmed PE ± DVT (22.8% of referrals). In VTE-positive cases ($n = 2343$), previous medical or surgical admissions were a common risk factor (10.9% and 14.3% respectively). In VTE cases with a recent history (<4 weeks) of surgery ($n = 310$), 136 (44%) had undergone orthopaedic surgery. In VTE cases with recent hospital admission, about 33% received thromboprophylaxis (aspirin, heparin or LMWH). In VTE cases with cancer (ongoing or within previous 6 months) ($n = 273/2343$, 11.7%), the most common cancers described were colorectal ($n = 30$), prostate ($n = 22$), pancreatic ($n = 21$) and leukaemia ($n = 16$). To date, 186 cases of suspected VTE in pregnancy have been recorded; additional data are being collated to characterize thromboprophylaxis patterns in pregnancy. Initial registry findings highlight the current inadequate provision for assessment of post-treatment outcomes (recurrence and complications) both in primary and secondary care. Focused efforts, using novel follow-up initiatives, are being undertaken to define outcomes for different populations of VTE-positive patients. Available outcome data will be analysed and presented. Previous interim data (August 2003) showed 333 patients with follow-up. Provisional findings on bleeding complications were: early (<5 d) in 0.6%; late (>5 d) in 1.5%.

169

A novel variant of acquired von Willebrand syndrome associated with systemic lupus erythematosus successfully managed with recombinant activated factor VIIa and rituximab

W. A. Lester,[1] P. Short,[1] C. Ryder,[2] T. Southwood[2] & M. D. Williams[1]

Departments of [1]Haematology, and [2]Rheumatology, Birmingham Children's Hospital NHS Trust, Birmingham, UK

Our patient was diagnosed with systemic lupus erythemastosus (SLE) aged 9 years. Spontaneous petechiae and symptomatic oropharyngeal mucosal bleeding were noted shortly after initial diagnosis. All laboratory investigations were normal with the exception of impaired *in vitro* aggregation of platelets in response to Ristocetin (RIPA). A mixture of our patient's platelet poor plasma and normal control platelet rich plasma showed a markedly reduced RIPA consistent with the presence of an inhibitor despite the patient having a normal platelet count, von Willebrand factor (VWF):Ag, VWF:RCo and factor VIII and normal VWF:Ag multimers. The patient required frequent inpatient admissions for bleeding but slowly improved with standard immunosuppressive therapy and monthly infusions of IV immunoglobulins (IVIg). 6 years after diagnosis the patient remained on oral prednisolone, methotrexate and cyclophosphamide but developed a life threatening mucocutaneous haemorrhage in her neck requiring intubation and intensive care. Laboratory investigations were identical to those at diagnosis. Bleeding failed to respond to infusions of platelets and intermediate purity plasma derived factor VIII, but transient haemostasis was observed after treatment with 90 μg/kg recombinant activated factor VIIa. Methylprednisolone, cyclophosphamide, IVIg and plasma exchange were administered during the first week with a subsequent reduction in haemorrhagic manifestations. Treatment was consolidated with rituximab and cyclophosphamide. 6 months later, there has been no recurrence of serious haemorrhage and the patient's plasma does not inhibit normal control RIPA. In summary, we report a clinically severe novel variant of acquired von Willebrand syndrome associated with SLE but with no depletion of platelets or clotting factors despite apparent inhibitor activity against VWF platelet interaction, which responds clinically to immunosuppressive therapy.

170

Acquired haemophilia associated with clopidogrel; report of two cases

M. A. Haj, H. Dasani & P. W. Collins

Arthur Bloom Haemophilia Centre, University Hospital of Wales, Cardiff, UK

Acquired haemophilia is a rare bleeding disorder caused by autoantibodies against factor VIII. Bleeding is often severe and may be life threatening. In 50% of patients, no underlying disorder is found, however, common associations are with autoimmune disease, malignancy, dermatological disorders, pregnancy and drugs. We report two female patients age 70 and 67 years, who recently presented with a history of excessive bruising and bleeding 2–3 months after commencing clopidogrel for peripheral vascular disease. One patient had had a documented normal aPTT at the time of starting clopidogrel whilst the other patient had not been tested. Investigation showed that both patients had a low factor VIII (3.9 and 1.0 IU/dl) with a detectable anti-factor VIII inhibitor (2.2 and 17.6 Bethesda units) respectively. There was no evidence of any other underlying cause. Both patients responded to discontinuation of clopidogrel and commencing high dose steroids. The factor VIII levels rose to 119 and 136 IU/dl and the inhibitors became undetectable (<0.4 Bethesda units) within 8 weeks of treatment. A possible link between acquired haemophilia and clopidogrel has not previously reported. Clopidogrel is a relatively new anti-platelet drug that is becoming increasingly common in patients with cardiovascular disease. Physicians who pre-

Samsung Bioepis Exhibit No. 1002
Page 1447

scribe clopidogrel should be aware of this possible association, particularly if patients develop bruising after starting this drug. Increased bruising should not be ascribed to the anti-platelet action of clopidogrel unless a coagulation screen has been found to be normal. Further investigation for an anti-factor VIII inhibitor should be undertaken if indicated.

171

Analysis of predisposing factors and six-month outcomes of DVT management in 668 patients treated with LMWH (tinzaparin) as outpatients

S. Rhodes, S. Bond & E. S. Green

Department of Haematology, Great Western Hospital, Swindon, UK

In Swindon, eligable patients with a suspected or proven DVT are treated as outpatients using a once daily low molecular weight heparin (LMWH). Prospective audit using an electronic database has been ongoing since 1998. As a result of this data analysis, we can identify patients at greater risk of recurrent thromboembolic disease and bleeding complications. As of August 2003, there was a total cohort of 717 patients: average age was 61 years, with 53% female patients. The major predisposing factors were recorded as immobility (32.5%), previous VTE (21.3%) and surgery (14.8%). Six-month follow-up data were available for 668 patients: 20 (3.0%) patients had confirmed thromboembolic complications, of which 18 (2.7%) occurred subsequent to the initial LMWH treatment phase (late). Bleeding complications occurred in 12 (1.8%), with four (0.6%) being classified as major (three gastrointestinal bleeds and one retroperitoneal haematoma). Of the 20 confirmed thromboembolic complications, two (10%) had no predisposing factor at initial presentation. Other risk factors were malignancy (six events), immobility (six events), surgery/trauma (four events), oral contraception/HRT use (three events), previous VTE (two events), family history of VTE (two events) and a long haul flight (one event). Six patients had more than one risk factor; two of the patients had two events and both of these patients had a malignancy. Incidence of thromboembolic and bleeding events at 6 months were low (comparable with clinical trial data and other UK audit data) in our centre. The most significant risk factors for recurrent thromboembolic complications were malignancy and immobility. The database has allowed analysis of the data over different time periods enabling us to show that the service has been extended to more patients but with a reduction in complication rates. The service is now beginning to manage selected patients with a confirmed PE on an ambulatory basis.

172

Heparin induced thrombocytopenia (HIT): a 5-year review at University Hospital Birmingham, UK

F. A. Wandroo & J. T. Wilde

Department of Haematology, University Hospital NHS Trust, Queen Elizabeth Hospital, Birmingham, UK

We performed a retrospective analysis of patients suspected of having heparin-induced thrombocytopenia during the 5-year period of January 1998 and January 2003.Of 15 evaluable patients who underwent HIT screening, eight (44%) patients (five males and three females) were found to have heparin/PF4 antibodies by ELISA assay. The median age was 69 years (range 26–73 years). Unfractionated heparin (UFH) had been given in three patients, low molecular weight (LMWH) heparin in three patients and in two patients both UFH and LMWH was used. Five patients received therapeutic doses of heparin (UFH-3, LMWH-1, both-1) and three patients received prophylactic doses (LMWH-2, both-1). Median date of onset of thrombocytopenia from the start of heparin was 5.5 d (range 2–20 d). The median platelet count nadir was 47×10^9/l (range 24–86). The patient who developed thrombocytopenia on day 2 had past history of heparin exposure over a year previously. Only one of eight patients (12.5%) developed a thrombotic event (a below knee DVT). Heparin was stopped in all except one patient who was not considered to have HIT clinically. Danaparoid was substituted in five patients and prostacycline in one patient. Two patients received no ongoing anticoagulation. The mean time of platelet recovery to $>100 \times 10^9$/l was 6 d. Two patients had persistent thrombocytopenia suggesting that there was likely to be another cause. Four patients subsequently died. No death was attributable to HIT. It is well established that the platelet count needs to be monitored in patients who are on UFH or LMWH beyond 5 d. Results of sensitive diagnostic tests such as the ELISA assay should be interpreted in the clinical context so as to accurately assess the probability of HIT. Of interest is the low incidence of clinically overt thrombosis in this group of patients with HIT.

173

Acquired FVIII inhibitor associated with nephrotic syndrome in a child

M. C. Mathias, K. Khair, I. Hann & R. Liesner

Department of Haematology, Great Ormond Street Children's Hospital, London, UK

Acquired factor VIII (FVIII) inhibitors usually occur in adults and may be triggered by pregnancy or malignancy. They usually present with rapid onset bruising and bleeding and if untreated are associated with a high mortality rate. We present a child from West Africa who developed steroid-resistant nephrotic syndrome at the age of 19 months, which was not initially associated with any thrombotic or haemorrhagic symptoms. When he was first seen in our centre he had been pretreated with cyclophosphamide and prednisolone. He was noted at this time to have an APTT of 121.2 s (27–38 s) with a 50:50 mix of 50.2 s and FVIII activity of <1 IU/dl. Although the Exner was positive on the first occasion the DRVVT and autoantibody screen were negative and anticardiolipin antibodies were within the normal range. He had a FVIII inhibitor of 143 Bethesda units (BU). He was found to be negative for the inversion 22 FVIII mutation and although further detailed genetic studies were carried out, no evidence of congenital haemophilia was ever found. There was no personal or family history of haemophilia and he had had an uncomplicated circumcision at 3 weeks of age. Over the subsequent 3 years this boy developed a target joint in one knee, but he was otherwise remarkably free from serious bleeding despite a maximum inhibitor titre of 1558 BU. Attempts to wean his immune suppression resulted in a rebound of his nephrotic syndrome and ultimately this necessitated repeated hospital admissions in Ghana, culminating in his death in the fourth year of his illness. We present a child with nephrotic syndrome complicated by a very high titre acquired FVIII inhibitor, which did not respond to prolonged immunosuppression, in whom there were remarkably few bleeding episodes.

174

The effect of citrate and citrate theophylline adenosine dipyridamole (CTAD) anticoagulants on the determination of plasminogen activator inhibitor-1 (PAI-1) antigen

M. S. Khan, C. Burgess, K. Khair, I. Hann & R. Liesner

Department of Haematology, Great Ormond Street Hospital, London, UK

PAI-1 deficiency is an extremely rare disorder characterized by a bleeding diathesis due to hyperfibrinolysis as a result of low activity and antigen. Forty-five samples, comprising 20 adult controls and 25 test samples from a children's haemostasis clinic were collected, as part of screening investigations for easy bruising, bleeding and/or non-accidental injury. All the samples were collected in duplicate in both citrate and CTAD anticoagulant bottles between 10 and 12 a.m. and by the same experienced haemophilia nurse to standardize any diurnal variation and phlebotomy technique. The aim was to determine

Samsung Bioepis Exhibit No. 1002
Page 1448

whether the samples with CTAD as anticoagulant were better suited to determine PAI-1 antigen than using 3.8% citrate as an anticoagulant. Earlier studies have shown that plasma determination of PAI-1 antigen must be performed in blood collected on an antiplatelet/anticoagulant mixture. CTAD inhibits the release of PAI-1 from platelets unlike citrate. Using the PAI-1 Tintelize antigen assay (Biopool, Sweden), the results showed a significantly higher antigen value with the citrate bottles than with the CTAD anticoagulant ($P < 0.001$). This is of major clinical significance when using the PAI-1 antigen assay as a screening test to eliminate PAI-1 deficiency in patients with a bleeding diathesis in the absence of any haemostatic dysfunction or when used as part of screening tests for exclusion of non-accidental injury in medico-legal cases. These are preliminary findings of a larger study of the evaluation of suitable assay for the determination of PAI-1 activity and antigen from blood plasma samples for routine laboratory use.

175

Increased circulating endothelial cells as an index of endothelial damage in congestive heart failure

A. Blann, A. Chong, B. Freestone & G. Lip

Department of Medicine and Haematology, Birmingham, UK

Background: Circulating endothelial cells (CECs) in the peripheral blood probably represent the most direct evidence of endothelial cell damage. CECs are increased in haematological diseases, myocardial infarction, unstable angina and critical limb ischaemia. As congestive heart failure (CHF) is associated with endothelial abnormalities, we hypothesized that CECs are raised in CHF, and that they would correlate with the more established plasma indices of endothelial perturbation, that is, von Willebrand factor (vWf) and soluble E-selectin (sE-sel).

Methods: We studied 30 patients with acute CHF, 30 patients with chronic stable CHF [all in sinus rhythm with ejection fraction (40%) and 20 controls]. Patients with acute CHF were venesected within 24 h of hospital admission. CECs were quantified using epifluorescence microscopy after immunomagnetic separation. Citrated plasma was analysed for sE-sel and vWf by ELISA.

Results: Levels of CECs, vWf and E-sel were significantly higher (all $P < 0.01$) in patients with CHF compared to controls, with no significant differences between acute and chronic CHF. CECs correlated with plasma vWf ($P < 0.0001$) and E-selectin ($P = 0.022$) but not ejection fraction or NYHA class. In stepwise multiple regression analysis, CHF was the only independent predictor of raised CECs ($P < 0.0001$).

Conclusion: CECs may be used as a novel measure of endothelial damage in CHF, which may have implications for the thrombotic risk associated with CHF and prognosis in this condition.

176

Abnormal vascular, platelet and coagulation markers in essential thrombocythaemia are not reversed by treatments that reduce the platelet count

A. Blann, G. Caine & D. Bareford

Department of Haematology and Medicine, City Hospital, Birmingham, UK

Essential thrombocythaemia (ET) is characterized by increased platelet counts, and abnormalities in plasma markers of platelet activation (e.g. P-selectin), vascular dysfunction (von Willebrand factor, E-sepectin) and coagulopathy (fibrinogen, D-dimers). Treatment centres on lowering platelet counts. We hypothesized that successful treatment of thrombocytosis would result in a reduction in selected specific markers of these processes, and therefore compared such levels in 32 ET patients on treatment, 13 patients not on treatment, and in 15 healthy age- and sex-matched controls. As expected, ET patients on treatment had a higher platelet count than the controls, and those patients untreated had higher numbers than those on treatment (all $P < 0.01$). However, fibrinogen (Clauss), soluble P-selectin, platelet P-selectin, von Willebrand factor and soluble VCAM (all ELISA) were all higher in both patient groups compared to controls (all $P < 0.05$), but there was no difference between patient groups. Levels of D-dimers were higher in those on treatment compared to the controls ($P < 0.05$). Overall, there were no differences in haemoglobin, white blood cell count, soluble E-selectin or soluble ICAM amongst the three groups. We conclude that successful treatment of thrombocytosis does not bring about normalization of additional markers of the pathophysiology of platelets, the endothelium or coagulation.

177

The effect of multi-factorial intervention on markers of endothelial damage/dysfunction and thrombogenesis in diabetes mellitus

H. S. Lim, A. D. Blann & G. Y. H. Lip

Haemostasis, Thrombosis and Vascular Biology Unit, University Department of Medicine, City Hospital, Birmingham, UK

Background: Endothelial abnormalities and a hypercoagulable state may contribute to increased cardiovascular risk in diabetes mellitus, particularly in patients with overt cardiovascular disease (OCVD). We sought to determine if a 'package of care' of intensified multi-factorial cardiovascular risk intervention could improve parameters of endothelial abnormality and hypercoagulability in diabetes, and if diabetic patients with OCVD would derive similar benefit as compared to those without.

Methods: We measured plasma von Willebrand factor (vWf, an index of endothelial damage/dysfunction), soluble E-selectin (sE-sel, marking endothelial activation) and tissue factor (TF, an initiator of coagulation) by ELISA in 94 patients with diabetes mellitus (38 with CVD and 56 without OCVD and 38 comparable controls). Thirty-three patients with and 31 without OCVD then participated in multi-factorial cardiovascular risk intervention over 1 year.

Results: Plasma levels of vWf, sE-sel and TF were significantly higher in diabetic patients compared to controls, with TF highest in patients with OCVD ($P < 0.01$ by ANOVA). Intensive multi-factorial intervention was associated with significant reductions in HbA1c, total and LDL cholesterol (all $P < 0.001$) associated with reductions in vWf in both patient groups (both $P < 0.01$). TF was reduced only in patients without OCVD ($P = 0.03$). There were no significant changes in sE-sel levels in either group.

Conclusion: Patients with diabetes mellitus demonstrate endothelial perturbation, which can be partially influenced by a 'package of care' of intensified multi-factorial cardiovascular risk intervention. These data suggest the need for earlier and more aggressive risk factor intervention.

178

What is the role of thrombophilia screening in eye disease?

J. Mainwaring,[1] M. Costin,[2] J. Needham,[1] H. Lewis,[1] C. Sandy,[1] S. Keightley,[1] D. Morsman[1] & S. Knight[1]

[1]*Department of Haematology and Ophthalmology, North Hampshire Hospital, Aldermaston Road, Basingstoke;* [2]*Department of Ophthalmology, Southampton General Hospital, Tremona Road, Southampton, UK*

Requests for thrombophilia screens and the apparent indications keep on increasing with significant workload pressures on haematology laboratories. There has been much debate on the merits of these screens with those in favour and those against. Now ophthalmic surgeons have been made aware of the potential link between retinal vein occlusions (RVOs) and thrombophilic defects especially the factor V Leiden mutation (FVL). During the past 12 months we have seen 167 patients in our thrombophilia clinic. This group comprised 90 females and 77 males with an age range of 13–80 (mean 52). The majority of referrals

Samsung Bioepis Exhibit No. 1002
Page 1449

were patients after completion of anticoagulation therapy for venous thromboembolic events (VTEs). There were also 12 ophthalmic referrals comprising nine females and three males. Reason for referrals were RVOs in seven, unexplained visual field loss in three and unexplained retinal infarcts in two. Comprehensive screens were performed and the findings were presence of anticardiolipin antibodies ± lupus inhibitor in four, no abnormality in four, raised homocysteine in one, type 1 protein C deficiency in one, type 2 antithrombin deficiency in one and heterozygous FVL in one. Several patients had other risk factors such as hypercholesterolaemia making it difficult to know the significance of the results. The majority received aspirin whether the screens were abnormal or not with good effect. The only exception was the patient with type 1 protein C deficiency who started long-term warfarin because of his increased risks of VTEs as a consequence of being a frequent long haul flyer. We as a group feel the jury is out as to whether thrombophilia testing is useful in these patients, bearing in mind the complexity and costs of the tests, and also will an abnormal result change management. Without doubt larger studies are needed to clarify the situation.

179

The effect of hormone replacement therapy on the expression of monocyte tissue factor

E. A. Wilson,[1] C. Bain,[2] M. D. McColl[3] C. Pearson,[1] I. A. Greer,[2] M. A. Lumsden[2] & I. D. Walker[1]

[1]Department of Haematology, Glasgow Royal Infirmary, Glasgow; [2]University Department of Obstetrics and Gynaecology, Glasgow Royal Infirmary, Glasgow, UK; [3]Department of Haematology, Crosshouse Hospital, Ayrshire, UK

Hormone replacement therapy (HRT) is associated with an increase in both venous and arterial thrombotic events during the first year of treatment. This could conceivably be mediated via an increase in monocyte tissue factor (TF) expression, which has been shown to be elevated in patients with previous idiopathic venous thromboembolism. A double-blind randomized control trial was undertaken to investigate TF expression during the first 3 months of treatment with oral HRT. Healthy postmenopausal women ($n = 32$, mean age 59) were randomized to either 1 mg oestradiol and 0.5 mg norethisterone ($n = 8$), 1 mg oestradiol and 5 mg dydrogesterone ($n = 14$) or placebo ($n = 10$). Monocyte TF was measured by flow cytometry on fresh blood incubated with lipopolysaccharide 40 μg/ml at 37°C, 5% CO_2 for 3 h. The sample was labelled with FITC-conjugated CD14 for monocyte identification and a PE-conjugated anti-TF antibody. The number of TF binding sites was calculated by comparing the fluorescence of the monocyte population with standard beads. There was no significant difference between TF expression at baseline and 3 months for the dydrogesterone group (mean ± SD, 855 ± 355 molecules/cell versus 1209 ± 656 molecules/cell), the norethisterone group (751 ± 426 molecules/cell versus 839 ± 347 molecules/cell), and the placebo group (787 ± 291 molecules/cell versus 1110 ± 489 molecules/cell). These results provide no evidence that the early increase in thrombotic events poststarting HRT is mediated via an increase monocyte tissue factor expression.

180

Self-management of anticoagulation: a randomized trial (SMART)

D. A. Fitzmaurice, E. T. Murray, F. D. R. Hobbs & D. MacCahon

Department of Primary Care, University of Birmingham, Birmingham, UK

Introduction: The increased demand for oral anticoagulation has led to a need for alternative models of care. Self-management (PSM), analogous to diabetic self-care has been proposed as one such alternative. This paper reports the first large-scale UK trial of PSM compared with routine care.

Method: All adult patients receiving warfarin from 50 general practices were invited to participate. Following consent patients were randomized to either control or intervention. Intervention patients were trained in 2 × 3 h sessions to measure INR and interpret their dose of warfarin. Patients completing training were followed up for 12 months.
Results: A total of 617 patients were randomized (337 intervention/280 control, from 2470 invited). Using intention to treat analysis, there was no significant difference in percent time in range (PSM 70% versus control 68%). There were no significant differences in adverse events. Cost-effectiveness data will be available for presentation.

181

A study of the effect of introduction of clinical guidelines on the management of major haemorrhage in patients on warfarin

A. Salamat,[1] S. E. Lessels,[1] J. Seaton[2] & H. G. Watson[1]

Departments of [1]Haematology and [2]Clinical Effectiveness, Aberdeen Royal Infirmary, Aberdeen, UK

The management of major haemorrhage in patients on warfarin is highly variable and often suboptimal. In an attempt to develop standardized, evidence-based care we developed a guideline based on BCSH recommendations. Practice, compared with the guideline was assessed for 14 months prior to and 12 months after the production of the guideline. In 82 patients (34 pre and 48 postguideline) over a 26-month period, indication for use of PCC, INR at time of request, PCC dose, use of the appropriate dose and route of administration of vitamin K, follow-up monitoring of coagulation status, and clinical thrombotic events were recorded. Eighty-two episodes of PCC use were recorded compared with 108 expected. Comparing the respective periods pre and postguideline the following were observed: of requests which were granted 100% and 92% were for appropriate indications, the dose of PCC was correct in 52% and 67% of cases, patients who received the wrong dose were more likely to be under-dosed, 73% and 80% respectively. Concomitant use of vitamin K improved from 65% to 79% and the dose was correct in 35% and 47% of occasions respectively. Monitoring of coagulation post-PCC was performed in only 65% and 53% of occasions respectively. One thrombotic event was recorded in the 82 treatment episodes. Introduction of a guideline has only marginally improved practice and verbal re-iteration by a specialist in haematology at the time of all PCC issues is now being evaluated.

182

Patient self-testing of INR is a safe and acceptable alternative to laboratory-based testing

K. E. Williams,[1,2] C. Gardiner,[1,2] I. J. Mackie,[1,2] S. J. Machin[1,2] & H. Cohen[1]

[1]University College London Hospitals; [2]Medicines and Healthcare Products Regulatory Agency, UK

Approximately 750 000 UK patients currently take warfarin and, as this is expected to increase this by approximately 10% annually, alternatives to hospital-based anticoagulation clinics are being explored. Following a satisfactory laboratory evaluation (MDA 01026) of the Roche Diagnostics CoaguChek S (CCS), we studied the accuracy and patient acceptability of self-testing for INR using the CCS. Patients were randomized to either self-testing or control groups. Both groups continued to have their anticoagulation monitored every 4 weeks in the hospital laboratory. Dosage adjustments were performed by the anticoagulant clinic staff using the laboratory INR. Eighty-four patients were randomized to the study; 44 to self-testing and 40 to the control group. Following training, and subject to satisfactory assessment, the self-testing group performed weekly INR measurements using the CCS over a 6-month period. Fifteen patients (18%) failed to complete the study; five were unable to obtain an adequate sample, three had deteriorating health, two moved from the area, one discontinued

Samsung Bioepis Exhibit No. 1002
Page 1450

warfarin and four demonstrated compliance problems. Of 31 self-testing patients who completed a questionnaire, 27 found the CCS easy to use, 27 were confident in the result they obtained and 24 preferred self-testing to the hospital anticoagulation clinic. When comparative INR data were analysed ($n = 234$), excellent agreement was obtained between the methods ($r = 0.95$), median INR CCS = 2.90 (IQR 1.15) laboratory = 2.96 (IQR 1.17) with a mean difference of 0.05. Overall 85% of results were within 0.5 INR units of each other. On four occasions differences of >1 unit INR were obtained, but in each case the patient's anticoagulation was poorly controlled (INR >4.5 by both methods) and the differences in INR would not have significantly altered patient management. We conclude that self-testing is a safe alternative to attending the hospital clinic and is acceptable to patients.

183

The role of medication adherence in warfarin anticoagulation control and its cognitive determinants

D. Engova,[1] C. Duggan[2] & P. MacCallum[3]

[1]Department of Practice and Policy, The School of Pharmacy, University of London; [2]Academic Department of Pharmacy, Barts and The London NHS Trust; [3]Department of Haematology, Barts and The London NHS Trust, London, UK

Non-adherence is frequently considered as a factor to explain fluctuating INRs in warfarin-anticoagulated patients. Others argue that patients on warfarin are more adherent than those on diuretics. We measured level of adherence to medication taking and regimen in warfarin-anticoagulated patients and explored determinants of this behaviour based on social cognition theories. A survey of a simple random sample of attendees of a single hospital-based anticoagulation clinic was conducted. Of the 162 respondents, typical of anticoagulated population on all background characteristics, 146 (92%) scored 29 or the maximum score of 30 on the Medication Adherence Report Scale. Calculation of adherence from warfarin plasma levels identified eight (5%) patients with too low plasma warfarin levels. A total of 160 (99%) reported taking warfarin at the same time each day, mostly in the evening (120, 76%). A total of 293 (59%) of 499 INR measurements were within the therapeutic range. On the 'effects of warfarin treatment' 7-question battery, 108 (67%) to 129 (80%) did not perceive warfarin a risk (four questions). Fifty-nine (37%) to 92 (57%) perceived warfarin to have a great deal of benefits, while 63 (39%) to 83 (52%) did not know if warfarin had benefits (three questions). Scores on beliefs about Medication Questionnaire General ranged from 16 to 39 with 141 (88%) of scores above the theoretical mean, suggesting acceptance of allopathic medication. Intrinsic Desire for Information scale scores were normally distributed, suggesting a range of desires for information within the sample. We have identified high adherence to medication taking and regimen in our sample although 41% of INRs were out of range; non-adherence was not the cause of INR fluctuation. Warfarin was not perceived as risk but there was uncertainty about benefits. Most patients considered allopathic medication as good. These general beliefs about medication were more important determinants of high adherence than specific beliefs. Influencing these opinions by providing information would have to be carefully considered and tailored as not all required information.

184

The effect of initiation of oral anticoagulation on protein Z and coagulation activation

D. McCollum,[1] R. C. Tait,[1] J. Conkie,[1] M. Wight,[1] M. D. McColl[2] & I. D. Walker[1]

[1]Glasgow Royal Infirmary, Glasgow; [2]Crosshouse Hospital, Ayrshire

Protein Z (PZ) is a vitamin K dependent glycoprotein with a similar structure to coagulation factors VII, IX, X and to protein C. During the initiation of warfarin therapy patients may be at an increased thrombotic risk. This study aims to determine whether the rate at which PZ falls contributes to this prothrombotic tendency and to compare the rates at which the levels of other vitamin K dependent factors fall. We correlated the changing levels of vitamin K dependent proteins with alterations in the level of the coagulation activation marker, prothrombin fragment F1 + 2. We recruited seventeen patients (M = 10 and F = 7; mean age = 68.6) with atrial fibrillation (AF) electively starting warfarin (5 mg induction regimen). None had prior history of thrombosis or bleeding. Patients with antiphospholipids were excluded. Samples were taken at baseline (day 1) and at days 2, 3, 4, 5, 6, 9, 16 and 30 for analysis of factors II, VII, IX, X, protein C, protein S, F1 + 2 and PZ antigen (ELISA assay). Fourteen of 17 had INRs >2 by day 9. PZ falls slowly but significantly during warfarinization, in a time frame similar to factor II and X (see table). The 5 mg anticoagulant regime did not cause a precipitous fall in protein C nor was there evidence of transient hypercoaguability as measured by the F1 + 2 level. To summarize, PZ drops relatively slowly during 5 mg warfarin induction regimen this slow fall is unlikely to contribute to transient hypercoaguability. This study suggests that a 5-mg daily warfarin outpatient-loading regime, is a safe and gentle approach to anticoagulation.

	Half-life decline (d)	Percentage of baseline at Nadir	Days to Nadir
Factor II activity	3.1	26	9
Factor VII activity	1.3	15	5
Factor IX activity	2.1	27	9
Factor X activity	2.5	19	9
Protein Z antigen	3.0	11	16

185

Bleeding complications of oral anticoagulant therapy: a prospective audit

E. S. Green,[1] S. Bond,[1] S. Rhodes,[1] R. James,[2] M. Taylor,[1] P. Rose[2] & C. Emmas[3]

[1]Swindon and Marlborough NHS Trust; [2]University Hospitals of Coventry and Warwickshire NHS Trust; [3]AstraZeneca, UK

Bleeding is the most serious complication of the use of oral anticoagulation in the prevention and treatment of thromboembolism. We designed a tool to prospectively audit the frequency and severity of over anticoagulation and bleeding in two anticoagulant services; a district hospital serving a population of 330 000 patients (3400 anticoagulant patients) and a University hospital with a population of 300 000 (4000 anticoagulant patients), both providing Anticoagulant practitioner-led inpatient and outpatient anticoagulant dosing. Data was collected over 10 months including INR at time of event, whether complications necessitated hospital attendance and/or admission, investigations carried out and treatment given. There were 347 events; 133 were asymptomatic high INRs (>8). The majority of these were treated with oral vitamin K as outpatients. There were 214 bleeding episodes, 54 major and 160 minor. The most common sites of bleeding were epistaxis (70/214), GI bleeding (45/214), haematoma (36/214) and haematuria (24/214). A total of 106 patients were admitted to hospital, constituting 0.61% of the total emergency medical admissions over this period. Patients spent a total of 887 d in hospital. Fourteen patients died, four directly as a result of the bleeding episode. INR stability in the period prior to the event was measured by ±1 standard deviation from the mean. Fifty-two of 347 patients were new at the time of the event (receiving anticoagulant therapy for less than 4 weeks), 164/347 were unstable, 121/347 were stable and 10/347 were GP monitored patients with no INR data available. At the time of event 68% of patients had an INR above target range (major bleed 72%; minor 66%). The most common factors contributing to the event were

Samsung Bioepis Exhibit No. 1002
Page 1451

concurrent illness (78/347), prescription of potentiating medication (65/347), increase in prescribed warfarin dose (21/347) or patients inadvertently exceeding their prescribed dose (15/347).

186

Health care costs of treating bleeding and other complications associated with warfarin treatment of atrial fibrillation and venous thromboembolism in the UK

S. Bond,[1] S. Rhodes,[1] R. James,[2] M. Talor,[1] E. S. Green,[1] P. Rose[2] & C. Emmas[3]

[1]Swindon and Marlborough NHS Trust, Swindon; [2]University Hospitals of Coventry and Warwickshire NHS Trust, Walsgrave, UK; [3]AstraZeneca UK, Luton, UK

Oral anticoagulation with warfarin is an effective therapy for the treatment and prevention of thromboembolic events in patients with atrial fibrillation (AF) and venous thromboembolism (VTE). However, bleeding is a common complication of warfarin therapy. This study assesses the cost of treating this complication from the perspective of the UK National Health Service (NHS). A prospective clinical audit of adverse events associated with warfarin treatment was carried out over a 10-month period at two hospital-based anticoagulation clinics monitoring 7400 patients. Resource use associated with the treatment of bleeding and asymptomatic high INR values (>8) was obtained from hospital records or from the patient where no hospital admission occurred. Events occurring in inpatients were excluded due to the difficulty in assessing which costs were due to the original admission. Event costs were calculated by multiplying the resource units by the corresponding published national 2002 reference costs. A total of 325 bleeding events and asymptomatic high INRs requiring treatment were recorded with a total cost of £367 850. Of these 124 were in patients treated for AF and 107 in VTE patients. The remaining events occurred in patients treated for other conditions and are not considered in this analysis. The average costs associated with treatment for any event were £1121 (95% CI: £675–£1567) in AF patients and £1032 (95% CI: £641–£1423) in VTE patients. The average costs associated with major bleeds ($n = 32$) (£4584, 95% CI: £3256–£5912) were significantly higher than the costs associated with minor bleeds ($n = 111$) (£715, 95% CI: £432–£998) or asymptomatic high INR values (£251, 95% CI: £118–£384) ($P < 0.001$). The average costs in those admitted to hospital (£3298, 95% CI: £2555–£4041) were greater than in those not admitted (£106, 95% CI: £83–129). Treatment of bleeding and asymptomatic high INR values associated with warfarin therapy results in a significant consumption of NHS resources.

187

Management of anticoagulated patients before and after elective urological surgery

E. Ong[1] & D. A. Tolley[2]

[1]Department of Urology, Aberdeen Royal Infirmary, Aberdeen; [2]Scottish Lithotriptor Centre, Edinburgh, UK

Literature suggests that the risk of bleeding associated with perioperative intravenous heparin therapy is higher than the risk of thromboembolism associated with subtherapeutic anticoagulation perioperatively. This audit is an initial step to establish an evidence-based guideline on perioperative management of anticoagulated patients undergoing elective urological surgery. Seventy-five questionnaires were sent to urologists and consultant physicians (haematologists and cardiologists) in the East of Scotland. This is to find out the established practice and the expert opinion of the consultant physicians. They were asked various questions on the perioperative management of anticoagulation. A review of literature was also performed to assess the current recommendation. Sixty-three per cent of urologists and 51% of physicians replied; 59% of urologists would stop warfarin 3–4 d preoperatively and 64% would admit the patients the day before surgery; 67% of urologists would operate with international normalized ratio (INR) less than 1.5 and majority would not use Vitamin K in elective surgery. Some urologists would convert warfarin into intravenous heparin in all warfarinized patients. All physicians think the conversion is unnecessary if the indication of warfarin therapy is atrial fibrillation or venous thromboembolism, although some physicians agree that it is required in prosthetic heart valves cases. Postoperatively, most urologists would re-start intravenous heparin within 4–6 h and re-start warfarin the day after surgery; 62% of urologists agree that patients can be discharged earlier if subcutaneous instead of intravenous heparin is used because this can be managed in the community. Literature review shows that the available guidelines are subjective. No optimal strategy has been identified, as the established practice is inconsistent. There is a need to establish an objective guideline in each unit or specialty to reduce the risk of bleeding or thromboembolism in these patients.

188

Monitoring low molecular weight heparin in pregnancy: the timing of the peak measurement

C. R. Shiach[1] & T. A. Johnston[2]

[1]Department of Haematology, Manchester Royal Infirmary; [2]Depatrment of Obstetric, St Mary's Hospital, Manchester, UK

The role of monitoring low molecular weight heparin (LMWH) by an anti-Xa assay remains disputed. Much confusion arises because different centres undertake this using different protocols. The timing of the peak measurement has been recommended at 2, 3 and 4 h in pregnant women. This study aimed to identify the true time of a peak LMWH level in pregnant women. Fourteen pregnant women took part in the study. Samples were taken at time 0 immediately prior to LMWH and then at 1, 2, 3, 4 and 6 h after LMWH. LMWH concentration was measured using a one stage clotting anti-Xa assay. Thirty-seven separate studies were done. Six women were studied only once. Eight women had studies two to six times during their pregnancy. Data showed that the mean time of peak LMWH is 4.1 h (95% CI 3.6–4.5). The median is 3.7 h (95% CI 3.4–4.4). The interquartile range is 3.2–4.8 h. Where a pregnant woman has no detectable LMWH at time 0 we change their dosing to a twice-daily regime. Further analysis of the data showed that there is no significant difference in the time that women reach peak LMWH level between women having once daily and those having twice daily heparin ($P = 0.68$) although those on once daily regimes have a wider scatter of results. This study shows that although pregnant women have increased rates of clearance of LMWH the time for LMWH to reach a peak is close to 4 h, which is very similar to results obtained in non-pregnant individuals. Even where women need twice daily dosing there is no change in the time of the peak LMWH level.

Poster Presentation

General Haematology and Transfusion

189

Results of a digital web-based exercise for UK NEQAS(H), blood cell and bone marrow morphology

E. Wells,[1] J. Ardern,[2] L. Hickman,[2] M. Brereton,[2] J. Burthem,[2] R. Bromilow,[1] L. Seal,[3] B. de la Salle[4], P. McTaggart,[4] J. Parker-Williams[4] & K. Hyde[2]

[1]Department of Biological Sciences, University of Salford; [2]Department of Haematology, Manchester Royal Infirmary, Manchester; [3]Department of Biological Sciences, Manchester Metropolitan University, Manchester; [4]UK National External Quality Assessment Scheme, NEQAS(H), UK

We present results from a fourth national web based morphology exercise. Participants of UK NEQAS(H), General Haematology Scheme

Samsung Bioepis Exhibit No. 1002
Page 1452

were invited to www.manlab.co.uk or via NEQAS(H) site to review Case 0301BF1, a myelodysplastic syndrome with complex morphology. Digital images were presented in three formats. Part 1 had 12 single static images with expert morphological comment and participant's consensus comments from the original national survey. Part 2 provided four images with interactive tutorial requiring participants to identify specific cells. Part 3, was comprised of nine 'Stitched' images that allowed movement across the image and a zoom facility. Of 426 participants 128 (30%) returned results to UK NEQAS(H). Part 1: Over 90% of responders gave positive opinion regarding image quality, access to the site, presentation of expert opinion and agreed that the single images represented the morphological features they had originally reported. Part 2: Over 95% responded positively to the educational element and commented that the tutorial element was a useful tool. Part 3: Of 128 responders 34 were unable to download the software necessary to view the 'Stitched' image due to Trust policy. Of the remaining 94 responders, 85 found image quality adequate and representative. A high proportion (71%) thought the Stitched image offered advantages over single static images, including ease of navigation, overview of morphology and magnification control. This positive feedback supports our intention to place images of all six annual blood film surveys on the WWW. We aim to expand the tutorial aspect by using specific surveys to highlight interesting morphology. Whilst software problems are to be addressed the development of 'Stitched' images into a virtual slide is ongoing. The potential educational aspect of digital morphology is creating keen interest; future development will provide a mechanism for continuing professional development as part of external quality assessment.

190

The hypergammaglobinaemia of Gauchers disease is not reversed by enzyme replacement therapy

D. A. Hughes, R. J. Baker, F. Wright & A. B. Mehta

Department of Academic Haematology, Royal Free Hospital, London, UK

Accumulation of glucosylceramide in Gaucher disease leads to characteristic storage cells and multiorgan pathology. Enzyme replacement therapy (ERT) with recombinant glucocerebrosidase has been successful in reversing symptoms relating to bulk storage, e.g. hepatosplenomegaly and bone marrow infiltration but its effects on immune system pathology are unknown. We have measured the immunoglobulin levels in 57 patients with Gaucher disease receiving ERT for up to 10 years. Twenty-eight of 57 patients (43.8%) were found to have levels of immunoglobulins significantly higher than those found in healthy adults. There were no sex or age related differences between those patients with elevated immunoglobulins (15 male; median age 44 years, 13 female; median age 48 years) and those with normal levels (13 male median age 41 years, 14 female; median age 44.5 years). Nineteen patients had elevation of a single immunoglobulin (eight IgM, seven IgG and five IgA), seven had elevation of two immunoglobulin classes (four IgA + IgG, three IgA + IgM) and one had elevation of all three immunoglobulin classes. Six patients had monoclonal gammopathy. Lymphocyte subset analysis of the peripheral blood of eight patients failed to show evidence of B-cell clonality. In patients with IgM polyclonal gammopathy levels of immunoglobulin normalized with ERT. This was not the case with IgG and IgA gammopathies which were unaffected by ERT even after 10 years of treatment. No patient with normal immunoglobulin levels prior to treatment developed a gammopathy after initiation of ERT. Our research suggests that in contrast to organomegaly, which is due to bulk storage of glucosyl ceramide the persistence of polyclonal gammopathy relates to constant immune stimulation which may occur at low levels of storage product not reversed by ERT. This may in turn contribute to the pathogenesis of haematological malignancy, which is notably elevated in Gaucher disease.

191

Abnormal distribution of cell surface receptors in glycosphingolipid storage disorders: an *in vitro* model of Gaucher's and Fabry disease

D. A. Hughes & A. B. Mehta

Department of Academic Haematology, Royal Free and University College Medical School, London, UK

Gaucher and Fabry Disease are lysosomal storage disorders resulting from deficiency of specific hydrolytic enzymes. Storage of un-degraded substrate results in characteristic pathologies but despite recent advances in the understanding of their natural history the pathophysiology underlying these conditions remains poorly defined. We have developed an *in vitro* assay of lysosomal storage in order to investigate the role of cellular interactions in the pathology of Gaucher and Fabry disease. Monocytes were selected from peripheral blood mononuclear cells of patients with Gaucher or Fabry disease and cultured in serum medium. Cells were cultured alone or with effete red cells to stimulate glycosphingolipid storage. The validity of the model was confirmed by measurement of chitotriosidase, an enzyme highly expressed in Gaucher disease. Over 7-d morphological differentiation into macrophages was accompanied by a 15-fold elevation in chitotriosidase in the supernatant of Gaucher cells compared to threefold elevation in controls. Cells from Gaucher and Fabry patients exhibited extensive cytoplasmic vacuolation. This was visible in only a subpopulation of cells from female carriers of Fabry disease (an X-linked condition) and in no cells from controls. The vacuolation was associated with an abnormal distribution of the adhesion and phagocytic receptors, the type-three complement receptor (CR3) and the mannose-6-phosphate receptor (MPR). In contrast to controls where the receptors were detected on the cell surface, the receptors of storage cells were located in a peri-nuclear compartment, defined as lysosomal by the expression of the LAMP-1 glycoprotein. Reduced cell surface expression of CR3 was further confirmed by our finding of reduced adhesion of Gaucher-derived monocytes to artificial substrata. Failure of recycling of cell surface receptors out of the lysosomal compartment is likely to be a consequence of disordered membrane trafficking due to substrate storage. This has implications for cellular interactions and also for the efficient delivery of recombinant enzyme, which is targeted to the prelysosomal compartment via the MPR.

192

Use of the galactomannan ELISA in the detection of invasive aspergillosis: a retrospective review and potential pitfalls in application

Z. Y. Lim,[1] A. Pagliuca,[1] J. Wade,[2] A. Ho,[1] S. Devereux[1] & G. J. Mufti[1]

Departments of [1]Haematological Medicine and [2]Medical Microbiology, Kings College Hospital, London, UK

Invasive aspergillosis (IA) remains a significant source of morbidity and mortality amongst haemato-oncology patients. The development of IA compromises treatment options and has a high economic burden. Much effort has been directed to the early detection and treatment of IA. However, the diagnosis and management of IA remains a challenge. Detection of galactomannan antigen in serum and body fluids has been intensively studied in recent years. An ELISA based test has been used in Europe for several years and recently the FDA approved it for use with a cut-off of 0.5 optical density (OD). Between October 2000 and September 2003, we analysed approximately 2500 serial samples from 290 haematology patients for the presence of galactomannan antigen using the Platelia *Aspergillus* EIA (ASPEIA) test kit. There were positive results in 46 patients (>0.5 OD). Results were charted and each individual highest test was correlated with clinical and radiological findings. The patients were assessed for diagnosis of IA based on the EORTC criteria. Of the 46 patients, five had probable and six had possible IA. Of the 244 patients with negative ASPEIA results, 15 had probable and 15 had possible IA. The sensitivity, specificity, positive predictive value (PPV)

Samsung Bioepis Exhibit No. 1002
Page 1453

and negative predictive values (NPV) were 26.8%, 85.9%, 23.9% and 87.7% respectively. The majority of our patients received oral itraconazole prophylaxis, which has been reported to reduce the sensitivity of the test. Half of the patients had also received piperacillin and tazobactam, which is known to cause false positive results. Negative ASPEIA test results (<0.5 OD) when evaluated together with radiological findings had good specificity and NPV. However the PPV is poor. Our findings suggest that ASPEIA tests should be interpreted with care, probably in conjunction with other diagnostic modalities. Newer techniques, such as fungal PCR may have an important role to play in future detection and monitoring of IA.

193

Only a minority of age eligible patients with myeloma undergo treatment with autologous transplantation. A population based study of the contributory factors. A combined survey on behalf of The Northern Ireland Regional Haematology Group (NIRHG) and The Newcastle Regional Haematology Group (NRHG)

T. C. M. Morris,[1] E. Ranaghan,[1] G. H. Jackson[2] & M. R. Velangi[2]
[1]The Northern Ireland Regional Haematology Group (NIRHG); [2]The Newcastle Regional Haematology Group (NRHG)

Although autologous transplantation is recognized as a standard of care for younger patients with myeloma, clinical trials do not represent fully the spectrum of patients who present with myeloma in this age group. The aim of this survey was to identify the number of patients eligible by age, who had failed to receive a transplant, the reason for this, what proportion of these patients might in different circumstances have received transplantation and to identify if there was unmet need for transplantation. The survey was carried out between January 2000 and December 2001. Of 418 patients with myeloma, 131 were aged <65 years (31.3%) and 43% of these were transplanted leaving a total of 75 patients in the survey. The main reasons for non-transplantation were co-morbid medical conditions 30%, early death 15%, patient decision 11%, refractory disease 12% and failed mobilization 11%. Only a small number of patients over 65 had transplants. Comparison of transplant rates for NIRHG and NRHG were lower than the UK national average obtained from the EBMT Annual Activity Survey suggesting the reduced transplantation rate may be a further marker of the poor health status of these populations. This study highlights the need for early diagnosis and effective non-toxic therapies for induction of patients with myeloma.

194

The impact of the National Screening Program on the affected births and β-thalassaemia mutations in transfusion-dependent thalassaemic patients in Taiwan

J. P. S. Chern,[1] K. H. Lin,[2] Y. N. Su,[3] M. Y. Lu,[2] S. T. Jou,[2] D. T. Lin,[2] S. C. Wang[4] & K. S. Lin[2]
[1]Department of Medicine, Tao-Yuan General Hospital, Department of Health, Tao-Yuan; [2]Department of Pediatrics, College of Medicine, National Taiwan University and National Taiwan University Hospital, Taipei; [3]Department of Medical Genetics, National Taiwan University Hospital, Taipei; [4]Department of Pediatrics, Chang-Hua Christian Hospital, Chang-Hua, Taiwan

Taiwan is a country where the prevalence rate of β-thalassaemia carrier is at least 1.1%. To prevent thalassaemia major, the Taiwan government has initiated a National Screening Program since 1993. We are now examining the differences before and after the initiation of National Screening Program. The data of the total number of patients with thalassaemia major are obtained from the register of Taiwan Thalassemia Association. The data about the affected birth rate and the affected births per year are obtained from Bureau of Health Promotion, Taiwan. Ninety-one patients with transfusion-dependent thalassaemia treated in National Taiwan University Hospital and Tao-Yuan General Hospital were included for analysis. Of which, DNA analysis of 86 patients were recorded. There are now 361 patients with transfusion-dependent thalassaemia in Taiwan. The affected births of thalassaemia major was 15 in 1991, 10 in 1992, and 17 in 1993 respectively. And the affected births have fallen to three per year since 2000. The affected birth rate per 100 000 births was 5.59 in 1994 and 1.21 in 2002. Fourteen patients treated in our hospitals were born after the initiation of National Screening Program. DNA analysis of them revealed a new mutation [IVS-1-5 (G-C)], which came from a foreign bride form Indonesia. Otherwise, most of the mutations were common β-thalassaemia mutations in Taiwan. Despite the great success in the National Screening Program for thalassaemia, there are still few doctors failed to detect the parents at risk. Hence, postgraduate education programmes for primary care physicians, health education for general population, update management guidelines and decision support should be offered by the national register system in the future.

195

Variable recovery of the 3rd international ferritin standard 94/572 shown by UKNEQAS haematinics survey of ferritin immunoassays

M. S. Hamilton, S. Blackmore & A. Lee
UKNEQAS Haematinics, Department of Haematology, Good Hope NHS Trust

A serum pool of low ferritin was spiked with 63 and 189 μg/l of the 3rd international serum ferritin standard 94/572 (supplied by National Institute for Biological Standards and controls) and distributed to laboratories participating in the UKNEQAS Haematinics external quality assurance scheme. A total of 283 laboratories returned data from 14 method groups. The mean recovery of added standard varied from 68.5% to 157.2%. Recoveries for most methods were within the acceptable range of 90–110% but two of the larger methods had more extreme recoveries of 157.2% (Abbott Architect) and approximately 70% (Bayer immuno 1).The variable recovery of some methods may reflect differences in antibody specificity and the use of two different international standards for primary calibration (spleen and liver ferritin). Current commercially available kits for ferritin are standardized against the 1st and 2nd international standard. Despite the differences in recovery observed intermethod comparison on monthly UKNEQAS surveys generally show close intermethod agreement with the all lab trimmed mean (ALTM) The biases of the methods relative to the ALTM do not seem consistent with either the standard used for primary calibration or the recovery of the 3rd international standard. The use of a single international standard is normally recommended for primary method calibration, but the variable results obtained by this study call into question the suitability of the third international standard for this purpose.

Method	*n*	Mean % recovery 94/572	95% CI	Mean % recovery 94/572	95% CI	Standard used to calibrate
Abbott architect	38	157.2	151.8–162.7	156.8	151.5–162	Nk
Abbott axsym	18	103.8	97.5–110.1	104.7	98.6–110.9	80/602 liver
Abbott IMX	8	103.1	98.3–107.9	103.2	100.3–106.2	Nk
Autodelphia	5	108.7	93.7–136.2	117.1	101.6–146.5	Nk
BayerImmuno1	14	69.0	67.6–70.5	75.5	74.0–77	80/602 liver
Beckman access	49	106.4	104.4–108.4	109.7	108.1–111.3	80/578 spleen
BayerACS180	7	107.6	96.8–118.4	111	101.2–120.7	Nk
Bayer Centaur	63	103.9	102.2–105.5	107.7	105.8–109.6	80/602 liver
DPC Immulite 1	4	89.1	86.5–90.5	85.3	74.8–87.8	Nk
DPC Imm2000	37	96.3	93.9–98.6	96.8	94.8–98.8	80/578 spleen
Olympus 600	2	73.8	69.8–77.8	86.8	85.2–88.4	Nk
Roche E170	21	93.1	88.5–97.7.	92.4	87.8–97.0	80/602 liver
Roche Elecsys	13	87.9	80.2–95.7	89.9	85.2–94.5	Nk
Tosoh AIA	4	68.5	66.7–70.6	69.3	65.8–71.9	Nk

Samsung Bioepis Exhibit No. 1002
Page 1454

196

Evaluation of DiffMaster Octavia for use on Wright–Geimsa stained blood smears

D. M. J. Pearson

Department of Haematology, Royal Hallamshire Hospital, Sheffield, UK

Introduction: Differential count of leucocytes in peripheral blood is traditionally performed manually with a light microscope. A Swedish company, CellaVision AB, provides a computerized system with decision support. The system, DiffMaster Octavia, automatically collects and preclassifies images of blood cells on a stained blood smear, presenting them to the user in an organized manner. The system has predominantly been used in laboratories analysing May–Grunwald–Giemsa stained blood smears. Since no major study on Wright–Giemsa staining smears has been performed with the system, the Haematology Department, Royal Hallamshire Hospital performed an evaluation based on the NCCLS H20-A protocol.

Methodology: Differential counts (200 leucocytes/smear) were performed on Wright–Giemsa stained smears in duplicate for each sample, using both manual light microscopy and the DiffMaster Octavia. Two examiners analysed one slide each per sample. The evaluation assessed accuracy, imprecision and clinical sensitivity/specificity. The imprecision evaluation was calculated both as intraassay and interassay variation. Interassay variation was calculated using the duplicate samples. Intraassay variation was determined for both methods in a separate study by analysing the same slides five times each by five Biomedical Scientists.

Results: Regression analysis showed no systematic difference between the methods. Imprecision was in general lower for DiffMaster Octavia. The clinical sensitivity and specificity evaluation showed equivalent results.

Conclusions: The results demonstrated that DiffMaster Octavia contributes to a higher quality in the haematology laboratory. The system makes analysis less susceptible to its inherent subjective variation. The DiffMaster Octavia clearly has the potential to move blood film morphology into a new era. Automated classification is dependent on smear and stain quality, there is more work to be done on this, when optimized, results are excellent. It provides an excellent reference for teaching and with higher classification rates it will undoubtedly have a role in managing morphology workload of large hospitals.

197

Introduction of an integrated care pathway for neutropenic sepsis

C. Harnett,[1] N. L. Rymes,[1] S. R. Smith,[1] P. Turner[2] & D. L. Turner[1]

Departments of [1]Haematology and [2]Microbiology, Torbay Hospital, Torquay, UK

Protocol driven care for the management of neutropenic sepsis is widely practised. With the perception of increased numbers of neutropenic patients being referred directly to A + E outside of 'office hours' an integrated care pathway was developed for the management of suspected neutropenic sepsis. The aim being to improve awareness, communication and documentation for junior doctors and nurse practitioners and to provide detailed guidance in the management of suspected neutropenic sepsis. The notes of all neutropenic patients admitted as an emergency to the inpatient haemato-oncology unit were retrospectively reviewed for the 6-month period beginning January 2003. There were 38 evaluable episodes and the care pathway was used in 18 (47%). All patients were receiving myelosuppressive chemotherapy-22 of 38 were NHL, two myeloma, two AML and 12 other solid tumour patients. Twenty-five patients were 7–14 d post-chemotherapy and 20 of those (80%) had grade 4 neutropenia. Eighteen (47%) patients were referred by their GP. Patients presented out of hours in 19 (50%) cases. Fourteen patients (37%) were seen by a doctor within 30 min of presentation. Of the 33 acute neutropenic episodes seven patients (21%) received antibiotics within 60 min, nine (27%) waited 60–120 min and 17 patients waited more than 2 h. As a result of this survey we aim to further educate both patients and GPs to phone earlier and not delay their admission until after office hours. We plan develop a direct nurse to on-call team referral pathway to further reduce treatment delays with the aim of delivering the first dose of antibiotics within 1 h of arrival.

198

The use and abuse of haematology investigations in a young stroke population

F. Ronsberg,[1] S. Ross,[2] M. Greaves[1] & H. G. Watson[3]

[1]Department of Medicine and Therapeutics, University of Aberdeen; [2]Acute Stroke Unit, Aberdeen Royal Infirmary; [3]Department of Haematology, Aberdeen Royal Infirmary, Aberdeen, Scotland, UK

Triggered by awareness of inconsistency and gross overuse of haematological investigation in medical patients, we conducted a retrospective study of 92 young (<65 years) patients with acute ischaemic stroke. All were objectively diagnosed. TIA and other causes of stroke were excluded. We reviewed and compared practice on an acute stroke unit (ASU) with management in a general medical ward (GM). The populations were similar; ASU, 50 patients, 58% men, mean age 57 years and GM 42 patients, 52% men, mean age 55 years. Notably, coagulation screens were performed in 46% and 40% of cases respectively (excluding subjects on warfarin). Other uninformative tests were often requested in both ASU and GM settings such as LFTs in 96% and 90%, and CRP in 82% and 79% respectively. Testing for MPD and antiphospholipid syndrome (APS) is indicated in this population as a positive diagnosis would alter prognosis and management. While most patients had a full blood count, few had appropriate investigation of APS either in hospital or following discharge. LA testing was performed on 6% ASU and 14.4% GM cases and anti-cardiolipin in 8% and 7% of cases respectively. The mean age of those tested for APA was lower than the overall population. Inherited thrombophilia was tested for in 10% of ASU and 4.7% of GM cases. The study confirms our anecdotal observations. Coagulation screens are performed inappropriately, often leading to additional irrelevant investigations. Cases of APS are probably overlooked. Testing for heritable thrombophilia was not overused. There is a clear need to ensure that testing is carried out to detect disorders that change management and that tests that are not clinically indicated are minimized.

199

Genetic screening for haemachromatosis – a cautionary tale

J. L. Starczynski, L. Hooper, G. Pratt, N. Ali, M. Hill & C. D. Fegan

Department of Haematology, Birmingham Heartlands Hospital, Birmingham, UK

Haemachromatosis is a common autosomal recessive genetic disorder of iron metabolism. Several single nucleotide polymorphisms (SNPs) within the HFE gene have been reported which are associated with the disease. C282Y was the first SNP reported with approximately 80% of patients with hereditary Haemachromatosis worldwide being homozygous for the polymorphism. Other SNPs including H63D and S65C have also been associated with the haemachromatosis phenotype. In our laboratory we had been routinely screening patients for the two common SNPs C282Y and H63D using a multiplex PCR (Stott *et al*,

Samsung Bioepis Exhibit No. 1002
Page 1455

Clinical Chemistry 1999;45:3, 426–428). However, conflicting results with another laboratory led us to investigate this method. In our laboratory two family members appeared homozygous for H63D but another laboratory had found these individuals to be heterozygous. Subsequent sequence analysis revealed heterozygosity for H63D and in addition heterozygosity for S65C. The presence of the S65C polymorphism appears to interfere with the BbrPI restriction enzyme site thus resulting in the anomaly. To determine the prevalence of the S65C genotype and its impact on our mutliplex method we re-analysed 250 patients for H63D and S65C by restriction fragment length polymorphisms (RFLP) with bcl-1 and hinf-1 restriction enzyme digests respectively. H63D was wild type in 79% of patients with 19% showing both alleles and 2% with homozygous genotype. S65C was wild type in 95.2% of patients with 4.8% heterozygous and no homozygous patients. There were five (2%) patients that showed compound heterozygosity for both H63D and S65C. On review all of these five cases had been mistakenly genotyped as homozygous for H63D. This clearly shows that the presence of the S65C polymorphism interferes with the BbrPI restriction enzyme digest leading to anomalous reporting of the H63D genotype. This study indicates that using the multiplex method described may lead to a potential error rate of up to 2% and our current practise is to screen for all three mutations.

200

Results of bone marrow biopsy patient satisfaction survey at the Royal Bournemouth Hospital

R. Hall

Department of Haematology, Royal Bournemouth Hospital, Bournemouth, UK

The 'Safety of Medazolam sedation for Bone Marrow Biopsy (BMB)' audit, presented last year at this meeting, yielded alarming results. These prompted us to set a standard of 'no routine medazolam usage for BMB'. A patient satisfaction survey was therefore constructed to assess levels of pain experienced by patients undergoing BMB to enable other analgesic approaches to be addressed. Patients less than 65 years of age who were having a BMB under local anaesthetic were targeted to complete the questionnaire. Thirty questionnaires were completed over a 3-month period. Questions included number of patient BMBs, a pain scale for patient completion and whether additional analgesia prohibiting driving after the procedure would be acceptable to patient. The results indicated that for 80% patients this was their only BMB; 80% patients indicated some level of discomfort on the pain scale of 1–10 but only 7% indicated a pain level >5. Only 23% patients indicated that they would like further analgesia. All these patients accepted the inability to drive after such medication. We therefore propose a proforma for patient assessment prior to BMB and oral analgesia in addition to local anaesthetic in selected cases. The full questionnaire and results will be presented.

201

The use of anagrelide for control of thrombocythaemia at the regional haematology unit, Belfast City Hospital

G. M. Benson, R. J. G. Cuthbert, F. G. C. Jones, P. J. Kettle & M. F. McMullin

Department of Haematology, Belfast City Hospital, Belfast BT9 7AB, UK

Anagrelide is currently available off license for the control of elevated platelet counts caused by primary marrow pathology. We wished to review the indications, side-effects and response in patients treated at BCH for control of elevated platelet counts with anagrelide.

Method: We accessed pharmacy records from January 2000 and identified 14 patients who had the relevant data collected retrospectively.

Results: Fourteen patients (2M:12F) with a mean age 61.5 years (range 27–82). Twelve of these had essential thrombocythaemia, one had polycythaemia vera and one had a primary myeloproliferative disorder 'NOS'. All but one patient had previously been treated with hydrozyurea (HU); one continues to be treated with both anagrelide and HU, while a further young female opted to be treated solely with interferon-α. Anagrelide was used as a second line agent for failure of adequate control with HU and also for those younger patients. Side-effects included headache, palpitations, fluid retention, diarrhoea and nausea, all of which declined over time suggesting long-term tolerability. Two patients required concomitant prescribing of recombinant erythropoietin. Only one patient suffered a thrombohaemorrhagic event, which occurred on HU alone with a platelet count over 500. The range of dose to maintain a normal platelet count was from 500 mcg to 7 mg daily in divided doses, taking on average 4.5 months to achieve a platelet count less than 400.

Discussion: The MRC PT1 trial comparing anagrelide and aspirin to hydroxyurea and aspirin recently reported an increase in serious adverse events in the anagrelide arm (6 out of 400 showed progressive myelofibrosis). It was recommended that patients switch from anagrelide to HU.

Conclusion: This cohort has been maintained on anagrelide as a second line agent or due to their young age with overall good control of the platelet counts and an acceptable side-effects profile.

202

Results of an open-label study of high-dose liposomal amphotericin B (AmBisome) in the treatment of confirmed fungal infections in high-risk patients

S. Kinsey,[1] G. Jackson,[2] T. Holyoake,[3] S. McCann[4] & B. Gibson[5]

[1]*Department of Paediatric Haematology, St James' Hospital, Leeds;*
[2]*Department of Haematology, Royal Victoria Infirmary, Newcastle;*
[3]*Department of Haematology, Glasgow Royal Infirmary, Glasgow, UK;*
[4]*Department of Haematology, St James' Hospital, Dublin, Ireland;*
[5]*Department of Haematology, Royal Hospital for Sick Children, Glasgow, UK*

Invasive fungal infections remain a significant cause of morbidity and mortality in immunocompromised patients. Liposomal Amphotericin B (AmBisome) has shown response rates of around 40–60% in the treatment of invasive filamentous fungal infections. The pharmacodymnamic properties of AmBisome suggest that it may be of greater efficacy at higher doses. We conducted a single arm, open label, multicentre trial to evaluate the efficacy of a high-dose regimen of AmBisome. Patients with proven or probable invasive fungal infection, as defined by the EORTC-MSG criteria, were treated with AmBisome 10 mg/kg/d for 5 d, followed by AmBisome 3 mg/kg/d for 9 d. A complete (CR) or partial (PR) response was considered a successful outcome. Thirty-one patients have been recruited to date and we report the results from the first 17 patients. There were nine male and eight female patients, aged 8–68 years (median – 36 years). Fourteen patients had an underlying haematological malignancy, nine (53%) had relapsed disease and 11 underwent stem cell transplantation (related allogeneic – six, MUD – two, autologous – three). Ten patients had proven infection diagnosed by histology (four patients) and culture (*Aspergillus* spp-5; Rhizopus Arrhizus-1) while seven had probable disease. At 14 d, the overall response rate to treatment was 68.7% (11/16 – data unavailable for one patient). Two patients had CR (12.5%) and nine patients had PR (56.2%) to treatment. All patients treated to day 14 were continued on AmBisome. The median treatment duration was 29 d (range 2–192 d). Survival rate at 12 weeks was 58.8% (10/17) with only two fungal related deaths reported. Nephrotoxicity rate (defined as an increase in creatinine greater than two times baseline) was 13%; in line with other studies using lower doses of AmBisome.

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Samsung Bioepis Exhibit No. 1002
Page 1456

High-dose AmBisome treatment is efficacious and well tolerated in the management of proven or probable invasive fungal disease.

203

Communication with patients diagnosed with haematological malignancy. On behalf of the Regional Multiprofessional Audit Group

C. A. MaCartney,[1] M. McAfee[2] & T. C. M. Morris[1]
[1]*Department of Haematology, Belfast City Hospital;* [2]*Clinical Audit Department, Belfast City Hospital, Belfast, UK*

In 2000 a regional oncology audit was carried out to identify patients and carers perspectives of cancer services in Northern Ireland in an aim to monitor recommendations set out by the Calman-Hine report in 1995 and the Campbell report in 1996. A subsequent audit in 2002 was performed to assess patients diagnosed with haematological malignancy. The report recommended equity of care, cancer services as patient centres, patients and carers views to be paramount and performance of clinical audit to measure effectiveness of the service provided. The questionnaire had been developed for the accreditation of cancer services in the Trent region audit and was adapted to include questions on Hickman line insertion. Five main areas were covered as follows; diagnosis, treatment, follow-up, quality of information sources and carers experiences. A total of 178 outpatients attending Belfast City Hospital Trust, Ulster and Community Hospitals Trust and United Hospital Trust completed a questionnaire in an interview type setting with the help of clinical audit staff. Informed consent was obtained. The project was supported by a grant from the Regional Multiprofessional Audit Group. Satisfaction scores were calculated for each main area and identified that patients were most satisfied with the explanation they received of necessary investigations, diagnosis and treatment and the ability to contact healthcare staff when required. There was less satisfaction with referral from the general practitioner, time spent waiting at the clinic before diagnosis, privacy during surgery and radiotherapy and explanation of side-effects of surgery. The Likert scale was used to indicate how good patients felt the information they had received was. Written information on diagnosis and treatment and complementary therapies. Carers were not satisfied in over 20% of cases with the opportunity to speak to the doctors about their concerns. Comparison of the previous regional audit of oncology patients and those diagnosed with haematological malignancy revealed similar areas of strength and weakness, but overall haematology patients were more satisfied with the care they received.

204

Peripheral blood leucocyte and lymphocyte subpopulations in elderly Kuwaiti subjects

S. A. Kaaba,[1] S. Alfadhli,[1] M. H. A. Burhamah[2] & H. Al Jafar[3]
[1]*Medical Laboratory Sciences, Kuwait University, Kuwait;* [2]*Department of Paediatrics, Al Sabah Hospital, Kuwait;* [3]*Department of Haematology, Al Amiri Hospital, Kuwait*

Immune senescence and alteration in the haematological parameters are thought to accompany advancing age. This may result in altered immune surveillance and alterations in the leucocyte populations, and may be important in the development of cancers and in the morbidity and mortality from infection that occur with relative higher frequency in the elderly. In the present study, we determined the relative and absolute numbers of immunocompetent cells, including CD2, CD3, CD19, CD4, CD8, CD56, HLADR, CD45RO, and CD45RA cells in the blood of 58 elderly Kuwaiti subjects (27 females, 31 males, average age 67.8 ± 6.9 years) and compared them with values found in 127 healthy adult subjects in the same population (63 females, 64 males, average age 27.9 ± 9.2 years). Hb levels in the elderly subjects was significantly lower when compared to adult controls ($P = 0.019$), while white blood cells (WBC) and absolute lymphocyte counts were comparable in the two groups. Absolute and relative CD2 and CD45RA cells, and absolute CD3 cells were not significantly different in the two groups. However, CD4, CD19, CD56, and relative CD3 and CD8 cells were significantly lower in the elderly compared to controls ($P < 0.001$, 0.002, 0.035, 0.001 and 0.001 respectively). In contrast, HLADR and total CD45RO cells were significantly higher in the elderly ($P < 0.001$). The CD4 to CD8 ratio was significantly lower in the elderly subjects ($P = 0.002$), and CD4 cells showed progressive decline with age. The results show alteration in both the B and T lymphocyte populations in older subjects. We speculate that these changes may be linked to some diseases associated with ageing.

205

Pseudothrombocytopenia (PTCP) apparent *in vitro* in ethylene diamine tetra acetic acid (EDTA), sodium citrate and heparin anticoagulants but not by flow cytometric analysis using CD61 antibody

M. Kaleelrahman, A. G. Kulasekararaj & L. A. Parapia
Department of Haematology, Bradford Teaching Hospitals NHS Trust, Bradford, UK

In vitro platelet aggregation due to ethylene diamine tetra acetic acid (EDTA)-dependent PTCP is usually reversible when repeated in sodium citrate or heparin. We would like to report the second case of PTCP in the world in which EDTA, sodium citrate and heparin failed in preventing platelet aggregation. Platelet counts using CD61 antibody revealed a more reliable result. An 80-year-old with no past history of bleeding problems was referred for investigation of thrombocytopenia prior to an elective herniorrhaphy and transurethral resection of benign prostatic hypertrophy. He had a past history of hypertension. He was on ramipril, aspirin, frusemide and tamsulosin. His Hb and WBC were normal and the platelet count was 7000/μl in EDTA, 2000/μl in citrate and 5000/μl in heparin. A blood film showed platelet clumping and no other abnormalities. Serum electrophoresis revealed an IgG-λ paraprotein level of 6 g/l. A bone marrow aspiration and biopsy showed no increase in megakaryocytes or plasma cells. Skeletal survey was normal. Rheumatoid factor, anti-nuclear and anti-cardiolipin antibodies were negative. His CD61 platelet count using a flowcytometry was 108 000/μl. His Platelet Function Analyser closure time and clotting profiles were normal. He underwent surgery uneventfully. The proposed mechanism in PTCP is an EDTA mediated IgG and/or IgM antibody which reacts with hidden cryptic antigens on platelet glycoprotein IIb/IIIa. We have not been able to explain the phenomenon in our patient. Since the failure to recognize EDTA-dependent PTCP may result in inappropriate treatment, identification of this is important. Measurement using CD61 in this situation is essential.

206

Blood film reports: how often are they acted upon?

T. Latham
Department of Molecular and Clinical Medicine, University of Edinburgh, Edinburgh, UK

It is familiar to all haematologists that advice provided with blood films is sometimes not followed, but there is little data available on the extent of non-concordance. We present a variety of methods for

Samsung Bioepis Exhibit No. 1002
Page 1457

investigating this and the results of a local audit. Blood film reports from a 2% sample of in-patient and clinic FBC requests from a major Edinburgh teaching hospital over a 2-week period included a request to telephone to register receipt of results. A 20% sample of non-responders was studied in greater detail to investigate reasons for non-concordance. Additionally, blood film reports issued over a 1-month period suggesting that a further sample for haematinic assays be sent were followed up to see if such a sample was subsequently received. Only 20/173 (12%) of study reports were registered as having been received. There were no replies from FBC samples with normal counts. Response rates were equally poor in all hospital departments. The detailed analysis of 38 non-responders suggested that the level of non-concordance was not explained by rapid discharge or movement of patients or by non-receipt of printed results. Out of 20 suggestions for further tests, only three samples (15%) were subsequently requested, despite blood being drawn again from 12 of the patients for other tests. None of the patients had had the relevant tests performed in the previous 2 years. This study suggests that non-concordance with blood film reports is may be unexpectedly extensive and may frequently lead to clinically indicated tests not being performed. The study has prompted investigation of the presentation of results and of ward organization factors, and we suggest this is an important area for audit in other hospitals.

207

Variability in platelet dose and incremental response in haematology patients in the UK

A. Norton,[1] J. P. Kerr,[2] S. O'Donovan,[3] D. Edmondson,[3] P. Cheesley,[3] M. Stear,[2] E. J. Massey[2,3], & M. F. Murphy[1,3]

[1]John Radcliffe Hospital, Oxford; [2]United Bristol Healthcare Trust, Bristol; [3]National Blood Service, UK

Morbidity and mortality due to haemorrhage in patients with thrombocytopenia has been markedly reduced since the introduction of platelet transfusion. Practice varies considerably and the optimal dose is not known. This study aimed to provide information about the current variability in adult transfusion doses (ATD) of platelets and the response achieved in patients with bone marrow failure due to treatment or disease. Platelet concentrates from three transfusion centres processed over a 6-week period were assessed for volume, platelet concentration and dose. A total of 3326 platelet concentrates were evaluated 1474 apheresis and 1852 derived from whole blood donations. The mean (range) volume of a pack was 206 (149–332) and 277 (172–361) ml for apheresis and pooled platelets respectively. The mean platelet concentrations were 1291 (233–2461) and 1092 (512–1791) $\times 10^9$/l. The mean number of platelets per unit was 265 (45–472) and 300 (158–439) $\times 10^9$. Twenty-three patients (12M, 11F, age 19–65 years) received 75 single and six double ATD platelet transfusions: seven AML, two ALL, four myeloma, two aplastic anaemia, one CML, one MDS (RAEB), one Hodgkins disease and five NHL. For single ATD platelet transfusions the mean dose of platelets was 274 (186–370) $\times 10^9$/unit, 3.8 (1.9–7.1) $\times 10^9$/kg patient body weight. The mean platelet increment at 20–24 h post-transfusion was 14 (−6–77) $\times 10^9$/l. For double ATD platelet transfusion, the mean platelet dose was 511 (373–593) $\times 10^9$/unit, 7.7 (5.3–9.6) $\times 10^9$/kg patient body weight. These transfusions yielded a mean platelet increment of 27 (0–79) $\times 10^9$/l. This study confirms considerable variability in the transfused dose. Clinical factors, notably infection and drug therapy, affect the incremental response to platelet transfusion, making it difficult to determine the optimal dose of platelets using this parameter. A better measure of the effectiveness platelet dose is haemorrhage. An international multicentre study to address this is in progress.

208

SHOT 6th Annual Report – errors remain the predominant finding

H. Cohen, H. Jones & D. Stainsby

SHOT Steering Group, SHOT Office, Manchester Blood Centre, Manchester, UK

The 6th SHOT report again showed a preponderance of 'incorrect blood component transfused' events, an increase of 21% over the previous 12 months. Almost all hospitals participate in SHOT, but over 50% submitted no incident reports, suggesting that transfusion hazards remain under-recognized and under-reported. Two-thirds of errors occurred in clinical areas, the commonest being failure to carry out the bedside check. One-third of errors occurred in hospital laboratories; 40% of 'wrong blood' events involved multiple errors. There were 32 reports of ABO incompatible red cell transfusions resulting in the deaths of two patients and major morbidity in four. Two patients suffered major morbidity due to ABO incompatible FFP. Twenty-one patients (of whom two died) received unnecessary transfusions on the basis of wrong blood count results. Nineteen RhD negative patients (including three young females) inadvertently received RhD positive transfusions, 13/19 due to laboratory errors. Special transfusion requirements were not met in 83 patients, 60 of whom were put at risk of TA-GvHD. Failure or inability to access patients' transfusion records and lack of communication in shared care were contributory factors. SHOT made the following recommendations: (1) Resources must be available to hospitals for improvements in transfusion safety. (2) Appropriate use of blood components must be strenuously promoted. (3) Blood administration training should be implemented and competency testing developed. (4) Blood should only be prescribed by authorized clinicians. (5) Blood transfusion teaching must be included in all relevant curricula. (6) Hospital transfusion laboratory staffing must be sufficient for safe practice. (7) Electronic aids to transfusion safety should be assessed and developed at national level. (8) Policies should be developed for communicating special transfusion needs where care is shared. (9) There is a need for a national body, with relevant expertise and resource, to advise government on priorities for improvements in transfusion safety.

209

Oral vitamin K does not affect vitamin K-dependent coagulation factors in normal individuals

S. Wright,[1] G. Westland,[1] R. R. Jeffrey[2] & H. G. Watson[1]

[1]Department of Haematology, Aberdeen Royal Infirmary; [2]Department of Cardiothoracic Surgery, Aberdeen Royal Infirmary, Aberdeen, UK

Recent data suggest that use of vitamin K in the peri-operative period reduces blood losses and transfusion requirements. The only likely mechanism, which could explain these observations is that vitamin K administration leads to augmentation of the levels of vitamin K dependent factors. We tested this hypothesis in a randomized, double-blind study comparing changes in levels of factor II, VII, IX and X in healthy volunteers given either 10 mg of oral vitamin K or placebo. Twenty healthy volunteers (10 men) had samples taken for assays of factors II, VII, IX and X before and 24 h after receiving 10 mg of vitamin K (Konakion MM) or a placebo. The groups were similar in age (38.7 years, SD 2.3: 41.1 years SD 3.8 respectively) and sex (five men in each group). All assays were performed by standard one-stage procedure on an Electra 1800C coagulometer. Analysis of change in coagulation factor levels, comparing the recipients of vitamin K with placebo, by two-tailed *t*-test, showed no significant difference for any factor. In well-nourished volunteers administration of vitamin K does not augment coagulation factor levels and therefore would not be expected to

Samsung Bioepis Exhibit No. 1002
Page 1458

reduce peri-operative blood loss and transfusion requirement (Barnette *et al*, *Acta Anaesthesiol Scand* 1997;41:78; Kaplon *et al*, *J Heart Lung Transplant* 1999;18:346).

210

The incidence and aetiology of anti-c, anti-E and anti-Kell antibodies in antenatal samples across West Yorkshire from January 2000 to June 2002: time to review blood selection guidelines for transfusion to females?

R. M. James[1,2] & S. MacLennan[2]

[1]*Paediatric Haematology and Oncology, St James' University Hospital;* [2]*National Blood Service, Leeds, UK*

Strategies to reduce haemolytic disease of the newborn (HDN) have concentrated on preventing the formation of anti-D and managing pregnancies complicated by anti-D. With the success of such work, the relative importance of other antibodies implicated in HDN has increased. This study looked at the incidence of anti-c, anti-E and anti-Kell in women attending antenatal clinics across West Yorkshire. Case notes were then reviewed to ascertain the incidence of blood product transfusion prior to the discovery of the antibody, indication for transfusion if transfused, parity when the antibody was discovered, and the presence of other antibodies. Overall, there were 26 cases of anti-c, 21 cases of anti-E and 23 cases of anti-Kell, involving a total of 58 women in 10 different hospitals in the study period. Notes were located for 53 (91%) of these women, accounting for 64 of the total 70 cases. Thirty-four cases (53%) had received at least one blood product transfusion prior to the discovery of the antibody. Transfusion related to an obstetric or gynaecological problem in 24 of the 27 cases for which an indication was recorded. There was no significant difference in parity or the presence of other antibodies between the previously transfused and the non-transfused group. Although uncommon, anti-c, anti-E and anti-Kell may all cause HDN. In particular, anti-c and anti-Kell may cause severe, life-threatening HDN. The high rate of prior transfusion suggests that the antibodies were induced as a consequence of transfusion in many of the cases, and were therefore potentially preventable. The question of whether females requiring transfusion should receive Rh and Kell matched blood products has been debated previously. However, given the advances in laboratory technology and data computerization, as well as the prevailing medico-legal climate, it may well be prudent to review current practice.

211

An audit of blood issue fridge activity

P. E. Patten, L. Meaney, H. Durrant, K. Eason & C. Harrison

Department of Haematology, Guy's and St Thomas Hospital, London, UK

We recently installed an automatic system for storing cross-matched blood prior to collection (DiaMed's Blood Audit and Release System). This system allows analysis of blood collection activity in our hospital. For seven consecutive days the following activities were recorded: load (L) – cross-matched unit loaded, temporary removal (TR) – unit removed for transfusion or storage in another facility, stock return (SR) – unused unit returned and permanent removal (PR). The number of units specified for each patient was also documented. A total of 2031 activities were recorded during this period, 58% of which occurred during 06.00–18.00 Monday–Friday and 28% was error data (separate analysis). A total of 645 units were loaded, of which only 455 (70.5%) subsequently underwent TR. A further 114 units (17.7%) were SR. Of single unit TR, a median of 5.4 (range 0–15) were removed in any 6-h period and occurred equally during all time periods; whereas multiple unit TR were predominantly 06.00–18.00 Monday–Friday. SR activity (114 units) occurred almost exclusively 06.00–12.00 Monday–Friday. Average PR was 46 units/d (range 29–82) with a peak of 82 units on Thursday 06.00–12.00. 81.15% of PR activity was 06.00–12.00 Tuesday–Saturday. Overall, there is a high cross-match workload. The data suggest that a total of 47.2% of units are not transfused following cross-match, with 29.5% never even removed from the fridge. The high rates of SR and PR on mornings following elective surgical lists suggest that excess numbers of units are requested for surgery, which is confirmed by returns data. Single unit TR occurs at all times; this implies single unit transfusions are given 24 h a day despite the likelihood that many of these could be avoided during patients' sleeping hours. Data from this system should allow better rationalization and planning of cross-match requests.

212

Defective high shear platelet function among platelet donors

P. Harrison,[1] H. Segal,[1] C. Furtado,[1] S. Verjee,[2] K. Sukhu[1] & M. Murphy[2]

[1]*Oxford Haemophilia Centre and Thrombosis Unit, Churchill Hospital;* [2]*National Blood Service, John Radcliffe Hospital, Oxford, UK*

As single donor plateletpheresis concentrates now account for about 40% of platelet concentrates in the UK, the platelet quality of individual donations is becoming increasingly important. The aim of this study was to investigate the prevalence of defective platelet function among 100 healthy UK platelet donors on three consecutive donation days. High shear platelet function testing was performed by the PFA-100 within citrated blood samples taken prior to plateletpheresis. Abnormal CADP closure times (CTs)were obtained in an average of 23% of donations but with no severe abnormalities > 200 s. Abnormal CEPI CTs were obtained in an average of 32% of donations with 14% between 200 and >300 s. An average of 16% donors were presenting with prolonged CEPI CTs only, indicative of an aspirin-like pattern. Of these 9% of all donors were presenting with a severe aspirin-like defect with prolonged CEPI CTs between 200 and 300 s and with the majority appearing transient in nature. An average of 4% of all donors were presenting with maximal CEPI CTs of >300 s. Platelet dysfunction as detected by the PFA-100 is surprisingly common within a voluntary platelet donor population. The majority of CEPI defects appeared transient in nature and are indicative of surreptitious intake of cyclooxygenase inhibitors (e.g. aspirin). Identification of defective platelet function within donors provides a simple means to eradicate defective platelets from being clinically utilized.

Samsung Bioepis Exhibit No. 1002
Page 1459

Oral Communication

Presidents Free Communications

213

Ximelagatran, an oral direct thrombin inhibitor, compared with current standard therapy for acute, symptomatic deep vein thrombosis, with or without pulmonary embolism: the THRIVE Treatment Study

J. Wimperis,[1] J.-N. Fiessinger,[2] M. V. Huisman,[3] B. L. Davidson,[4] H. Bounameaux,[5] C. W. Francis,[6] H. Eriksson,[7] T. Lundström,[8] S. D. Berkowitz,[9] P. Nyström[8] & J. S. Ginsberg[10]

[1]Department of Haematology, Norfolk & Norwich Hospital, Norwich, UK; [2]Department of Vascular Medicine, Hôpital Européen Georges Pompidou, Paris, France; [3]Department of General Internal Medicine, Leiden University Medical Centre, Leiden, The Netherlands; [4]Swedish Medical Centre and University of Washington School of Medicine, Seattle, WA, USA; [5]Department of Internal Medicine, University Hospital of Geneva, Geneva, Switzerland; [6]Department of Medicine, University of Rochester, Rochester, NY, USA; [7]Department of Medicine Sahgrenska University Hospital/Östra, Göteborg, Sweden; [8]AstraZeneca R&D, Mölndal, Sweden; [9]Clinical Development AstraZeneca LP, Wilmington, DE, USA; [10]Department of Medicine, McMaster University, Hamilton, ON, Canada

Ximelagatran (Exanta™, AstraZeneca), an oral direct thrombin inhibitor, has a rapid onset of action and predictable pharmacokinetics without food or alcohol interactions and a low potential for drug interactions. In this international, randomized, double-blind, double-dummy trial, 2489 patients with acute deep vein thrombosis (DVT), of whom 37% had locally confirmed pulmonary embolism (PE), received oral ximelagatran 36 mg bid for 6 months or s.c. enoxaparin 1 mg/kg bid for a minimum of 5 d followed by warfarin (target INR 2.0–3.0) for 6 months. The aim was to determine whether ximelagatran is non-inferior to enoxaparin/warfarin in preventing recurrent venous thromboembolism (VTE), by comparing Kaplan–Meier estimates of the cumulative risk of an event at 6 months. At baseline, bilateral compression leg ultrasonography and ventilation-perfusion lung scanning were performed. An independent committee adjudicated all VTE recurrences, bleeding events and mortality. Ximelagatran was administered to 1240 patients and enoxaparin/warfarin to 1249. Demographics of both groups were similar (mean age, 57 ± 16 years; 53% men). Comparison of the outcome of clinical endpoints between the two treatment groups demonstrated non-inferiority of ximelagatran versus enoxaparin/warfarin (table). Incidence of serum alanine aminotransferase elevations (>3 times the upper limit of normal) was 9.6% and 2.0% for patients receiving ximelagatran and enoxaparin/warfarin respectively. During 6 months of treatment, oral ximelagatran, administered in fixed doses of 36 mg bid without coagulation monitoring, was non-inferior to enoxaparin/warfarin in preventing recurrent VTE in patients with acute DVT with or without PE. Ximelagatran was associated with a favourable outcome with respect to major bleeding.

Event	Ximelagatran, *n* (%*)	Enoxaparin/warfarin, *n* (%*)	Absolute difference ximelagatran–enoxaparin/warfarin %* (95% CI)
Recurrent VTE (ITT)	26 (2.1)	24 (2.0)	+0.2** (−1.0; +1.3)
Recurrent VTE (OT)	23 (2.0)	17 (1.5)	+0.5 (−0.6; +1.6)
Major bleeding (OT)	14 (1.3)	26 (2.2)	−1.0 (−2.1; +0.1)
Recurrent VTE and/or major bleeding (OT)	37 (3.2)	43 (3.7)	−0.5 (−2.0; +1.0)
All-cause mortality (ITT)	28 (2.3)	42 (3.4)	−1.1 (−2.4; +0.2)
All-cause mortality (OT)	7 (0.7)	10 (0.9)	−0.2 (−1.0; +0.5)

*Estimated cumulative risk; **rounded figure. CI, confidence interval; ITT, intention-to-treat; OT, on-treatment.

214

Polycythaemia vera and pregnancy: a review of treatment and outcome of 16 pregnancies in 8 women

S. E. Robinson,[1] D. Radia,[1] M. Pocock,[2] J. Duncan,[3] A. Mumford,[4] S. Machin,[5] S. Bewley,[6] B. J. Hunt[1] & C. Harrison[1]

[1]Department of Haematology, Guy's and St Thomas Trust, London,[2]Department of Haematology, Royal Devon and Exeter Hospital, Exeter; [3]Department of Haematology, Royal Surrey Hospital, Brighton; [4]Department of Haematology, Bristol Royal Infirmary, Bristol; [5]Department of Haematology, University College London Hospital; [6]Womens Health, Guy's and St Thomas Trust, London, UK

Polycythaemia vera (PV) is a rare condition in women of childbearing age, the literature to date comprises only 20 case reports of pregnancies. We report 16 pregnancy outcomes in eight women, age 17–38 years, who had PV during pregnancy. The pregnancies were divided into standard antenatal care (group A) or intervention relating to their PV (aspirin 75 mg, ±LMWH, vitamin C and E, with uterine artery Doppler assessment) group B. In group A there was only 1/7 live births; this was an elective delivery at 34/40 due to placental insufficiency and foetal distress. First trimester miscarriage occurred in three (42%), The remaining three (42%) resulted in still-birth, at 25 and 29 weeks. Two were complicated by intrauterine growth retardation (IUGR) and oligohydramnios with placental infarcts in one. The third was a twin pregnancy also affected by IUGR, twin 1 was dead on ultrasound scanning and twin 2 died within 24 h of caesarean section. All nine patients in group B received aspirin; four had venesection to attain packed cell volume (PCV) < 0.45 and four received prophylactic LMW heparin (two antenatally). There were eight live births (89%), seven at term (78%), and one first trimester miscarriage (11%). Pregnancies with PV are high-risk with increased incidence of first trimester miscarriage, stillbirth, IUGR and premature delivery. Although maternal outcome is good, foetal outcome may be poor. There was no correlation between PCV, platelet count or results of thrombophilia screening and pregnancy outcome. Patients who received aspirin 75 mg, regular monitoring ± LMW heparin had a good outcome with 89% live births compared with 14% in the standard antenatal care group. A prospective study is required to develop treatment further and substantiate initial findings.

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Samsung Bioepis Exhibit No. 1002
Page 1460

215

Genomic breakpoint cloning and a functional assay of DNA topoisomerase II cleavage reveal translocation mechanism underlying formation of t(15;17) in therapy-related acute promyelocytic leukaemia

A. R. Mistry[1,2], C. A. Felix,[2] R. Whitmarsh,[2] A. Mason,[1] A. Reiter,[3] B. Cassinat,[4] A. Parry,[4] C. Walz,[3] J. L. Wiemels,[5] M. Segal,[5] L. Ades,[6] I. A. Blair,[7] N. Osheroff,[8] T. J. Littlewood,[9] M. Lafage-Pochitaloff,[10] N. C. P. Cross,[11] C. Chomienne,[4] E. Solomon,[1] P. Fenaux[5] & D. Grimwade[1,12]

[1]Department of Medical and Molecular Genetics, GKT School of Medicine, London, UK; [2]Children's Hospital of Philadelphia, PA, USA; [3]III. Medizinische Universitätsklinik, Mannheim, Germany; [4]Hôpital St Louis, Paris, France; [5]University of California, San Francisco, CA, USA; [6]Hôpital Avicenne, Bobigny, France; [7]University of Pennsylvania, Philadelphia, PA, USA; [8]Vanderbilt University, Nashville, TN, USA; [9]John Radcliffe Hospital, Oxford, UK; [10]Institut Paoli-Calmettes, Marseille, France; [11]Wessex Regional Genetics Laboratory, Sailsbury, UK; [12]Department of Haematology, University College London Hospitals, London, UK

Mechanisms underlying formation of chromosomal translocations that represent a critical step in leukaemogenesis through the generation of oncogenic chimaeric fusion proteins, are poorly understood. However, a significant proportion of AML cases arise following exposure to chemotherapeutic agents targeting DNA topoisomerase II, e.g. etoposide and mitoxantrone. For acute promyelocytic leukaemia (APL), which is characterized by the t(15;17)(q22;q12-21) generating the PML-RARα oncoprotein, recent studies have highlighted an increase in secondary cases following exposure to mitoxantrone for treatment of prior breast carcinoma. Drugs targeting DNA topoisomerase II act by damaging DNA by disrupting the cleavage-religation equilibrium, increasing the concentration of DNA topoisomerase II covalent complexes; however, their precise role in the pathogenesis of secondary leukaemias has not been firmly established. We characterized the genomic breakpoint junctions in five cases of therapy-related APL (t-APL). Induction of DNA topoisomerase II cleavage complexes by various chemotherapeutic DNA topoisomerase II inhibitors was examined in the normal homologues of the *PML* and *RARA* breakpoint sequences using an *in vitro* assay that entails trapping of the cleavage complexes and mapping of the cleavage sites at the sequence level. All four cases of mitoxantrone-related t-APL occurring following breast carcinoma treatment were found to have genomic breakpoints clustered within a 7-bp region of *PML* intron 6, consistent with a damage hotspot and corresponding precisely to a functional DNA topoisomerase II cleavage site induced by mitoxantrone. Functional DNA cleavage sites induced by etoposide and its metabolites were also identified at *PML* and *RARA* breakpoint junctions in a further case of t-APL arising following etoposide. Analysis of breakpoint junctions revealed regions of micro-homology and minimal processing, consistent with involvement of the non-homologous end-joining (NHEJ) pathway. This study directly implicates DNA topoisomerase II in mediating DNA cleavage at translocation breakpoints in t-APL, with the t(15;17) being generated through aberrant repair mediated by the NHEJ pathway.

216

T-cell-mediated colitis and bone destruction in a murine model of autoimmunity are both driven by RANK/RANK LIGAND interactions and modulated by recombinant Fc-OPG

A. J. Ashcroft,[1] S. M. Cruickshank,[2] P. I. Croucher,[3] M. J. Perry,[4] S. Rollinson,[1] J. M. Lippitt,[3] J. A. Child,[5] C. R. Dunstan,[6] S. R. Carding[2] & G. J. Morgan[1]

[1]Academic Unit of Haematology and Oncology, School of Medicine, University of Leeds, Leeds; [2]University of Leeds, Leeds; [3]University of Sheffield, Sheffield; [4]University of Bristol, Bristol; [5]United Leeds Teaching Hospitals, Leeds, UK; [6]Amgen Inc., Thousand Oaks, CA, USA. Present address: ANZAC Research Institute, Concord, Australia

Deregulation and hyper-activation of T cells is linked to bone destruction in auto-immune and malignant diseases such as ATLL due to the expression of RANKL. We have studied Interleukin 2-deficient (IL2−/−) mouse model of autoimmunity to assess the role of RANKL in bone and other organs targeted by T-cell mediated inflammation. IL2−/− mice spontaneously develop a haematopoietic disorder characterized by lymphoid hyperplasia with an infiltrate of activated T cells in bone, spleen, lymph nodes, and particularly the gut, resulting in severe colitis. Bone physiology was evaluated in IL2−/− mice and controls at 4, 7, and 9 weeks of age corresponding to; no clinical disease, moderate, and severe disease respectively. Femora and tibia were assessed by X-ray, densitometry, dynamic and static histomorphometry, and TRAP staining. Bone-marrow mRNA was assessed by Taqman RT-PCR for RANK/RANKL/OPG expression and serum for RANKL/OPG by ELISA. Purified (>93%) IL2−/− T cells were adoptively transferred into Rag1−/− mice to assess reproducibility of bone disease. An IL2−/−/TCR β−/− hybrid animal was generated to assess if IL2 acted on bone solely via CD4 T cells. Once bone disease in IL2−/− mice was described, animals were treated, alongside controls, with Fc-OPG.

Results: IL2−/− mice develop significant osteopenia between 4 and 9 weeks of age ($P < 0.001$); have abnormal bone formation ($P < 0.01$), have raised osteoclast numbers ($P = 0.041$), OPG/RANK/RANKL mRNA levels, and serum RANKL ($P = 0.007$). Bone disease and colitis can be transferred to Rag1−/− mice using IL2−/− T cells ($P = 0.001$), IL2−/−/TCR−/− mice are disease free. Fc-OPG reverses bone disease evident in IL2−/− mice ($P < 0.001$) and prevents colitis. This anti-inflammatory effect is associated with loss of colonic dendritic cells.

Conclusion: Our results demonstrate that activated T cells induce bone disease and colitis in the IL2−/− mouse, the methods by which pathology is produced, and an effective treatment. We also demonstrate a novel anti-inflammatory role of osteoprotegerin and its mechanism of action (Fc-OPG kindly supplied by Amgen Inc.).

217

Diagnosis and outcome prediction for childhood acute leukaemia by gene expression profiling

F. W. van Delft,[1] T. Bellotti,[2] L. K. Jones,[1] N. Patel,[1] I. Hann,[3] M. Hubank,[2] J. Sargent,[4] S. Daw,[5] A. Gammerman,[2] V. Saha,[1] V. Chauhan,[1] S. Senderovich,[1] H. Kempski,[3] D. Fletcher[3] & C. Taylor[4]

[1]CR-UK, Children's Cancer Group, Royal London Hospital, London; [2]Royal Holloway University, Department of Computer Sciences, Egham, Surrey; [3]Great Ormond Street, Institute of Child Health, Guilford Street, London; [4]Haematology Research Department, Pembury Hospital, Pembury, Kent; [5]University College London Hospital, London, UK

Successful treatment of patients with leukaemia depends on early identification of patients at risk for disease relapse. Risk stratification has achieved this. Although the majority of patients are cured, the

Samsung Bioepis Exhibit No. 1002
Page 1461

mechanism of relapse is poorly understood. Drug resistance plays a major role in recurrence of disease and is predictive for outcome. To address disease relapse and to develop more accurate stratification, we have prospectively analysed the gene expression pattern of leukaemic blasts with the use of oligonucleotide microarrays for classification purposes and to unravel genes involved in drug resistance. Children diagnosed with acute leukaemia at the Royal London, GOS and UCL Hospitals have been included in this study from February 2002 onwards. Ethical approval and written consent were obtained. The expression profile of diagnostic leukaemic cells was determined using oligonucleotide microarrays (U133A, Affymetrix). Data was analysed with Micro Array Suite 5.0. Concurrently, chemosensitivity was determined in these blasts, using the ATP assay. The LC50 for all drugs used was calculated. We classified patients as either sensitive or resistant. We used GeneSpring 5.0.3 to analyse the microarray data. Statistical group comparison discriminated expression profiles between patients with ALL and AML. Disease phenotype was predicted in three patients with unclassified leukaemia. Similarly, we have defined molecular signatures for subtypes of ALL, e.g. hyperdiploidy, TEL-AML1, T-ALL and E2A-PBX1. Next we correlated expression data with drug chemosensitivity profiles in order to define outcome predictors. Gene sets were identified that are predictive for sensitivity to Dexamethasone, Vincristine, and Asparaginase. This kind of analysis also provides us with an insight into the mechanism of drug resistance, which has the potential to identify new drug targets. In conclusion, we find expression analysis an accurate diagnostic tool in classification of childhood acute leukaemia. Correlation with chemosensitivity and treatment response promises to add a prognostic feature to this technology.

218

Haploidentical transplantation – a single centre experience

J. Motwani, D. Chandra, S. Kirk, P. J. Darbyshire & S. E. Lawson
Department of Haematology, Birmingham Children's Hospital, Birmingham, UK

In children without matched related or unrelated donors, haploidentical family members are the only source of stem cell graft. However, haploidentical haematopoietic stem cell transplantation (HSCT) is usually associated with poorer outcomes. We present 35 consecutively treated paediatric patients (19 males, 16 females) using a fludarabine-based regimen consisting of cyclophosphamide, anti-lymphocyte globulin and busulphan or total body irradiation. They received mobilized peripheral blood stem cells (PBSC) haploidentical stem cell grafts from parental (31) or sibling (three) donors. Twenty-eight children underwent HSCT for underlying malignant diseases [relapsed ALL (14), relapsed AML (five), JMML (two), MDS (three), Haemophagocytic lymphohistiocytosis (HLH) (three), biphenotypic leukaemia (one)] and seven for underlying non-malignant diseases [Fanconi anaemia (three), primary immunodeficiencies (two), osteopetrosis (one), adrenoleukodystrophy (one)]. The median follow up for this patient group was 17 months (1–55). We observed engraftment in 100% patients with rejection in 2.8%. Acute graft-versus-host disease (GvHD) was observed in 30% patients but only two children needed treatment. Of the 33 evaluable patients, 100 d survival was observed in 30 (90%). Opportunistic infections were seen in 43% patients, of which 26% succumbed to their infections. The infections were CMV (six), HSV (one), RSV (two), EBV (one), Mycobacterium kansasii (one), HZV (two), Aspergillosis (three), Adenovirus (one), Parainfluenzae type 3 (one). The latter four patients died. At the time of last follow up, 60% patients were alive, well and disease free. We observed 14 deaths: eight (relapse or disease progression), one (VOD), one (radiation toxicity) and four (infection related). We conclude that using fludarabine-based conditioning regimen and CD34-selected PBSC graft, engraftment is achievable in majority of children receiving haploidentical donors, with low incidence of severe GvHD. Infection-related deaths were relatively low and exclusively in malignant diseases. Relapse remains the major cause of treatment failure and novel approaches like donor lymphocyte infusions to prevent disease recurrence are urgently needed.

Oral Communication
Haematological Malignancies

219

Circulating BCR-ABL specific CD8$^+$ cells are detectable in chronic myeloid leukaemia patients and healthy subjects

N. M. Butt,[1] L. Wang,[1] S. E. Christmas,[2] H. Abu-Eisha[2] & R. E. Clark[1]
[1]*Department of Haematology, University of Liverpool;* [2]*Department of Immunology, University of Liverpool, Liverpool, UK*

The fusion oncoprotein BCR-ABL that characterizes chronic myeloid leukaemia (CML) is a leukaemia-specific neoantigen, which may be immunogenic *in vivo*. KQSSKALQR and GFKQSSKAL peptide sequences spanning the b3a2 BCR-ABL junction have binding affinity for HLA-A3 and B8 respectively, and we have shown the presence of KQSSKALQR on the CML cell surface. Here we investigate circulating BCR-ABL specific CD8 positive T cells directed against the BCR-ABL junction using fluorochrome-labelled tetramers of HLA-A3 with KQSSKALQR and of HLA-B8 with GFKQSSKAL. Three groups consisting of HLA-A3 and/or HLA- B8 positive healthy donors, CML patients with the relevant HLA type and the b3a2 transcript and a negative control group that did not possess the relevant HLA types and/or transcript were studied. Fresh peripheral blood mononuclear cells were isolated from subjects and stained with the relevant tetramer and anti-CD8 and assessed by FACS analysis. Eight of 12 CML patients studied serially on at least three occasions had BCR-ABL junction specific CD8$^+$ T cells. Specific T cells were more likely to be found in patients with a low leukaemic burden as assessed by quantitative real time PCR ($P = 0.03$). Three of 18 healthy donors had BCR-ABL junction specific T cells. No specific T cells were detected in any of the negative control subjects. An in-vitro dendritic cell protocol was used to expand BCR-ABL specific CTL and assessed by radiochromium release and tetramer. Radiochromium release confirmed specific CTL expansion in two of seven healthy donors and one of six CML patients whilst tetramer assessment demonstrated specific CD8$^+$ T cells in three of seven and two of six subjects respectively. Failure to detect these tetramer positive cells by radiochromium release may represent the lower sensitivity of this assay or a genuine dysfunctional CD8$^+$ T-cell phenotype. The occurrence of specific T cells in some healthy donors might represent an immune response to occult BCR-ABL rearrangements.

220

Rapid and quantitative detection of mutations of the FLT3 gene in AML

V. Walsh,[1] A. F. Gilkes,[1] M. Sweeney,[1] S. J. Austin[1] R. E. Gale[2] & K. I. Mills[1]
[1]*Department of Haematology, Cardiff, Wales;* [2]*Department of Haematology, University College Hospital, London, UK*

The most common single mutation observed in approximately one-third of AML cases is an activating mutation of the tyrosine kinase receptor FLT3. The presence of the mutation is associated with adverse prognosis in terms of increased risk of disease relapse. The majority of mutations (25–35% of AML patients) occur as an internal tandem

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Samsung Bioepis Exhibit No. 1002
Page 1462

duplication (ITD) within the juxtamembrane region. A further 3–7% of patients have a mutation within the tyrosine kinase domain (TKD) of FLT3. The identification of the type of mutation, and population size, may have prognostic implications. We have used the Agilent 2100 Bioanalyser to assay PCR reactions for either the ITD or the TKD mutations. The 'chips' used within the Bioanalyser separate RNA or DNA through glass etched micro channels filled with gel matrix, nucleic acid is stained and laser detected. The PCR primers and protocol for the ITD analysis were as previously published (Kottaridis *et al*, *Blood* 2001;98), but within our laboratory the reactions were analysed on the Bioanalyser using a DNA 500 chip. We detected the presence of an ITD in 27% of the 125 AML samples, with the clone population varying from 1.1% to 98%. A separate PCR reaction using primers flanking the TKD domain was used, in combination with an ECO RV or Hinf I (Smith *et al*, MTAL Workshop, 2003) digestion, to detect and quantitate the different mutations. Distinctive digestion and/or heteroduplex patterns were observed that identified D835, D835del, I836del or D839G mutation. In total, TKD mutations were observed in 16% (13.6% D835 mutations, 2.4% I836del, 0% D839G) of samples examined, with clone population from 3.9% to 46.3%. The D835/6 mutation rate was higher than previously reported and may be due to the efficiency and sensitivity of the 2100 BioAnalyser for the analysis of FLT3 mutations.

221

Expression of AML1-ETO in human CD34$^+$ cells blocks granulocytic differentiation and increases self-renewal

A. Tonks,[1] L. Pearn,[1] A. J. Tonks,[2] T. Hoy,[1] J. Fisher,[1] A. K. Burnett[1] & R. L. Darley[1]

[1]Department of Haematology, UWCM, Cardiff; [2]Department of Medical Microbiology, UWCM, UK

The t(8;21) translocation that encodes the AML1-ETO fusion protein (aka RUNX1-CBF2T1), is one of the most frequent translocations in AML. Although its role in leukaemogenesis is unclear, the fusion gene is believed to dysregulate the expression of genes that are important for normal differentiation and proliferation of haemopoeitic progenitors. Here, we describe the effect of AML1-ETO on granulocyte and monocyte development. Expression of AML1-ETO in human CD34$^+$ cells was achieved by retroviral infection of these cells using a vector co-expressing GFP. The effect of AML1-ETO on myeloid growth and development was initially studied in bulk liquid culture, in the presence of IL-3, SCF and G/GM-CSF. Exogenous expression of AML1-ETO in these cells had an initial inhibitory effect on myeloid growth ($P < 0.01$). Despite this, AML1-ETO expressing cells continued to proliferate >80 d, resulting in a 5×10^3-fold expansion over that observed for control cells (which stopped growing around day 28). In addition, expression of AML1-ETO was able to suppress granulocytic colony formation by 45% when compared to control cultures. In contrast, there was no observed decrease in monocytic colony forming efficiency in cells expressing AML1-ETO. Morphologically, granulocyte terminal differentiation in AML1-ETO expressing cells was inhibited by 83% ($P < 0.01$) giving rise to a build-up of early to intermediate granulocytes, which maintained this appearance throughout the prolonged period of expansion. Phenotypically, these cells were GFP$^+$, CD13$^+$, M-CSF-R$^-$ and showed developmental irregularities such as the failure to lose expression of CD34$^+$ or significantly upregulate CD11b in the same manner as control cells ($P < 0.01$). However, there was no significant difference in CD14 expression in monocytic cells expressing AML1-ETO (GFP$^+$, CD13$^+$, M-CSF-R$^+$). Taken together, these results suggest that expression of AML1-ETO suppressed the differentiation of granulocytic cells and allowed extensive self-renewal to take place supporting a causal role for t(8;21) translocations in leukaemogenesis.

222

The relationship between *FLT3* mutation status, biological characteristics and outcome in patients with acute promyelocytic leukaemia

D. Grimwade[1,2], R. Gale,[2] R. Hills,[3] P. Kottaridis,[2] D. Swirsky,[4] A. Gilkes,[5] E. Nugent,[1] K. Mills,[5] K. Wheatley,[3] E. Solomon,[1] A. K. Burnett[5] & D. C. Linch[2], on behalf of the NCRI Adult Leukaemia Working Party, UK

[1]Department of Medical and Molecular Genetics, GKT School of Medicine, London; [2]Department of Haematology, University College London Hospitals, London; [3]Clinical Trials Unit, University of Birmingham, Birmingham; [4]HMDS, Leeds; [5]Department of Haematology, University of Wales, Cardiff, UK

The majority of cases of acute promyelocytic leukaemia (APL) are characterized by the t(15;17) leading to formation of the PML-RARα fusion protein. Evidence to date suggests that PML-RARα is insufficient for leukaemogenesis, although the precise nature of cooperating events implicated in generating the full disease phenotype remain uncertain. Potential candidates include activating mutations of FLT3, which could confer a survival and proliferative advantage thereby complementing the differentiation block induced by PML-RARα. The aetiological and clinical significance of *FLT3* mutations in APL is not firmly established. We studied 157 cases of *PML-RARA* positive APL (median age 37 years) treated with ATRA and anthracycline-based chemotherapy in the MRC AML 10/12 trials. *FLT3* mutation was detected in 75/157 cases (48%): 60 length mutations (ITDs), 18 activation loop mutations (D835/I836), including three cases with ITD+D835. Presence of ITDs was correlated with M3v subtype, bcr 3 *PML* breakpoint and expression of reciprocal *RARA-PML* transcripts. The latter association could indicate that expression of the reciprocal fusion product, which has been proposed to contribute to genomic instability predisposes to FLT3 ITD formation, or alternatively FLT3 ITD and t(15;17) could arise through common mechanisms. Microarray analysis using Affymetrix U133A GeneChips undertaken in 25 cases revealed distinct gene expression profiles between cases with ITDs, D835 and wild-type FLT3, suggesting differences in downstream targets according to the nature of the FLT3 mutation. There was a trend towards poorer prognosis in APL with FLT3 mutations (57% survival at 5 years versus 69% wild type, $P = 0.08$), reflecting a higher incidence of induction death (21% vs. 8%, $P = 0.03$); although there was no evidence of effect for *FLT3* mutation status on outcome after stratification for WBC. Nevertheless, the high frequency of activating mutations of FLT3 in cases with WBC above 10×10^9/l (40/51, 78%) provides a rationale for future evaluation of FLT3 inhibitors as a component of induction therapy particularly in this higher risk group of patients.

223

Haemorrhagic cystitis and BK virus: association and treatment

C. L. Wong, C. Williams, R. Chopra & K. Mutton

Adult Leukaemia Unit, Christie Hospital, Manchester, UK

Objectives: To determine the association between BK virus and haemorrhagic cystitis (HC) and to ascertain the effectiveness of Cidofovir in the treatment of BK-associated HC.

Method: We conducted a prospective study of 44 patients undergoing chemotherapy, PBSCT and BMT. The association between HC and BK virus was studied by monitoring the urinary excretion of BK virus using electron microscopy and polymerase chain reaction. Urine dipstick analysis was performed daily on all patients.

Samsung Bioepis Exhibit No. 1002
Page 1463

Result: HC occurred in eight of 44 (18.1%) patients (seven had allogeneic PBSCT/BMT and one had chemotherapy). BK viruria (>1 positive sample) was detected in all the eight (100%) patients with HC and in six (16.7%) of the remaining 36 patients without HC. BK viruria was detected in 14 of 44 (31.8%) patients undergoing various treatments. Eight of 14 (57%) developed HC whilst none of the 30 BK-negative patients developed this complication. The occurrence of HC and BK viruria was related to the source of the PBSC/BM ($P < 0.0123$). HC occurred in seven of 17 (41.2%) allogeneic PBSCT/BM recipients whilst none of the eight autologous PBSCT recipients developed HC. Among the 17 recipients of allogeneic PBSCT/BMT, BK viruria was detected in 12 (70.6%) patients. Seven of the 12 (58.3%) patients developed HC. Occurrence of BK viruria (but not HC) was also shown to be related to the primary disease ($P = 0.0046$). However, the incidence of HC and BK viruria were unrelated to treatment regimes and occurrence of graft-versus-host-disease. The association between HC and BK was shown to be statistically significant ($P < 0.0001$). Treatment of four of the eight patients with HC associated with BK viruria with Cidofovir resulted in resolution of HC and a reduction in the BK virus load. None of these patients demonstrated any side-effects to Cidofovir.

Conclusion: We conclude that there is a significant association between BK virus and HC. In the event of failed conservative measures, Cidofovir may be used to treat BK-associated HC. However, patients should be monitored closely for side effects to this drug.

224

Sodium valproate, when combined with all-trans retinoic acid, induces apoptosis and histone acetylation in primary blasts from patients with acute myeloid leukaemia (AML)

C. Bradbury,[1] C. Craddock[1] L. O'Neill[2] M. Drayson,[1] B. Turner[2] & C. Bunce[3]

[1]Department of Haematology, University Hospital NHS Trust; [2]Division of Immunity and Infection, University of Birmingham; [3]School of Biosciences, University of Birmingham, Birmingham, UK

There is increasing evidence that the abnormal patterns of gene expression documented in AML can be associated with perturbations in chromatin modelling, raising the possibility that modulators of chromatin structure may be of therapeutic benefit. We and others have examined the potential use of sodium valproate (SV), a potent histone deacetylase inhibitor, in AML. Although SV is able to enhance the response of AML blasts to ATRA *in vitro* previous studies have not correlated the cellular response with changes in histone acetylation and methylation. Blasts from eight patients with AML were exposed to SV (1 mmol/l) and ATRA (100 nmol/l) at levels of both drugs, which are achievable clinically. Treatment with either drug alone had little impact on survival or differentiation of primary AML blasts. In contrast combined therapy with both SV and ATRA resulted in a marked reduction in the numbers of surviving cells together with an increased proportion of cells in the sub-G1 gate on cell cycle analysis, indicative of induced apoptosis. Immunophenotypic analysis demonstrated that myeloid differentiation markers were induced in cells surviving exposure to both agents for 5 d. Histone acetylation was studied after electrophoretic separation of histones and immunostaining with antibodies for specific histone acetylation events. Treatment with 1 mmol/l SV was associated with increased acetylation, which was detectable by 1 h and sustained for 18 h. Concurrent changes in histone methylation were observed. Treatment with ATRA did not alter histone acetylation patterns when used either alone or in combination with SV. In summary, our results confirm that combined SV and ATRA treatment of AML blasts results in both reduced cell survival and a shift towards a more mature postmitotic phenotype. These events are associated with SV mediated alterations in patterns of histone acetylation. These data provide the basis for an ongoing Phase II trial of combined SV and ATRA therapy in patients with relapsed AML.

Oral Communication

Cellular & Molecular Biology

225

Epigenetic inheritance and leukaemogenesis: lessons from the M-CSF receptor gene

G. A. Follows[1,2], H. Tagoh,[1] P. Lefevre,[1] S. Melnik,[1] S. Richards,[2] G. J. Morgan[2] & C. Bonifer[1]

[1]Molecular Medicine Unit, Leeds University; [2]Academic Unit of Haematology and Oncology, Leeds General Infirmary, Leeds, UK

Complex epigenetic mechanisms direct the inheritance of information from mother to daughter cells, which consequently determines specific patterns of gene expression. It is now appreciated that deregulation of these mechanisms is a recurring feature in the establishment and maintenance of oncogenic transformation. We have studied epigenetic aspects of the regulation of the human M-CSF receptor gene (c-FMS), as it is a target for AML1 and AML1-ETO. We have shown that the promoter and elements within the second intron (FIRE) bind transcription factors *in vivo* and macrophage differentiation leads to increased DNaseI hypersensitivity over FIRE. Using chromatin immunoprecipitation assays we have shown that in t(8;21) cells, the two elements within the second intron, FIRE and FIRE-1kb, are targets for AML1/ETO, which closely follows HDAC1 recruitment. Leukaemic t(8;21) cell lines express low levels of c-FMS mRNA, yet displayed strong DNaseI hypersensitivity over the intronic elements. We showed that AML1-ETO repressed FIRE activity, and this associated with increased methylation and decreased acetylation of histone H3 lysine 9 at key regulatory elements. DMS *in vivo* footprinting experiments of c-FMS cis-regulatory elements with leukaemia patient samples showed that CD34+ blast cells had transcription factor complexes bound at FIRE independent of the presence of t(8;21). This did not induce DNA cytosine methylation, as the presence of AML1-ETO did not associate with increased CpG methylation at any c-FMS regulatory element. Despite having a bound complement of transcription factors resembling highly expressing macrophages, the leukaemic cells, however, showed DNA topology more closely resembling non-expressing Hela cells, as evidenced by *in vivo* UV footprinting. It appears, therefore, that binding AML1-ETO to the c-FMS second intron leads to repression of gene expression through 'active' repression rather than assembly of the gene into epigenetically silent heterochromatin. We postulate that this may contribute to the monocytic differentiation block observed in these leukaemias.

226

Inhibition of the internal tandem duplicated FLT3 receptor by tyrosine kinase inhibitor PKC412 alters DNA repair kinetics and down-regulates DNA repair enzymes, RAD51, DNA PK, PARP, and MSH2

H. M. Hunter,[1] C. H. Seedhouse,[2] M. Pallis[1] & N. H. Russell[1,2]

[1]Nottingham City Hospital NHS Trust; [2]Nottingham University, Nottingham, UK

The presence of the FLT3 receptor ITD mutation confers a worse DFS and OS in AML. Increased relapse risk may result from upregulated

Samsung Bioepis Exhibit No. 1002
Page 1464

DNA repair following chemotherapeutic agent exposure. The mutated FLT3 receptor signals via STAT5, a transcriptional activator for genes including the DNA repair enzyme RAD51. We investigated whether inhibition of FLT3 would alter DNA repair following subtoxic doses of daunorubicin. Cell lines MV4-11 (homozygous for FLT3 ITD) and HL60 (FLT3 wild type (WT) and six-patient AML samples (three ITD, three WT) were incubated with PKC412 for 24 h, then exposed to cytostatic doses of daunorubicin. DNA damage was assessed by comet assay before and during 24 h of repair. In comparison with controls MV4-11 and one-third ITD patient samples showed loss of early (within 1 h) repair when treated with PKC412, however this was not seen in HL60 or WT patient samples. Phosphorylated STAT5 and total cellular phosphotyrosine were measured by flow cytometry. PKC412 incubation resulted in reduction in phosphoSTAT5 and Phosphotyrosine in MV4-11 (39.5% and 65% respectively) with no response in HL60s. This was confirmed in patient samples. Mean reduction for ITDs 50.5% and 11.1%; and for WTs 0% vs. 5.8% respectively. Quantitative PCR was performed on cDNA made from RNA extracted from cell line and patient samples before and after incubation with PKC412. RAD51, DNA PK, PARP, and MSH2 levels were down-regulated in MV4-11s in contrast to HL60s. RAD51 and DNA PK also down-regulated, in ITD but not WT patient samples. In summary incubation with PKC412 resulted in a decrease in early DNA repair following sublethal DNA damage by daunorubicin at doses which down-regulated phosphoSTAT5 and total phosphotyrosine levels, and which resulted in a reduction in DNA repair enzyme mRNA levels. This would suggest that FLT3 mutations might confer their poor prognosis in part via upregulation of DNA repair following cytotoxic drug exposure.

227

An SCL +19 enhancer targets haematopoietic progenitors and endothelium in the mouse embryo and postnatal mice

L. Silberstein,[1] M. J. Sanchez,[3] G. J. Hoffman,[2] S. Piltz,[1] A. R. Green[1] & B. Gottgens[1]

[1]Department of Haematology, University of Cambridge, UK; [2]Department of Histopathology, University of Cambridge, UK; [3]Centro Andaluz de Biologia del Desarrollo, Pablo de Olavide University, Sevilla, Spain;

The stem cell leukaemia (SCL) gene is a basic loop-helix transcription factor with a critical role in normal haematopoiesis and angiogenesis. The SCL gene is normally expressed in haematopoietic stem cells, mast cells, erythroid cells, megakaryocytes and endothelium. Aberrant expression of the SCL gene leads to T-cell acute lymphoblastic leukaemia, whereas SCL−/− mice die due to the absence of haematopoiesis. Hence, temporal and spatial regulation of SCL expression is essential. Our laboratory has previously characterized a 5.5 kb enhancer located 3′ of the SCL transcription start site, which is capable of targeting the expression of β-galactozidase (lacZ) reporter gene to embryonic endothelium and haematopoietic stem cells in the foetal liver and the bone marrow. Subsequent experiments showed that a 641-base pair core enhancer gave an identical pattern of lacZ expression in the embryo. However, it was unclear if this element (later referred to as the +19 enhancer) was capable of maintaining reporter gene expression into the adulthood, since no lacZ activity was observed in postnatal mice. Using a transgenic construct, which contains a eukaryotic reporter gene, human placental alkaline phosphatase, we show that the +19 enhancer targets haematopoietic progenitors and endothelium in the embryo and in the adult mouse. Phenotypic analysis of the transgene-positive cells in the foetal liver and the bone marrow by fluorescence-activated cell sorting demonstrates that they are present within the c-kit-bright population. It is therefore likely that the +19 enhancer targets haematopoietic stem cells. Long-term reconstitution experiments are currently underway to confirm this hypothesis.

228

Differential HOX gene expression in differentiating myeloid cell lines

A. Kwasniewska, C. Bradley, G. McGonigle, D. Grier & A. Thompson

Department of Haematology, Queen's University, Belfast, UK

In mammalian cells the structure of chromatin dictates the accessibility of chromosomal DNA to transcription factors. Regulation of the acetylation and methylation status of core histone proteins results in local chromatin remodelling. In neoplastic cells this causes the silencing of genes encoding anti-oncogenic transcription factors. Aberrant expression of transcription factors, e.g. the PML-RARα fusion protein, recruit transcriptional repressors including histone deacetylases (HDACs) which condense chromatin into inaccessible units. *Hox* genes are master regulators of developmental processes and play a crucial role in haematopoiesis. Global down-regulation of *Hox* gene expression has been reported in PML-RARα patients, suggesting that *Hox* genes are direct targets of the particular HDAC-repressor complex associated with this fusion protein. A real-time PCR approach was undertaken to evaluate the short-term effects of two HDAC inhibitors, trichostatin A (TSA), and sodium valproate (NaV) alone and in combination with all-trans retinoic acid (ATRA) in a panel of leukaemic cell lines (HL-60, NB4, KG1a, K562, Kasumi-1, Mono Mac 6 and U937). Up-regulation of *CD86* was used as a marker of differentiation, and histological studies were performed to confirm the differentiating capacity of these treatments. *CD86* was found to be up-regulated significantly in the HL60, Mono Mac 6 and NB4 cell lines (>15-fold) within 8 h of combined TSA plus ATRA treatment. NaV plus ATRA induced greater expression of a number of *Hox* genes from the *HOXB* and *HOXD* cluster (particularly *B1*, *D8* and *D10*) in comparison with TSA plus ATRA treatment, suggesting these genes are repressed by an HDAC-containing complex in the normal state. Other *Hox* genes remained unaffected by the treatments, including *HOXA9*, indicating a degree of specificity of these treatments. Clinical trials involving HDAC inhibitors with differentiation agents are currently ongoing and monitoring of *Hox* gene expression during such treatments may prove beneficial.

229

CD8-independent, MHC class I-restricted antigen recognition by murine CD4 T cells via transferred T-cell receptors

A. Tsallios,[1] S. Xue,[1] H. J. Stauss[1] & E. C. Morris[1,2]

[1]Department of Immunology, Imperial College; [2]Department of Haematology, Royal Free and University College Hospitals Medical School, London, UK

CD4 T helper cells can play a critical role in the anti-tumour immune response. Cytokines secreted by CD4 T cells can have a direct effect on tumour cells and provide help for CTL priming and effector function. In this study we tested if it was possible to generate MHC-class I-restricted helper T cells by retroviral TCR gene transfer into CD4 lymphocytes. We used a TCR that recognizes the influenza virus A nucleoprotein (NP366–379) peptide in the context of murine D^b MHC class I. Murine splenocytes were isolated from

Samsung Bioepis Exhibit No. 1002
Page 1465

C57Bl6 mice ($H2^b$) and activated with conconavalin A and IL-7, and after 48 h transduced with the pMX-TCRα-IRES-TCRβ retroviral vector. The transduced splenocytes were then cultured in the presence of IL2 for a further 48 h before staining with anti-murine CD4, CD8 and V-β-11 antibodies and sorting into CD4 V-β-11 positive and CD8 V-β-11 positive populations. Sorted cells were expanded for a further 48–72 h prior to functional assays. The sorted TCR-transduced cells were tested for γ-interferon secretion in response to BM-derived dendritic cells (DCs) pulsed with NP peptide, an irrelevant peptide (MDM100) or no peptide. Secreted γ-interferon was measured by ELISA. The results are shown in the table below. Antigen-specific γ-interferon secretion was observed by both CD8-sorted cells (100% purity by FACS) and CD4 sorted cells (99.93% purity). The results indicate that it is feasible to generate CD4 helper T cells that are specific for peptide epitopes presented in the context of MHC class I.

Effector cells	Peptide (10 μmol/l)	Gamma Interferon (ng/ml) at +6 h	Gamma interferon (ng/ml) at +24 h
CD8 – F5TCR	NP	32.07	185.4
CD8 – F5TCR	MDM100	0.04	0.236
CD8 – F5TCR	No peptide	0.07	0.364
CD4 – F5TCR	NP	2.04	178.5
CD4 – F5TCR	MDM100	0.09	0.082
CD4 – F5TCR	No peptide	0.161	0.156

230

JUNE-1 is a novel gene involved in erythroid differentiation

S. Rainey,[1] V. Smyth,[1] Y. Zhengqiang,[1] J. P. Stewart,[1] E. Dunlop,[1] A. Banham[2] & T. R. J. Lappin[1]

[1]*Department of Haematology, Queen's University, Belfast;* [2]*Nuffield Department of Clinical Laboratory Sciences, University of Oxford, Oxford, UK*

The process of erythropoiesis is initiated by the action of erythropoietin (EPO) on erythroid progenitors, inducing a complex cascade of reactions leading to red blood cell development. In addition to erythroid progenitors, receptors for EPO have been found on tumour cells; an important finding, since EPO is used to treat anaemia in cancer patients. However, relatively little is known about genes that are regulated by EPO. The aim of our group is to investigate genes induced by EPO to give a fuller understanding of the downstream effects of this hormone in cellular proliferation and differentiation. Using differential display technology we identified a novel EPO induced gene that we have named *JUNE-1*. We have cloned full-length cDNAs encoding human and murine *JUNE-1*. The intron/exon structure of the gene has been analysed, and its chromosomal localization with respect to known polymorphic markers determined. Murine *JUNE-1* mRNA is expressed in all tissues examined so far, including tissues involved in haematopoiesis, such as spleen and kidney. The mRNA expression pattern of human *JUNE-1* is under investigation, and it has so far been shown to be highly expressed in a variety of leukaemia and lymphoma cell lines. A monoclonal antibody to human *JUNE-1* is in development, which will be used in protein expression and functional studies. Several potential binding sites for haematological transcription factors have been identified in the promoter region. The C-terminal region contains a *p*lant *h*omeo *d*omain (PHD), indicating a possible role in DNA or chromatin binding, with putative actions in transcriptional regulation or chromatin remodelling. This is supported by the presence of a nuclear targeting motif in the protein sequence. These observations are being further explored using assays such as yeast-two-hybrid and ChIP analysis, to further delineate the functions of *JUNE-1* in processes including erythroid differentiation, proliferation, and tumour biology.

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Samsung Bioepis Exhibit No. 1002
Page 1466

Annex 1

Pfizer Inc's opposition statement against EP 1 720 571 B1 (ALEXION PHARMACEUTICALS, INC.)
"Method of treating hemolytic disease"

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1 Introduction

This constitutes opponent Pfizer Inc's statement under Rule 76 (c) EPC, namely the statement of the extent to which European Patent number EP 1 720 571 B1 ("the Patent") is opposed and the grounds on which the opposition is based as well as an indication of facts, evidence and arguments presented of these grounds.

The following documents are introduced into the proceedings:

	Document	**Publication date**
D1	Hillmen et al, The New England Journal of Medecine 2004;350:552-9	Feb 5, 2004
D2	Medscape Medical News, Dec 13, 2002, Jane Salodof MacNeil	Dec 2002
D3	Hillmen et al, British Journal of Hematology 121 (Suppl. 1) 87-95, 2003, Abstract 257	April 2003
D4	Hillmen et al, Blood. 100(11). November 16, 2002. Abstract No. 154	Nov 2002
D5	Hillmen et al, Journal of The American Society of Hematology 102(11), 2003, Abstract 1858	Nov 2003
D6	Rother et al, Molecular Immunology. 40(2003), p 197, Abstract 54	Sept 2003
D7	Rosse et al, American Society of Hematology – Education program, January 2004 (2004-01), pages 48-62	Jan 2004
D8	Biotechnology News, 17 DEC 2004, 24(30), p.10	Dec 2004
D9	U.S. Patent No. 6,355,245	2002
D10	Thomas et al, Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401, 1996	1996
D11	Drugs of the Future 2004, 29(7): 673-676	July 2004
D12	Hillmen et al, British Journal of Haematology, 125 (Suppl.1),1-72, Abstract 5	April 2004
	Decision	
	T1979/09	Sept 2011

All the claims of the Patent are opposed.

The grounds on which the Patent is opposed are:
Article 100(c)/Article 123(2) EPC: Added subject-matter
Article 100(a)/Article 54 EPC: Lack of novelty
Article 100(a)/ Article 56 EPC: Lack of inventive step
Article 100(b)/Article 83 EPC: Insufficiency of disclosure

Samsung Bioepis Exhibit No. 1002
Page 1467

2- The patent

2.1 Formalities

The Patent was granted on European Patent Application No.05712607.0, which was derived from PCT Patent Application No. PCT/US2005/003225 ("the PCT Application" or "the Application as filed"), which was filed on Feb 03, 2005 ("the Filing Date") and published as WO2005/074607. Priority was claimed from US Patent Application No. 10/771,552 ("Priority Application") filed on Feb 03, 2004 ("Claimed Priority Date").

2.2 The Claims

There are eight claims in the Patent, two of which are independent claims, namely claims 1 and 5. The remaining claims are all dependent, directly or indirectly, on at least one of the independent claims.

3- Requests

It is requested that the Patent be revoked in its entirety.

Oral proceedings are requested if the Patent cannot be revoked on the basis of the written submissions alone. If oral proceedings take place and either the Patentee or any other party chooses to speak in French or German, simultaneous translation into English is requested.

4- Added subject matter (Art 100(c)/Art 123(2) EPC)

According to Art.123(2) EPC a European patent application may not be amended in such a way that it contains subject-matter which extends beyond the content of the application as filed. It is therefore, according to Art.100(c) EPC, a ground of opposition that a European patent contains subject-matter that extends beyond the content of the application as filed. According to the Guidelines for examination (H-IV, 2.2), this legal principle is to be interpreted to mean that "An amendment should be regarded as introducing subject-matter which extends beyond the content of the application as filed, and therefore unallowable, if the overall change in the content of the application (whether by way of addition, alteration or excision) results in the skilled person being presented with information which is not directly and unambiguously derivable from that previously presented by the application, even when account is taken of matter which is implicit to a person skilled in the art".

The combination of features in claims 1 and 5 of the opposed patent is not directly and unambiguously disclosed by the application as filed, thereby contravening Art.123(2) EPC.

Claims 1 and 5 of the opposed patent were introduced into the application by way of amendment and contain, in combination, the following features:

(1) the use of eculizumab
(2) the treatment of a patient afflicted with paroxysmal nocturnal hemoglobinuria
(3) intravenous administration
(4) administration of 600 mg each week for four weeks

(5) administration of 900 mg a week later
(6) administration of 900 mg biweekly thereafter
(7) a breakthrough in serum hemolytic activity when the patient is treated under a dosing schedule according to (4) to (6)
(8) administration of 900 mg every 12 days

There is no mention in the claims of the application as filed of any of features (2) to (8), these claims relating to the treatment of hemolytic disease in general terms and not mentioning any features relating to the route of administration or dosage regimen. Therefore, to be allowable according to Art.100(c) EPC, the combination of features (1)-(8) must be directly and unambiguously derivable from the description.

The description discloses feature (2) on page 11, lines 5-6: "The present disclosure relates to a method of treating paroxysmal nocturnal hemoglobinuria ("PNH") and other hemolytic diseases in mammals". Within this broad umbrella, the description contemplates the treatment of a variety of different diseases and symptoms such as abdominal pain, fatigue, dyspnea, insomnia, hemoglobinuria, anemia, erectile dysfunction, dysphagia, thrombosis (page 23, lines 7-17).

As for feature (1), it is disclosed that a large number of compounds of varying pharmacology can be used in this method. These compounds bind to or otherwise block the generation and/or activity of one or more complement components (page 11, lines 24-25). From this broad class, anti-C5 antibodies are described as particularly useful (page 12, lines 1-3) and h5G1.1-mAb (eculizumab) is described as a particularly useful anti-C5 antibody (page 18, lines 12-20).

Regarding feature (3), the selected compound can be administered via a number of routes, one of which is "intravenous infusion by injection" (page 20, lines 4-8). However, the term "intravenous administration" as used in the claim, covers both injection into a vein using a hypodermic needle and the use of a catheter and therefore has a broader meaning than "intravenous infusion by injection".

There is no disclosure in the general description of the invention of any specific dosage or dosage regimen. On the contrary, it is disclosed that the dose selected should vary considerably depending on the compound selected, the manner of administration, the particular symptoms of the patient, the overall health, condition, size and age of the patient, and the judgment of the prescribing physician (page 19, lines 17-23; page 20, lines 1-3). The general teaching of the application, therefore, is that the dose to be administered should be chosen by the physician on a patient-by-patient basis.

In fact, the only basis for features (4)-(8) comes from the specific Example of the application as filed presented on pages 32-39. In this specific, detailed example, eleven patients from a particular patient group (PNH patients that were transfusion-dependent and hemolytic) were treated weekly with a 600 mg intravenous infusion of eculizumab for four weeks, then with 900 mg eculizumab one week later (by an unspecified route) and then with 900 mg eculizumab on a bi-weekly basis (page 32, lines 5-16; also by an unspecified route). Two of the patients exhibited breakthrough in serum hemolytic activity during this biweekly phase of the treatment which was controlled by reducing the dosing interval from fourteen to twelve days (page 35, lines 8-17).

Samsung Bioepis Exhibit No. 1002
Page 1469

There is therefore no unambiguous disclosure of the claimed invention in the description. Rather, the features of the claim are drawn separately and variously from different parts of the disclosure, some of which are contradictory, requiring the creation of intermediate generalisations and/or selections.

The subject-matter of claim 1 is not directly and unambiguously derivable from the general part of the description since:

(a) It is necessary to select a pharmacological class (anti-C5 antibody) from a list of such classes (compounds that bind to or otherwise block the generation and/or activity of one or more complement components); a specific compound (eculizumab) from a list of compounds within that class; the disease PNH as the hemolytic disease to be treated from a list of several diseases; and a route of administration (intravenous) from several possible routes.

(b) There is no disclosure of "intravenous administration" in general terms, only the disclosure of "intravenous infusion by injection".

(c) There is no disclosure of any specific dosage regimen, only the disclosure that dosage will vary depending on the exact clinical situation to be addressed.

The subject-matter of claim 1 is not directly and unambiguously derivable from the Example since:

(a) the example is a specific disclosure that contains several other essential features that are missing from claim 1. In particular, the patients in this study were transfusion-dependent and hemolytic – this is a specific patient sub-group and the invention has not been shown to work in other sub-groups. Claim 1 therefore represents a generalisation of the actual teaching of the Example. Moreover, the specific meaning ascribed to the term "transfusion-dependent" on page 32, lines 7-9 should be specified in the claim. Likewise, the infusion time of "approximately 30 minutes" (page 32, lines 12-14) is not specified in the claim, despite being an essential feature of the Example. Finally, the breakthrough occurred in the extension study between weeks 12 and 64 (page 32 lines 17-20; page 35, lines 8-9) whereas the claim is not so limited and covers treatment regimens where the shorter 12 day treatment cycle is initiated in the initial 12 week acute phase of treatment.

(b) In the Example the patients received "a weekly 600 mg intravenous infusion" of eculizumab which implies (i) the drug was administered at time points one week apart and (ii) a catheter-based intravenous infusion was used to administer the drug. The corresponding features of the claim are "intravenous administration" which covers the use of a catheter and a direct injection and administration of "600 mg each week for four weeks" which implies that the drug was administered once within each of four successive calendar weeks but not necessarily at weekly intervals.

(c) In the Example, the route by which the drug was administered following the initial four week dosing is not specified whereas in the claim the route is specified as the intravenous route.

It is furthermore not appropriate to combine features from the general part of the description and the Example since:

Samsung Bioepis Exhibit No. 1002
Page 1470

(a) This would create an intermediate generalisation – the features of the Example are detailed and specific and associated with each other and to take one or more out of this narrow context and combine it with features of the invention described in general terms would create embodiments of the invention which were not contemplated in the application as filed.

(b) The general part of the description and the Example are not compatible teachings in that the general part of the description clearly contemplates a treatment in which the dosage is selected on a patient-by-patient basis according to the exact clinical situation presented whereas the Example teaches that the same, specific dosage should be administered to each patient regardless of the particular symptoms, overall health, condition, size and age of the patient.

Therefore, claims 1 and 5 of the patent include subject matter which is not directly and unambiguously derivable from the description or claims of the application as filed and are not allowable according to Art.123(2) and Art.100(c) EPC. The dependent claims, which all contain the features of claims 1 and 5, also contravene these provisions.

5- Lack of novelty (Article 100(a)/Art 54 EPC)

5.1 – The subject matter of claim 1 and 5 is not entitled to the priority

According to Art.87(1) EPC, the priority of an earlier application may be claimed if it discloses "the same invention". According to the Guidelines for Examination (F-VI, 1.3) and G2/98, this is to be interpreted to mean that the subject matter of a claim in a European patent may enjoy the priority of a previous application only if the skilled person can derive the subject-matter of the claim directly and unambiguously, using common general knowledge, from the previous application as a whole.

The combination of features in claims 1 and 5 of the opposed patent is not directly and unambiguously disclosed in the priority application (US patent application No. 10/771,552 filed on 03 February 2004), in contravention of Art.87(1). The claims are therefore not entitled to the priority date.

Claims 1 and 5 of the opposed patent contain, in combination, the following features:

(1) the use of eculizumab
(2) the treatment of a patient afflicted with paroxysmal nocturnal hemoglobinuria
(3) intravenous administration
(4) administration of 600 mg each week for four weeks
(5) administration of 900 mg a week later
(6) administration of 900 mg biweekly thereafter
(7) a breakthrough in serum hemolytic activity when the patient is treated under a dosing schedule according to (4) to (6)
(8) administration of 900 mg every 12 days

There is no mention in the claims of the priority application of any of features (2) to (8), these claims relating to the treatment of hemolytic disease in general terms and not mentioning any

Samsung Bioepis Exhibit No. 1002
Page 1471

features relating to the route of administration or dosage regimen. Therefore, for the priority claim to be valid, the combination of features (1)-(8) must be directly and unambiguously derivable from the description.

The description of the priority application discloses feature (2) on page 4, lines 13-14: "Paroxysmal nocturnal hemoglobinuria ("PNH") and other hemolytic diseases are treated in accordance with this disclosure". Within this broad umbrella, the description contemplates the treatment of a variety of different diseases and symptoms such as hemoglobinuria, anemia, fatigue, low red blood cell count, erectile dysfunction, dysphagia and thrombosis (page 22, lines 12-17).

As for feature (1), it is disclosed that a large number of compounds of varying pharmacology can be used in this method. These compounds bind to or otherwise block the generation and/or activity of one or more complement components (page 11, lines 7-9). From this broad class, anti-C5 antibodies are described as particularly useful (page 11, lines 9-11) and h5G1.1-mAb (eculizumab) is described as a particularly useful anti-C5 antibody (page 17, line 23 – page 18, line 8).

Regarding feature (3), the selected compound can be administered via a number of routes, one of which is "intravenous infusion by injection" (page 19, lines 15-18). However, the term "intravenous administration" as used in the claim, covers both injection into a vein using a hypodermic needle and the use of a catheter and therefore has a broader meaning than "intravenous infusion by injection".

There is no disclosure in the general description in the priority application of any specific dosage or dosage regimen. On the contrary, it is disclosed that the dose selected should vary considerably depending on the manner of administration, the particular symptoms of the patient, the overall health, condition, size and age of the patient, and the judgement of the prescribing physician (page 19, lines 12-14). The general teaching of the application, therefore, is that the dose to be administered should be chosen by the physician on a patient-by-patient basis.

In fact, the only basis for features (4)-(8) comes from the specific Example of the invention presented on pages 30-35. In this specific, detailed example, eleven patients from a particular patient group (PNH patients that were transfusion-dependent and hemolytic) were treated weekly with a 600 mg intravenous infusion of eculizumab for four weeks, then with 900 mg eculizumab one week later (no route of administration specified) and then with 900 mg eculizumab on a bi-weekly basis (page 30, lines 6-16; no route of administration specified). One of the patients exhibited breakthrough in serum hemolytic activity during this biweekly phase of the treatment which was controlled by reducing the dosing interval from fourteen to twelve days (page 32, lines 13-19).

There is therefore no unambiguous disclosure of the claimed invention in the description. Rather, the features of the claim are drawn separately and variously from different parts of the disclosure, some of which are contradictory, requiring the creation of intermediate generalisations and/or selections.

The subject-matter of claim 1 is not directly and unambiguously derivable from the general part of the description since:

Samsung Bioepis Exhibit No. 1002
Page 1472

(a) It is necessary to select a pharmacological class (anti-C5 antibody) from a list of such classes (compounds that bind to or otherwise block the generation and/or activity of one or more complement components); a specific compound (eculizumab) from a list of compounds within that class; the disease PNH as the hemolytic disease to be treated from a list of several diseases; and a route of administration (intravenous) from several possible routes.

(b) There is no disclosure of "intravenous administration" in general terms, only the disclosure of "intravenous infusion by injection".

(c) There is no disclosure of any specific dosage regimen, only the disclosure that dosage will vary depending on the exact clinical situation to be addressed.

The subject-matter of claim 1 is not directly and unambiguously derivable from the Example since:

(a) the example is a specific disclosure that contains several other essential features that are missing from claim 1. In particular, the patients in this study were transfusion-dependent and hemolytic – this is a specific patient sub-group and the invention has not been shown to work in other sub-groups. Claim 1 therefore represents a generalisation of the actual teaching of the Example. Moreover, the specific meaning ascribed to the term "transfusion-dependent" on page 30, lines 8-9 should be specified in the claim. Likewise, the infusion time of "approximately 30 minutes" (page 30, lines 13-14) is not specified in the claim, despite being an essential feature of the Example.

(b) In the Example the patients received "a weekly 600 mg intravenous infusion" of eculizumab which implies (i) the drug was administered at time points one week apart and (ii) a catheter-based intravenous infusion was used to administer the drug. The corresponding features of the claim are "intravenous administration" which covers the use of a catheter and a direct injection and administration of "600 mg each week for four weeks" which implies that the drug was administered once within each of four successive calendar weeks but not necessarily at weekly intervals.

(c) In the Example, the route by which the drug was administered following the initial four week dosing is not specified whereas in the claim the route is specified as the intravenous route.

It is furthermore not appropriate to combine features from the general part of the description and the Example since:

(a) This would create an intermediate generalisation – the features of the Example are detailed and specific and associated with each other and to take one or more out of this narrow context and combine it with features of the invention described in general terms would create embodiments of the invention which were not contemplated in the application as filed.

(b) The general part of the description and the Example are not compatible teachings in that the general part of the description clearly contemplates a treatment in which the dosage is selected on a patient-by-patient basis according to the exact clinical situation presented whereas the Example teaches that the same, specific dosage

Samsung Bioepis Exhibit No. 1002
Page 1473

should be administered to each patient regardless of the particular symptoms, overall health, condition, size and age of the patient.

Therefore, claims 1 and 5 of the patent include subject matter which is not directly and unambiguously derivable from the priority application and are not entitled to the priority date. The validity of these claims is consequently to be considered as of the filing date of 03 February 2005.

5.2 – Features of claims 1 and 5 as granted

Claim 1 as granted contains the following features:

(1) the use of eculizumab
(2) the treatment of a patient afflicted with paroxysmal nocturnal hemoglobinuria
(3) intravenous administration
(4) administration of 600 mg each week for four weeks
(5) administration of 900 mg a week later
(6) administration of 900 mg biweekly thereafter
(7) a breakthrough in serum hemolytic activity when the patient is treated under a dosing schedule according to (4) to (6)
(8) administration of 900 mg every 12 days

The same numbering can be applied to claim 5 which comprises the same features as claim 1. The above numbering of the features of claims 1 and 5 will be used in the following sections.

5.3 – D1 (Hillmen et Al, 2004) anticipates the subject matter of claims 1 to 8

D1 published on Feb 5, 2004 and is therefore part of the state of the art under Art 54(2) EPC against claims which are not entitled to the priority date.

- Claims 1 and 5

On page 552, paragraph entitled "Methods", D1 describes a trial where 11 patients with PNH **(2)** received infusions **(3)** of Eculizumab **(1)** (600mg) every week for 4 weeks **(4)**, followed one week later by a 900-mg dose **(5)** and then by 900 mg every other week **(6)** through week 12.

On page 555, last sentence to page 556, first sentence, D1 describes one patient from the trial in whom eculizumab level fell below 35µg per milliliter at week 12, hemolytic activity returned **(7)** and lactate deshydrogenase levels increased transiently. The dosing frequency was increased from 900 mg every 14 days to 900 mg every 12 days **(8)**, reestablishing complete complement blockade during the ongoing 12-month extension study (data not shown).

Therefore, D1 anticipates claims 1 and 5 which lack novelty.

Samsung Bioepis Exhibit No. 1002
Page 1474

- Claims 2, 3, 6 and 7

On page 554, third full sentence, D1 describes that patients from the trial disclosed above "who were taking stable doses of immunosuppressive drugs (e.g., cyclosporine), warfarin, and iron supplements were permitted to continue them".

Therefore, D1 anticipates claims 2, 3, 6 and 7 which lack novelty.

- Claims 4 and 8

On page 555, figure 3A, the percentage of type III erythrocytes in patients from the above mentioned study is greater than 10% for all patients.

Therefore, D1 anticipates claims 4 and 8 which lack novelty.

6- Lack of Inventive Step (Article 100(a)/ Art 56 EPC)

As argued above the opponent is of the opinion that the subject matter of all claims lacks novelty. Should the Opposition Division (OD) be of a different opinion, the opponent reserves the right to use D1 cited in the novelty section for assessment of inventive step.

D7 (see page 53, last 5 lines first column and first 4 lines of second column), D8 (see 1st paragraph), D11 (see page 675, sentence bridging columns and first whole sentence in second column) and D12 (second column, lines 20 to 29) also disclose reduction of dosing interval of Eculizumab to prevent breakthrough of serum hemolytic activity and could also be used for assessment of inventive step.

As detailed below, in the unlikely case that the OD would consider the subject matter of claims entitled to the priority, we submit that none of the granted claims is inventive over the prior art published before the priority date.

6.1 Use of Eculizumab for the treatment of PNH was known at the priority date.

D2 published on Dec 13, 2002 and is therefore part of the state of the art under Art 54(2) EPC against all claims.[1]

D2 is an article discussing results from clinical trials conducted with Eculizumab. As mentioned in the 2nd paragraph of D2, 11 patients were administered Eculizumab for 3 months. D2 (paragraph 12 – emphasis added) further discloses, that "the PNH trial was conducted with Alexion support at two sites in the U.K. Patients received 600 mg of eculizumab each week for four weeks, after which the dose was switched to 900 mg every two weeks."

D3 (Hillmen et al.) is an abstract published in the British Journal of Haematology on April 9, 2003 and is therefore part of the state of the art under Art 54(2) EPC against all claims. D3 discloses that (emphasis added) "Eleven transfusion-dependent PNH patients have

[1] The Examiner confirmed that D2 is prior art in Section 2 of the Communication dated 21 September 2010 in prosecution

completed a 3-month pilot study receiving 600 mg Eculizumab by intravenous infusion over 30 min weekly for four doses and then 900 mg every other week."

D4 (Hillmen et al) is an abstract published on Nov 16, 2002 and is therefore part of the state of the art under Art 54(2) EPC against all claims. D4 discloses that "Ten transfusion-dependent PNH patients have been entered into an ongoing open label Pilot study of eculizumab and all patients will have completed the study by November 2002. Patients receive 600 mg eculizumab by intravenous infusion over 30 minutes weekly for 4 doses and then 900 mg every other week".

D6 (Rother et Al) is an abstract published in Sept 2003 and is therefore part of the state of the art under Art 54(2) EPC against all claims. D6 discloses that "Eleven transfusion dependent PNH patients have completed an open-label pilot study of eculizumab. Patients received 600 mg eculizumab by intravenous infusion over 30 min weekly for four doses, followed by 900 mg 1 week later, and then 900 mg every other week for the remainder of the trial."

It was therefore very well established at the priority date that Eculizumab could be used for treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered intravenously to the patient under the following dosing schedule: 600 mg each week for four weeks; 900 mg 1 week later and 900 mg on a biweekly basis.

6.2 Reducing the dosing interval to prevent potential breakthrough in patients treated with 900mg Eculizumab biweekly was known before the priority date.

D5 (Hillmen et al) is an abstract published on Nov 16 2003 and is therefore part of the state of the art under Art 54(2) EPC against all claims.

D5 relates to the results of the extension of the study disclosed in D4. On lines 5 to 8, D5 discloses that,

> *"We previously reported that eculizumab was very effective at acutely controlling intravascular hemolysis in a 3-month Pilot study of transfusion-dependent PNH patients. We now report that all 11 patients from the initial study entered an extension trial of 900mg eculizumab by i.v. infusion over 30 minutes every other week for one year."*

The pilot study mentioned in the above paragraph is the pilot study discussed lines 8 to 10 of D4. This is consistent with the disclosure of D4, which also mentions that the patients who have completed the pilot study have all entered into an extension study which results are reported in D5.

Therefore the patients from the study discussed in D5 have received 600 mg eculizumab by intravenous infusion over 30 minutes weekly for 4 doses and then 900 mg every other week during the pilot study disclosed in detail in D4. Then they entered into the extension study and continued to receive 900 mg eculizumab by i.v. infusion over 30 minutes every other week (see D5, l.7-8).

On page 510a, 1st col., l.8-12, D5 discloses that

> *"Two patients experienced transient breakthroughs in complement activity associated with increased LDH, hemoglobinuria and mild symptoms, these resolved immediately upon next dose of eculizumab and further breakthroughs were prevented by a slight decrease in the dosing interval."*

It was therefore very well established at the priority date that breakthroughs in serum hemolytic activity observed in patients afflicted with PNH and treated with Eculizumab under a dosing schedule according to items (i) to (iii) of claim 1 as granted could be prevented by slightly decreasing the dosing interval.

The difference between the subject matter of claim 1 and the disclosure of D5 is that the reduced dosing interval is 12 days.

The effect of this difference is to prevent further breakthrough in serum hemolytic activity in patients which exhibit such breakthrough when treated under a dosing schedule according to items (i) to (iii) of claim 1 as granted.

The objective technical problem to be solved is to identify a reduced interval allowing prevention of further breakthrough in serum hemolytic activity in said patients.

The man skilled in the art, starting from D5 and aiming at reducing the interval between two 900mg doses would have tried different intervals shorter than 14 days and would have determined very easily that a 12 days interval is suitable for preventing further breakthrough in serum hemolytic activity in patients exhibiting breakthrough when treated under a dosing schedule according to items (i) to (iii) of claim 1 as granted. As shown in figure 5 of the patent, this is exactly what has been done during the trial described in the examples of the patent. Figure 5 shows the effect of the dosing interval on hemoglobinuria in one of the two patients exhibiting breakthrough of hemolysis activity. When Eculizumab is administered every 14 days, hemoglobinuria is observed before the next dose is administered (see Fig.5 urine grade at day 13). The dosing interval was reduced to 13 days and hemoglobinuria was still observed on the last day of the interval (see Fig.5 urine grade at day 41). Therefore, the dosing interval was reduced to 12 days and no further hemoglobinuria event is observed in Fig.5.

The skilled person, observing a breakthrough in hemolytic activity in a patient administered with 900mg every 14 days, would have applied the teaching of D5 and reduced the dosing interval until it reaches the 12 days dosing interval such that further breakthrough were prevented. Indeed such a reduction in dosing interval represents a routine modification.

We make reference to T1979/09. Section 7 of the reasons of the decision concern an auxiliary request which included a dosage regimen. Reasons 7.3.1 states that:

> *"It is well known that in designing dosage regimen the two major parameters that can be adjusted are the dose itself, i.e. the quantity of drug administered, and the dosing frequency, i.e. the time interval between doses."*

Samsung Bioepis Exhibit No. 1002
Page 1477

Further, in Reasons 7.3.2 the Board stated that:

> *"The board considers that it is within the competence of the skilled person to find out the optimal dosage regime. Consequently, the replacement of a dosage regime by another dosage regime for the same purpose is considered to be a matter of routine experimentation and cannot be seen as involving an inventive step."*

This decision is applicable to the present situation - the use of the defined dosage schedule would have been considered routine to the skilled person in view of the prior art and faced with the technical problem.

Therefore claim 1 lack inventive step over D5 alone or in combination with any one of D2, D3, D4 or D6, or over any of D2, D3, D4 or D6. Claim 5 which relates to the same features also lack inventive step for the same reasons.

The additional features of dependant claims 2 to 4 and 5 to 8 do not confer inventive step the claimed use.

7- Insufficiency of disclosure (Art 100(b)/Art 83 EPC)

7.1 Claims 1 to 8 contravene Article 83 because they lack essential features for the alleged technical effect

Eculizumab is an essential feature of claims 1 to 8. The structure of Eculizumab is not disclosed in the application as filed. On page 8, lines 8-9, the patent discloses that h5G1.1-mAb is currently undergoing clinical trials under the tradename Eculizumab. On the same page, lines 5 to 8, the patent further mentions that methods for the preparation of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1 are described in U.S. Patent No. 6,355,245 (D9) and "Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chain Fv", Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401, 1996 (D10).

However, as detailed below, the complete sequence of Eculizumab or h5G1.1 mAb is not disclosed in these two documents.

D9 is a patent assigned to Alexion Pharmaceutical, Inc which relates to C-5 specific antibodies for the treatment of inflammatory diseases.

In example 7, D9 discloses a mouse monoclonal antibody 5G1.1. In example 11 (columns 42 to 44), D9 lists a number of humanized, chimeric and non humanized scFv, Fd and light chains. D9 does not comprise any reference to Eculizumab or h5G1.1 mAb nor does it disclose the sequence of a full length humanized antibody.

D10 is a publication from Alexion Pharmaceuticals relating to humanized anti-C5 antibody and scFv. D10 discloses the cloning of the variable region genes of the mouse mAb 5G1.1 (see page 1390), the humanization of 5G1.1 variable regions and the construction and expression of various Fab and scFv (see page 1391). The sequences of humanized variable regions are provided in Figure 1. The construction of a humanized antibody h5G1.1 called

h5G1.1 HuG4 is also discussed on page 1396 (paragraph bridging col.1 and 2) and on page 1399, col.2. However, the exact sequence of the humanized antibody is not disclosed in D10.

In view of the above, none of the patent, D9 or D10 provides a detailed description of the structure of h5G1.1 mAb or Eculizumab.

Therefore, the man skilled in the art would not be able to carry out the invention as he would not be able to prepare the specific antibody Eculizumab, which is an essential feature of the claims 1 to 8.

Therefore claims 1 to 8 are not sufficiently disclosed in the sense of Article 83 EPC.

7.2 Claims 1 to 8 contravene Article 83 because the invention is not disclosed in a manner sufficiently clear and complete.

Claim 1 as granted comprises the feature that the "patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii)".

The term "breakthrough in serum hemolytic activity" is neither defined in claim 1 nor in the general disclosure of the application as filed. This term is used in the example section (see page 35, lines 9-10) and in particular in connection with two patients who did not sustain levels of Eculizumab necessary to consistently block complement. Again, no definition of this term is provided in the example. On page 35, lines 11 to 15, the application as filed discloses that "breakthrough of complement blockade resulted in hemoglobuniria, dysphagia and increased LDH and AST which correlated with the return of the hemolytic activity". These events (hemoglobinuria, dysphagia and increased LDH and AST) seem to be associated to some extent with serum hemolytic activity. However, it is not clear if one, two, three or four of these events are necessary and/or sufficient to characterize a breakthrough according to claim 1.

Claim 1 does not provide any indication relative to the characterization of "breakthrough in serum hemolytic activity". In the absence of additional guidance from the description, the skilled person would not be able to determine when a patient exhibits breakthrough in serum hemolytic activity and as a consequence would not be able to determine if and when the dosing interval should be reduced to 12 days.

Therefore, the skilled person would not be able to carry out the subject matter of claims 1 to 8 which therefore contravene Art.83 EPC.

Samsung Bioepis Exhibit No. 1002
Page 1479

SECOND DECLARATION BY DR BRUCE ANDRIEN

I, Bruce Andrien, Director, Early Assay Development and Pharmaceutical Development, of Alexion Pharmaceuticals, Inc., of 100 College Street, New Haven, CT 06510, United States of America, make the following factual declaration concerning the light chain sequence of eculizumab:

1. I am Director, Early Assay Development and Pharmaceutical Development, of Alexion Pharmaceuticals, Inc. ("Alexion"), as shown in my curriculum vitae, a copy of which was attached with my declaration of 23 May 2017.

2. Through my work at Alexion over the past 17 years, I have become very familiar with the protein sequences and structures of antibodies.

3. I understand that EP 16195355.9 is a European patent application whose parent international application was filed on March 16, 2007.

4. I understand the pending claim 1 of EP 16195355.9 reads as follows:

> *"An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4."*

5. I understand that SEQ ID NO: 4 in EP 16195355.9 reads as follows:

> SEQ ID NO: 4 - Eculizumab Light chain
>
> MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDRVTITCGASENIYGAL
> NWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
> NVLNTPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASWCLLNNFYPREAKV
> QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
> LSSPVTKSFNRGEC

6. I understand that EP 16195355.9 also refers to an antibody comprising a light chain variable region that consists of SEQ ID NO: 3.

> SEQ ID NO: 3 - Eculizumab V_L
>
> MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDRVTITCGASENIYGAL
> NWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
> NVLNTPLTFGQGTKVEIKRT

7. I have been asked to comment on whether one skilled in the art of antibody structure would have immediately recognized that the Applicant of EP 16195355.9 did not intend to seek protection for an antibody comprising the whole of SEQ ID NO: 4, or the whole of SEQ ID NO: 3.

8. In my Declaration of 23 May 2017, I explained why one skilled in the art in 2007 would have immediately recognized that the signal peptide is included in SEQ ID NO: 4 and would have found it straightforward and simple to delineate the signal peptide from the active N-terminus of the light chain in SEQ ID NO: 4. The same applies to the signal peptide and active N-terminus of the light chain variable region in SEQ ID NO: 3.

9. One skilled in the art would have also known in 2007 that the signal peptide portion of SEQ ID NO: 4, or SEQ ID NO: 3, would not be present in the mature antibody.

10. As described, for example, in Alberts et al. (1994) (attached as Exhibit A), secretory proteins are initially produced as precursor polypeptides. Each precursor polypeptide contains a signal peptide that directs the polypeptide into the endoplasmic reticulum for further transport and secretion. Where the signal peptide is present at the N-terminus of the polypeptide, it is cleaved off after being transported into the endoplasmic reticulum.

11. It was known in 2007 that an antibody heavy or light chain protein is initially produced as a precursor polypeptide containing a signal peptide, as explained in my Declaration of 23 May 2017. It was also well known that, as for other secretory proteins, the signal peptide of the antibody heavy or light chain directs the polypeptide into the endoplasmic reticulum and is cleaved off during transport of the antibody heavy or light chain into the endoplasmic reticulum. As a result of this cleavage, the mature antibody comprising a heavy chain and a light chain never comprises a signal peptide in either chain.

12. Thus, one skilled in the art would immediately have recognized that the Applicant of EP 16195355.9 intended to obtain protection for an antibody that contains only the mature portion of the light chain shown in SEQ ID NO: 4 (i.e., residues 23 to 214 of SEQ ID NO: 4), or SEQ ID NO: 3 (i.e., residues 23 to 109 of SEQ ID NO: 3), and not for an antibody that contains the whole of SEQ ID NO: 4, or SEQ ID NO: 3.

DECLARED at New Haven, CT
United States of America

This 15th Day of August 2018

Bruce A. Andrien Jr.
Bruce A. Andrien Jr., Ph.D.

Europäisches Patentamt
European Patent Office
Office européen des brevets

European Patent Office
80298 MUNICH
GERMANY

Questions about this communication ?
Contact Customer Services at www.epo.org/contact

J A Kemp
14 South Square
Gray's Inn
London WC1R 5JJ
ROYAUME UNI

Date
07.05.2018

Reference	Application No./Patent No.
	16195355.9 - 1112 / 3167888
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Summons to attend oral proceedings pursuant to Rule 115(1) EPC

You are hereby summoned to attend oral proceedings arranged in connection with the above-mentioned European patent application.

The matters to be discussed are set out in the communication accompanying this summons (EPO Form 2906).

The oral proceedings, which will not be public, will take place before the Examining Division

on 05.02.19 at 09.00 hrs at the EPO,
PschorrHöfe, Bayerstr. 34, 80335 Munich

No changes to the date of the oral proceedings can be made, except on serious grounds (see OJ EPO 2009, 68). If you do not appear as summoned, the oral proceedings may continue without you (R. 115(2) EPC, see also OJ EPO 2008, 471).

Your attention is drawn to Rule 4 EPC, regarding the language of the oral proceedings, and to Special edition No. 3 OJ EPO 2007, L.1, concerning the filing of authorisations for company employees and lawyers acting as representatives before the EPO.

The final date for making written submissions and/or amendments (R. 116 EPC) is 04.01.19.

The actual room number as well as the waiting room numbers will be given to you by the porter in the foyer at the above EPO address.

Parking is available in the underground car park, accessible only via the entrance "Grasserstrasse 2/6". On presentation of the summons to oral proceedings at one of the porters' lodges in the PschorrHöfe, the parking ticket will be revoked.

Please note that the oral proceedings will always take place as scheduled unless you are otherwise informed. The EPO will generally not send a reminder or a further communication prior to the date of oral proceedings.

1st Examiner:	2nd Examiner:	Chairman:
Collura A	Terenzi C	Orlando M

Registered letter with advice of delivery
EPO Form 2008 01.18 [ORAL03=9999] (02/05/18)

to EPO postal service: 02.05.18

Samsung Bioepis Exhibit No. 1002
Page 1482

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Office européen des brevets

European Patent Office
80298 MUNICH
GERMANY
Tel: +49 89 2399 0
Fax: +49 89 2399 4465

Application No.: 16 195 355.9

Preparation for oral proceedings - Instructions to Support Service

Oral proceedings are to be held in connection with the above patent application

1. The matters to be discussed are set out in the annex (Form 2906)

2. Dispatch the summons using Form 2008/2310 and Form 2906 for the parties to attend on:

Day 05.02.2019 Time 09:00

Archibus

Conference Room 9999 booked

ORAL 01, 02, 03 and 05 coded

02.05.18 m
Date Initials

2.1 Parties' submissions in preparation for the oral proceedings, if any, should be made no later than

1 month(s)

before the date of the oral proceedings
(transfer to Form 2008.1 /2310.1)

2.2 Encode ORAL(04)

coded

02.05.18 m
Date Initials

2.3 Dispatch Form 2008.7 /2310.7 to division

02.05.18 m
Date Initials

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3. ☐ Arrange for the following special equipment to be provided in the conference room:

....
Date Initials

4. Return the dossier to primary examiner after dispatching the summons

....
Date Initials

26.04.18
Date

Orlando, Michele	Terenzi, Carla	Collura, Alessandra	
Chairman	2nd examiner	1st examiner	Legal member

Enclosure(s):

Samsung Bioepis Exhibit No. 1002
Page 1484

For the Examining Division

Annexes:
Confirmation of receipt (Form 2936)
Communication (EPO Form 2906)

Samsung Bioepis Exhibit No. 1002
Page 1485

The examination is being carried out on the **following application documents**

Description, Pages

1-4, 6-43, 45-55	as originally filed			
44	received on	10-07-2017	with letter of	10-07-2017
5	filed in electronic form on			16-01-2018

Sequence listings, SEQ ID NO

1-10	received on	10-07-2017	with letter of	10-07-2017

Claims, Numbers

1, 2	filed in electronic form on	16-01-2018

Drawings, Sheets

1/4-4/4	as originally filed

Reference is made to the documents already cited in the course of the procedure.

Moreover, the applicant should be aware of documents D8 and D9 filed by Third Parties on 12.12.17 which may be discussed in the course of the procedure and of WO2005/074607 (D10) which is the publication pamphlet of EP1720571, referred to in D9.

1. Articles 76(1) and 123(2) EPC

The present application does not meet the requirements of Article 76(1) EPC for the following reasons.

The applicant amended page 5 of the description of application as filed (which is the same as the description of th e parent application), and introduced a limitation to the length of the sequence of the indicated SEQ ID NO:3 and SEQ ID NO:4.

The applicant also amended the claims and introduced the same limitation to the length of the SEQ ID NO:4 in present claim 1.

In particular, instead of the full length of the sequences 3 and 4 provided in the application, the specific embodiments disclosed on page 5 and claimed in claim 1 are presently directed to:

(i) an antibody having a heavy chain variable region consisting of SEQ ID NO:1 and a light chain variable region consisting of residues 23-109 of SEQ ID NO: 3; and

(ii) an antibody (as claimed) comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO:4.

In his letter dated 16.01.2018, the applicant explained that the amendments that he had carried out were to be considered the correction of an obvious mistake which met the requirements of Rule 139 EPC.

As already stated in the official communication from the examining division dated 17.11.17, it cannot be excluded by the skilled reader that the SEQ ID NO: 3 and 4 as filed in the parent application and in the application as originally filed were actually those for which protection was sought.

Moreover, in the original and parent application no disclosure was present of the fact that sequences 3 and 4 did not have to include the leader peptide.

For the reasons given above, the examining division concludes that the subject-matter of the present application does not meet the requirements of Articles 76(1) and 123(2) EPC.

2. Article 54 EPC

2.1 Eculizumab is considered to have been available to the public before the filing date of the present application

a) In the official communication dated 17.11.17, the examining division expressed its opinion on the fact that, since D1-D5 are all publications referring to eculizumab and since in none of these documents it was stated that the product had not been available for public use or that the working groups using it had been bound to a confidentiality agreement with Alexion Pharmaceuticals, it had to be concluded that the structure of the antibody was available to the public at the time of filing of the present application.

Moreover, the examining division pointed out that any of the scientists working with eculizumab could have performed a sequence listing on the antibody with the general knowledge available in the art. Reference was also made to D7, which disclosed most of the sequence of the light and heavy chains of 5G1.1 (which is eculizumab, see D4) and explained how the sequence was found.

Samsung Bioepis Exhibit No. 1002
Page 1487

b) On 12.12.17, Third Party observations concerning the present application were received by the EPO.

In the letter it was pointed out that the patentee himself, in opposition proceedings for a granted patent relating to eculizumab (i.e. EP1720571 filing date 03.02.2005; priority date 03.02.2004, in which the applicant was the patentee) had stated that the sequence for eculizumab had been submitted to Chemical Abstracts Services (CAS) and entered STN database on 14.02.1999 (see also the document D6 cited in the European Search Report).

In particular the applicant, at the time of the opposition proceedings against EP1720571, had argued against the objection of lack of sufficiency of disclosure raised by the opponent and based on the allegation that the complete structure of eculizumab was neither disclosed in the application as filed nor in any of the prior art documents cited by the opponent.

c) With a letter dated 16.01.2018, the applicant provided his arguments in response to the objections raised both by the examining division and by Third Parties.

The applicant acknowledged that on 14.02.1999 the sequence of eculizumab was entered in CAS Registry, but that the sequence was not correct.

In support of his arguments, the applicant provided the correspondence between Mr Saxe, who was the primary intellectual property counsel at Alexion Pharmaceuticals, and Mr Bishop, from the CAS Registry database. From this correspondence, it can be seen that Alexion had filed a sequence of eculizumab which contained some mistakes and that the corrected sequence was substituted to the wrong one in May 2009.

The applicant also:

(i) provided evidence of the fact that the marketing approval from the FDA for eculizumab was received by Alexion on 16.05.2007, i.e. after the filing date of the present application;

(ii) stated that when eculizumab was made available for clinical trials, all physicians and participants were required to maintain Alexion's confidential and/or proprietary information in strict confidence. For that reason the skilled person couldn't have obtained and analysed eculizumab to determine its structure; and

(iii) affirmed that the third party observation filed on 12.12.2017 were merely circumstantial and did not provide evidence that eculizumab was publically available at the date of filing of the application.

The examining division considered all the arguments provided in respect to the disputed question of whether eculizumab was publically available or not at the filing date of the present application.

The opinion of the examining division is the following:

1. The fact that Alexion has received the marketing authorisation for eculizumab from the FDA on 16.03.2007 does not necessarily mean that the antibody was not available to the public or that is structure was not known before the filing/priority of the present application.

2. It is clear from Mr. Saxe's declaration that Alexion Pharmaceuticals had intended to submit the correct sequence of eculizumab to CAS and that the incorrect sequence was submitted only inadvertently.

This means that (i) there was no interest in keeping the substance secret after the date of submission to CAS and (ii) anybody to whom the correction of the mistake in the sequence would not have been obvious could have contacted Alexion Pharmaceuticals Inc. to obtain the correct sequence.

Even if it were now purported that Alexion Pharmaceuticals would have never disclosed the correct sequence to anybody who had asked them for it, that would not amount to a valid defence since under the legal principle of "*nemo potest venire contra factum proprium*" Alexion Pharmaceuticals would be bound to its declared intention to lay the compound open under the CAS rules in order to have other patents be regarded as sufficiently disclosed.

The statement from the applicant acting as patentee in EP1720571 (see D9, page 14, item 5.1.2) confirms the fact that he considered the structure of eculizumab to have been disclosed at the filing date of the present application. To be noted is also the fact that the date of the applicant's statement in D9, namely the 30.12.2013, is later than the date in which the correction to the eculizumab structure was requested to CAS.

3. The many patent and non-patent literature documents published before the priority date and relating to the use of eculizumab can be regarded as evidence that the compound was well-known and available to the public before the priority date of the patent application. Among these documents, different published patent applications belonging to the applicant can be seen (see for example D8 filed by Third Party on 12.12.2017 and in particular WO2005/074607 published on 18.08.2005, which is the international publication pamphlet of EP1720571).

4. In respect to the statement of the applicant that the physicians and participants to the studies referred to in the prior art were bound to an agreement of secrecy and confidentiality, a proof of the confidentiality agreement between the company and each participant/physician involved in the studies should be provided.

For the reasons given above, the examining division concludes that the structure of eculizumab was available to the public at the filing/priority date of the present application.

2.2 The present application lacks novelty in view of the disclosure of D1-D5

As already pointed out in the European Searching Opinion and in the official communication from the examining division dated 17.11.17, the disclosures of D1-D5 teaching eculizumab and its use in the treatment of paroxysmal nocturnal hemoglobinuria are considered novelty destroying for the subject-matter of the claimed invention.

2.3 The present application also lacks novelty over the disclosure of D10

The examining division considers that the present application also lacks novelty over the disclosure of D10, which is a prior art document by Alexion Pharmaceuticals relating to eculizumab, for which the applicant explicitly stated that the structure of the antibody had been made available to the public on 14.02.1999.

3. Article 56 EPC

The present application does not meet the requirements of Article 56 EPC, because the subject-matter of the claims 1-2 is not inventive.

As a matter of fact, claims which are not novel cannot be considered to involve any exercise of an inventive step.

4. Article 83 EPC

The attention of the applicant is drawn to the fact that in case a negative conclusion on Articles 123(2) and 76(1) EPC and a positive conclusion on Article 54 EPC will be taken by the examining division in the further prosecution of the case (in particular at the oral proceedings), an objection under Article 83 EPC would be likely to arise.

In the description of the invention (see page 5 last paragraph), the applicant stated that eculizumab is a specific embodiment of the described invention.

In the application as originally filed, as well as in the parent application, sequences 3 and 4 were disclosed for identifying respectively the light chain variable region and the light chain of eculizumab.

On 16.01.2018, the applicant amended the description of the invention and the subject-matter of the claims and stated that the original application referred to the wrong sequences 3 and 4, which erroneously included the leader peptide.

The application has therefore been amended in order to assign a different meaning than one originally meant to the SEQ ID NO:3 and the SEQ ID NO:4.

Therefore, in the situation identified above, following the statements of the applicant in his letter dated 16.01.18, i.e. that (i) the sequences filed with the original application were not those identifying the structure of eculizumab, and (ii) that the structure of eculizumab was not available to the public at the date of filing of the present application, it would follow that the present application was not sufficiently disclosed at the relevant date.

Indeed the skilled person, at the filing date, wouldn't have known which structure was to be assigned to eculizumab.

5. Procedure

It is not at present apparent to the examining division which part of the application could serve as basis for a new and allowable set of claims.

Refusal of the application under Art. 97(2) EPC should therefore be expected.

However, since the applicant has requested oral proceedings, a summons is dispatched with the present communication.

Any item with respect to novelty and inventive step as well the fulfilment of any requirements of the EPC which will become relevant in the course of the procedure will be discussed during oral proceedings.

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 720 571 B9

(12) **CORRECTED EUROPEAN PATENT SPECIFICATION**

(15) Correction information:
Corrected version no 1 (W1 B1)
Corrections, see
Claims EN 1, 5

(48) Corrigendum issued on:
24.10.2012 Bulletin 2012/43

(45) Date of publication and mention of the grant of the patent:
13.06.2012 Bulletin 2012/24

(21) Application number: **05712607.0**

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(51) Int Cl.:
A61K 39/395 *(2006.01)* ***C07K 16/18*** *(2006.01)*

(86) International application number:
PCT/US2005/003225

(87) International publication number:
WO 2005/074607 (18.08.2005 Gazette 2005/33)

(54) **METHOD OF TREATING HEMOLYTIC DISEASE**

VERFAHREN ZUR BEHANDLUNG EINER HÄMOLYTISCHEN ERKRANKUNG

PROCEDE DE TRAITEMENT DE MALADIES HEMOLYTIQUES

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IS IT LI LT LU MC NL PL PT RO SE SI SK TR

(30) Priority: **03.02.2004 US 771552**

(43) Date of publication of application:
15.11.2006 Bulletin 2006/46

(60) Divisional application:
12169343.6
12169508.4
12169513.4
12169519.1
12169526.6

(73) Proprietor: **ALEXION PHARMACEUTICALS, INC.**
Cheshire, CT 06410 (US)

(72) Inventors:
- **BELL, Leonard**
  Woodbridge, CT 06525 (US)
- **ROTHER, Russell, P.**
  Oklahoma City, OK 73151 (US)

(74) Representative: **Vossius & Partner**
Siebertstrasse 4
81675 München (DE)

(56) References cited:
- **ROSSE WENDELL F ET AL: "Immune-mediated hemolytic anemia." HEMATOLOGY / THE EDUCATION PROGRAM OF THE AMERICAN SOCIETY OF HEMATOLOGY. AMERICAN SOCIETY OF HEMATOLOGY. EDUCATION PROGRAM 2004, January 2004 (2004-01), pages 48-62, XP002497825 ISSN: 1520-4391**
- **JANE SALODOF MACNEIL: "Eculizumab could be paroxysmal nocturnal hemoglobinuria breakthrough" 19000101, [Online] 13 December 2002 (2002-12-13), pages 1-2, XP009106338 Medscape Medical News Retrieved from the Internet: URL:http://bcbsma.medscape.com/viewarticle /446336> [retrieved on 2008-09-24]**
- **"Alexion receives FDA, European approval for orphan drug status of new treatment for paroxysmal nocturnal hemoflobinuria" TRANSPLANT NEWS, [Online] 15 January 2004 (2004-01-15), XP002497241 Retrieved from the Internet: URL:http://findarticles.com/p/articles/mi_ m0YUG/is_1_14/ai_n17208612> [retrieved on 2008-09-25]**
- **HALL CLAIRE ET AL: "Primary prophylaxis with warfarin prevents thrombosis in paroxysmal nocturnal hemoglobinuria (PNH)" BLOOD, AMERICAN SOCIETY OF HEMATOLOGY, US, vol. 102, no. 10, 15 November 2003 (2003-11-15), pages 3587-3591, XP002482083 ISSN: 0006-4971**

Note: Within nine months of the publication of the mention of the grant of the European patent in the European Patent Bulletin, any person may give notice to the European Patent Office of opposition to that patent, in accordance with the Implementing Regulations. Notice of opposition shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

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(Cont. next page)

EP 1 720 571 B9

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- HILLMEN PETER ET AL: "Effect of eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria." THE NEW ENGLAND JOURNAL OF MEDICINE 5 FEB 2004, vol. 350, no. 6, 5 February 2004 (2004-02-05), pages 552-559, XP009057948 ISSN: 1533-4406
- FITCH JC ET AL: 'Pharmacology and biological efficacy of a recombinant, humanized, single-chain antibody C5 complement inhibitor in patients undergoing coronary artery bypass graft surgery with cardiopulmonary bypass' CIRCULATION vol. 100, no. 25, December 1999, pages 2499 - 2506, XP002209328
- DATABASE PUBMED 16464755 HILL A ET AL: 'Improvemnet in the symptons of smooth muscle dystonia during eculi therapy in paroxysmal nocturnal hemoglobinuria', XP003000273 & HAEMATOLOGICA vol. 90, no. 12 SUP, December 2005, page ENTIRE ABSTRACT
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- KAPLAN M.: 'ECULIZUMAB' CURRENT OPINION IN INVESTIGATIONAL DRUGS, PHARMAPRESS, US vol. 3, no. 7, 01 July 2002, pages 1017 - 1023, XP009045356 ISSN: 1472-4472

Samsung Bioepis Exhibit No. 1002
Page 1493

Description

BACKGROUND

Technical Field

[0001] Described is a method of treating a hemolytic disease such as, for example, paroxysmal nocturnal hemoglobinuria ("PNH"), by administering a compound which binds to, or otherwise blocks, the generation and/or activity of one or more complement components.

Background of Related Art

[0002] Paroxysmal nocturnal hemoglobinuria ("PNH") is an uncommon blood disorder wherein red blood cells are compromised and are thus destroyed more rapidly than normal red blood cells. PNH results from a mutation of bone marrow cells resulting in the generation of abnormal blood cells. More specifically, PNH is believed to be a disorder of hematopoietic stem cells, which give rise to distinct populations of mature blood cells. The basis of the disease appears to be somatic mutations leading to the inability to synthesize the glycosyl-phosphatidylinositol ("GPI") anchor that is responsible for binding proteins to cell membranes. The mutated gene, PIG-A (phosphatidylinositol glycan class A) resides in the X chromosome and can have several different mutations, varying from deletions to point mutations.

[0003] PNH causes a sensitivity to complement proteins and this sensitivity occurs in the cell membrane. PNH cells are deficient in a number of proteins, particularly essential complement-regulating surface proteins. These complement-regulating surface proteins include the decay-accelerating factor ("DAF") or CD55 and membrane inhibitor of reactive lysis ("MIRL") or CD59.

[0004] PNH is characterized by hemolytic anemia (a decreased number of red blood cells), hemoglobinuria (the presence of hemoglobin in the urine particularly evident after sleeping), and hemoglobinemia (the presence of hemoglobin in the bloodstream). PNH-afflicted individuals are known to have paroxysms, which are defined here as incidences of dark-colored urine. Hemolytic anemia is due to intravascular destruction of red blood cells by complement components. Other known symptoms include dysphagia, fatigue, erectile dysfunction, thrombosis and recurrent abdominal pain.

[0005] Hemolysis resulting from hemolytic diseases causes local and systemic nitric oxide (NO) deficiency through the release of free hemoglobin. Free hemoglobin is a very efficient scavenger of NO, due in part to the accessibility of NO in the non-erythrocyte compartment and a 10^6 times greater affinity of the heme moiety for NO than that for oxygen. The occurrence of intravascular hemolysis often generates sufficient free hemoglobin to completely deplete haptoglobin. Once the capacity of this hemoglobin scavenging protein is exceeded, consumption of endogenous NO ensues. For example, in a setting of intravascular hemolysis such as PNH, where LDH levels can easily exceed 2 - 3 times their normal levels, free hemoglobin would likely obtain concentrations of 0.8 -1.6 g/l. Since haptoglobin can only bind somewhere between 0.7 to 1.5 g/l of hemoglobin depending on the haptoglobin allotype, a large excess of free hemoglobin would be generated. Once the capacity of hemoglobin reabsorption by the kidney proximal tubules is exceeded, hemoglobinuria ensues. The release of free hemoglobin during intravascular hemolysis results in excessive consumption of NO with subsequent enhanced smooth muscle contraction, vasoconstriction and platelet activation and aggregation. PNH-related morbidities associated with NO scavenging by hemoglobin include abdominal pain, erectile dysfunction, esophageal spasm, and thrombosis.

[0006] The laboratory evaluation of hemolysis normally includes hematologic, serologic, and urine tests. Hematologic tests include An examination of the blood smear for morphologic abnormalities of RBCs (to determine causation), and the measurement of the reticulocyte count in whole blood (to determine bone marrow compensation for RBC loss). Serologic tests include lactate dehydrogenase (LDH; widely performed), and free hemoglobin (not widely performed) as a direct measure of hemolysis. LDH levels, in the absence of tissue damage in other organs, can be useful in the diagnosis and monitoring of patients with hemolysis. Other serologic tests include bilirubin or haptoglobin, as measures of breakdown products or scavenging reserve, respectively. Urine tests include bilirubin, hemosiderin, and free hemoglobin, and are generally used to measure gross severity of hemolysis and for differentiation of intravascular vs. extravascular etiologies of hemolysis rather than routine monitoring of hemolysis. Further, RBC numbers, RBC (i.e. cell-bound) hemoglobin, and hematocrit are generally performed to determine the extent of any accompanying anemia rather than as a measure of hemolytic activity per se.

[0007] Steroids have been employed as a therapy for hemolytic diseases and may be effective in suppressing hemolysis in some patients, although long term use of steroid therapy carries many negative side effects. Afflicted patients may require blood transfusions, which carry risks of infection. Anticoagulation therapy may also be required to prevent blood clot formation. Bone marrow transplantation has been known to cure PNH, however, bone marrow matches are often very difficult to find and mortality rates are high with such procedure.

JANE SALODOF MACNEIL ("Eculizumab could be paroxysmal nocturnal hemoglobinuria breakthrough", 19000101,

Samsung Bioepis Exhibit No. 1002
Page 1494

[Online] 13 December 2002 (2002-12-13), pages 1-2, Medscape Medical News)
is a report from 13.12.2002 based on Abstract 154 presented on 9.12.2002 at the 44th Annual Meeting of the American Society of Hematology (ASH) and refers to the use of Eculizumab for the treatment of the symptoms of PNH, in particular hemolysis. The therapy reduced the need for blood transfusions by 68%.

KAPLAN M: "ECULIZUMAB", CURRENT OPINION IN INVESTIGATIONAL DRUGS, PHARMAPRESS, US, vol. 3, no. 7, 1 July 2002 (2002-07-01), pages 1017-1023 describes clinical trials using eculizumab for a variety of diseases.

[0008] It would be advantageous to provide a treatment which safely and reliably eliminates and/or limits hemolytic diseases, such as PNH, and their effects.

Summary

[0009] The invention is defined by the claims.

[0010] Paroxysmal nocturnal hemoglobinuria ("PNH") and other hemolytic diseases are treated using a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Suitable compounds include, for example, antibodies which bind to or otherwise block the generation and/or activity of one or more complement components, such as, for example, an antibody specific to complement component C5. In particularly useful embodiments, the compound is an anti-C5 antibody selected from the group consisting of h5G1.1-mAb (eculizumab), h5G1.1-scFv (pexelizumab) and other functional fragments of h5G1.1. It has surprisingly been found that the described methods provide improvements in the PNH subject within 24 hours of administration of the compound. For example, hemolysis is significantly reduced within 24 hours of administration of the compound as indicated by resolution of hemoglobinuria.

[0011] The complement-inhibiting compound can be administered prophylactically in individuals known to have a hemolytic disease to prevent, or help prevent the onset of symptoms. Alternatively, the complement-inhibiting compound can be administered as a therapeutic regimen to an individual experiencing symptoms of a hemolytic disease.

[0012] A method of increasing the proportion of complement sensitive type III red blood cells and therefore the total red blood cell count in a patient afflicted with a hemolytic disease is contemplated. The method comprises administering a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components to a patient afflicted with a hemolytic disease. By increasing type III red blood cell count, symptoms such as fatigue and anemia also can be alleviated in a patient afflicted with a hemolytic disease.

[0013] Described is a method of rendering a subject afflicted with a hemolytic disease less dependent on transfusions or transfusion-independent by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. It has surprisingly been found that patients can be rendered transfusion-independent in accordance with the present methods. Unexpectedly, transfusion-independence may be maintained for twelve months or more, long beyond the 120 day life cycle of red blood cells. Transfusion-independence can be maintained for two years or more. Treatment for six months or more is required for the evaluation of transfusion independence given the long half life of red blood cells.

[0014] Also described is a method of treating a nitric oxide (NO) imbalance in a subject by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of nitric oxide (NO). NO homeostasis may be restored wherein there is a resolution of symptoms attributable to NO deficiency.

[0015] Also described is a method of treating thrombosis in a subject by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

[0016] Also described is a method of treating fatigue in a subject afflicted with a hemolytic disease by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

[0017] Also described is another a method of treating erectile dysfunction in a subject afflicted with a hemolytic disease by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

[0018] Also described is a method of treating abdominal pain in a subject afflicted with a hemolytic disease by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components

Samsung Bioepis Exhibit No. 1002
Page 1495

and compounds which block the activity of one or more complement components.

[0019] Also described is a method of treating a subject afflicted with a hemolytic disease by administering: 1) one or more compounds known to increase hematopoiesis (for example, either by boosting production, eliminating stem cell destruction or eliminating stem cell inhibition) in combination with 2) a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. Suitable compounds known to increase hematopoiesis include, for example, steroids, immunosuppressants (such as, cyclosporin), anti-coagulants (such as, warfarin), folic acid, iron and the like, erythropoietin (EPO) and antithymocyte globulin (ATG), antilymphocyte globulin (ALG), EPO derivatives, and darbepoetin alfa (commercially available as Aranesp® from Amgen, Inc., Thousand Oaks, CA (Aranesp® is a man-made form of EPO produced in Chinese hamster ovary (CHO) cells by recombinant DNA technology)). Erythropoietin (EPO) (a compound known to increase hematopoiesis), EPO derivatives, or darbepoetin alfa may be administered in combination with an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G 1.1-scFv and other functional fragments of h5G1.1.

[0020] Described is furthermore a method of treating one or more symptoms of hemolytic diseases in a subject where the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10% before or during treatment, by administering a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components, said compound being administered alone or in combination with one or more compounds known to increase hematopoiesis, such as EPO, EPO derivatives, or darbepoetin alfa.

[0021] Also described is a method of treating one or more symptoms of hemolytic diseases in a subject having a platelet count above 40,000 per microliter, by administering a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components, said compound being administered alone or in combination with one or more compounds known to increase hematopoiesis, such as EPO, EPO derivatives, or darbepoetin alfa.

[0022] Also described is a method of treating one or more symptoms of a hemolytic diseases in a subject having a reticulocyte count above 80×10^9 per liter, by administering a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components, said compound being administered alone or in combination with one or more compounds known to increase hematopoiesis, such as EPO, EPO derivatives, or darbepoetin alfa.

Brief Description of the Drawings

[0023]

Figure 1 A reports biochemical parameters of hemolysis measured during treatment of PNH patients with an anti-C5 antibody.

Figure 1 B graphically depicts the effect of treatment with an anti-C5 antibody on lactate dehydrogenase (LDH) levels.

Figure 2 shows a urine color scale devised to monitor the incidence of paroxysm of hemoglobinuria in PNH patients.

Figure 3 is a graph of the effects of eculizumab treatments on patient paroxysm rates, as compared to pre-treatment rates.

Figure 4 shows urine samples of PNH patients and measurements of hemoglobinuria, dysphagia, LDH, AST, pharmacokinetics (PK) and pharmacodynamics (PD) reflecting the immediate and positive effects of the present methods on hemolysis, symptoms and pharmacodynamics suitable to completely block complement.

Figure 5 graphically depicts the effect of anti-C5 antibody dosing schedule on hemoglobinuria over time.

Figures 6a and 6b are graphs comparing the number of transfusion units required per patient per month, prior to and during treatment with an anti-C5 antibody: Figure 6a depicts cytopenic patients; and Figure 6b depicts non-cytopenic patients.

Figure 7 shows the management of a thrombocytopenic patient by administering an anti-C5 antibody and erythropoietin (EPO).

Figure 8 graphically depicts the pharmacodynamics of an anti-C5 antibody.

Figure 9 is a chart of the results of European Organization for Research and Treatment of Cancer questionnaires ("EORTC QLC-C30") completed during the anti-C5 therapy regimen addressing quality of life issues.

Figure 10 is a chart depicting the effects of anti-C5 antibody treatments on adverse symptoms associated with PNH.

Samsung Bioepis Exhibit No. 1002
Page 1496

Detailed Description

[0024] Described is a method of treating paroxysmal nocturnal hemoglobinuria ("PNH") and other hemolytic diseases in mammals. Specifically, the methods of treating hemolytic diseases, which are described herein, involve using compounds which bind to or otherwise block the generation and/or activity of one or more complement components. These methods have been found to provide surprising results. For instance, hemolysis rapidly ceases upon administration of the compound which binds to or otherwise blocks the generation and/or activity of one or more complement components, with hemoglobinuria being significantly reduced after treatment. Also, hemolytic patients can be rendered less dependent on transfusions or transfusion-independent for extended periods (twelve months or more), well beyond the 120 day life cycle of red blood cells. In addition, type III red blood cell count can be increased dramatically in the midst of other mechanisms of red blood cell lysis (non-complement mediated and/or earlier complement component mediated e.g., Cb3). Another example of a surprising result is that symptoms resolved, indicating that NO serum levels were increased enough even in the presence of other mechanisms of red blood cell lysis. These and other results reported herein are unexpected and could not be predicted from prior treatments of hemolytic diseases.

[0025] Any compound which binds to or otherwise blocks the generation and/or activity of one or more complement components can be used in the described methods. A specific class of such compounds which is particularly useful includes antibodies specific to a human complement component, especially anti-C5 antibodies. The anti-C5 antibody inhibits the complement cascade and, ultimately, prevents red blood cell ("RBC") lysis by the complement protein complex C5b-9. By inhibiting and/or reducing the lysis of RBCs, the effects of PNH and other hemolytic diseases (including symptoms such as hemoglobinuria, anemia, hemoglobinemia, dysphagia, fatigue, erectile dysfunction, recurrent abdominal pain and thrombosis) are eliminated. or decreased.

[0026] Described is also that soluble forms of the proteins CD55 and CD59, singularly or in combination with each other, can be administered to a subject to inhibit the complement cascade in its alternative pathway. CD55 inhibits at the level of C3, thereby preventing the further progression of the cascade. CD59 inhibits the C5b-8 complex from combining with C9 to form the membrane attack complex (see discussion below).

[0027] The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of bacterial and viral pathogens. There are at least 25 proteins involved in the complement cascade, which are found as a complex collection of plasma proteins and membrane cofactors. Complement components achieve their immune defensive functions by interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions. A concise summary of the biologic activities associated with complement activation is provided, for example, in The Merck Manual, 16th Edition.

[0028] The complement cascade progresses via the classical pathway, the alternative pathway or the lectin pathway. These pathways share many components, and while they differ in their initial steps, they converge and share the same "terminal complement" components (C5 through C9) responsible for the activation and destruction of target cells. The classical complement pathway is typically initiated by antibody recognition of and binding to an antigenic site on a target cell. The alternative pathway is usually antibody independent, and can be initiated by certain molecules on pathogen surfaces. Additionally, the lectin pathway is typically initiated with binding of mannose-binding lectin ("MBL") to high mannose substrates. These pathways converge at the point where complement component C3 is cleaved by an active protease to yield C3a and C3b.

[0029] C3a is an anaphylatoxin (see discussion below). C3b binds to bacteria and other cells, as well as to certain viruses and immune complexes, and tags them for removal from the circulation. (C3b in this role is known as opsonin.) The opsonic function of C3b is generally considered to be the most important anti-infective action of the complement system. Patients with genetic lesions that block C3b function are prone to infection by a broad variety of pathogenic organisms, while patients with lesions later in the complement cascade sequence, i.e., patients with lesions that block C5 functions, are found to be more prone only to Neisseria infection, and then only somewhat more prone (Fearon, in Intensive Review of Internal Medicine, 2nd Ed. Fanta and Minaker, eds. Brigham and Women's and Beth Israel Hospitals, 1983).

[0030] C3b also forms a complex with other components unique to each pathway to form classical or alternative C5 convertase, which cleaves C5 into C5a and C5b. C3 is thus regarded as the central protein in the complement reaction sequence since it is essential to all three activation pathways (Wurzner, et al., Complement Inflamm. 8:328-340, 1991). This property of C3b is regulated by the serum protease Factor I, which acts on C3b to produce iC3b (inactive C3b). While still functional as an opsonin, iC3b can not form an active C5 convertase.

[0031] The pro-C5 precursor is cleaved after amino acid 655 and 659, to yield the beta chain as an amino terminal fragment (amino acid residues +1 to 655 of the sequence) and the alpha chain as a carboxyl terminal fragment (amino acid residues 660 to 1658 of the sequence), with four amino acids (amino acid residues 656-659 of the sequence) deleted between the two. C5 is glycosylated, with about 1.5-3 percent of its mass attributed to carbohydrate. Mature C5 is a heterodimer of a 999 amino acid 115 kDa alpha chain that is disulfide linked to a 655 amino acid 75 kDa beta chain.

Samsung Bioepis Exhibit No. 1002
Page 1497

C5 is found in normal serum at approximately 75 μg/ml (0.4 μM). C5 is synthesized as a single chain precursor protein product of a single copy gene (Haviland et al. J. Immunol. 1991, 146:362-368). The cDNA sequence of the transcript of this gene predicts a secreted pro-C5 precursor of 1658 amino acids along with an 18 amino acid leader sequence (see, U.S. patent 6,355,245).

[0032] Cleavage of C5 releases C5a, a potent anaphylatoxin and chemotactic factor, and leads to the formation of the lytic terminal complement complex, C5b-9. C5a is cleaved from the alpha chain of C5 by either alternative or classical C5 convertase as an amino terminal fragment comprising the first 74 amino acids of the alpha chain (i.e., amino acid residues 660-733 of the sequence). Approximately 20 percent of the 11 kDa mass of C5a is attributed to carbohydrate. The cleavage site for convertase action is at, or immediately adjacent to, amino acid residue 733 of the sequence. A compound that binds at, or adjacent, to this cleavage site would have the potential to block access of the C5 convertase enzymes to the cleavage site and thereby act as a complement inhibitor.

[0033] C5b combines with C6, C7, and C8 to form the C5b-8 complex at the surface of the target cell. Upon binding of several C9 molecules, the membrane attack complex ("MAC", C5b-9, terminal complement complex-TCC) is formed. When sufficient numbers of MACs insert into target cell membranes, the openings they create (MAC pores) mediate rapid osmotic lysis of the target cells. Lower, non-lytic concentrations of MACs can produce other proinflammatory effects. In particular, membrane insertion of small numbers of the C5b-9 complexes into endothelial cells and platelets can cause deleterious cell activation. In some cases activation may precede cell lysis.

[0034] C5a and C5b-9 also have pleiotropic cell activating properties, by amplifying the release of downstream inflammatory factors, such as hydrolytic enzymes, reactive oxygen species, arachidonic acid metabolites and various cytokines. C5 can also be activated by means other than C5 convertase activity. Limited trypsin digestion (Minta and Man, J. Immunol. 1977, 119:1597-1602; Wetsel and Kolb, J. Immunol. 1982, 128:2209-2216) and acid treatment (Yammamoto and Gewurz, J. Immunol. 1978, 120:2008; Damerau et al., Molec. Immunol. 1989, 26:1133-1142) can also cleave C5 and produce active C5b.

[0035] As mentioned above, C3a and C5a are anaphylatoxins. These activated complement components can trigger mast cell degranulation, which releases histamine and other mediators of inflammation, resulting in smooth muscle contraction, increased vascular permeability, leukocyte activation, and other inflammatory phenomena including cellular proliferation resulting in hypercellularity. C5a also functions as a chemotactic peptide that serves to attract pro-inflammatory granulocytes to the site of complement activation.

[0036] Any compounds which bind to or otherwise block the generation and/or activity of any of the human complement components may be utilized Antibodies specific to a human complement component are useful herein. Some compounds include antibodies directed against complement components C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, Factor D, Factor B, Factor P, MBL, MASP-1, and MASP-2, thus preventing the generation of the anaphylatoxic activity associated with C5a and/or preventing the assembly of the membrane attack complex associated with C5b.

[0037] Also useful may be naturally occurring or soluble forms of complement inhibitory compounds such as CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH. Other compounds which may be utilized to bind to or otherwise block the generation and/or activity of any of the human complement components include, but are not limited to, proteins, protein fragments, peptides, small molecules, RNA aptamers including ARC187 (which is commercially available from Archemix Corp., Cambridge, MA), L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, molecules which may be utilized in RNA interference (RNAi) such as double stranded RNA including small interfering RNA (siRNA), locked nucleic acid (LNA) inhibitors, peptide nucleic acid (PNA) inhibitors, etc.

[0038] Functionally, one suitable class of compounds inhibits the cleavage of C5, which blocks the generation of potent proinflammatory molecules C5a and C5b-9 (terminal complement complex). Preferably, the compound does not prevent the formation of C3b, which subserves critical immunoprotective functions of opsonization and immune complex clearance.

[0039] While preventing the generation of these membrane attack complex molecules, inhibition of the complement cascade at C5 preserves the ability to generate C3b, which is critical for opsonization of many pathogenic microorganisms, as well as for immune complex solubilization and clearance. Retaining the capacity to generate C3b appears to be particularly important as a therapeutic factor in complement inhibition for hemolytic diseases, where increased susceptibility to thrombosis, infection, fatigue, lethargy and impaired clearance of immune complexes are pre-existing clinical features of the disease process.

[0040] Particularly useful compounds for use are antibodies that reduce, directly or indirectly, the conversion of complement component C5 into complement components C5a and C5b. One class of useful antibodies are those having at least one antigen binding site and exhibiting specific binding to human complement component C5. Particularly useful complement inhibitors are compounds which reduce the generation of C5a and/or C5b-9 by greater than about 30%. Anti-C5 antibodies that have the desirable ability to block the generation of C5a have been known in the art since at least 1982 (Moongkamdi et al. Immunobiol. 1982, 162:397; Moongkamdi et al. Immunobiol. 1983, 165:323). Antibodies known in the art that are immunoreactive against C5 or C5 fragments include antibodies against the C5 beta chain

Samsung Bioepis Exhibit No. 1002
Page 1498

(Moongkamdi et al. Immunobiol. 1982, 162:397; Moongkamdi et al. Immunobiol. 1983, 165:323; Wurzner et al. 1991, supra; Mollnes et al. Scand. J. Immunol. 1988, 28:307-312); C5a (see for example, Ames et al. J. Immunol. 1994, 152: 4572-4581, U.S. Pat. No. 4,686,100, and European patent publication No. 0 411 306); and antibodies against non-human C5 (see for example, Giclas et al. J. Immunol. Meth. 1987, 105:201-209). Particularly useful anti-C5 antibodies are h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1. Methods for the preparation of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1 are described in U.S. Patent No. 6,355,245 and "Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chain Fv", Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401, 1996. The antibody h5G1.1-mAb is currently undergoing clinical trials under the tradename eculizumab.

[0041] Hybridomas producing monoclonal antibodies reactive with complement component C5 can be obtained according to the teachings of Sims, et al., U.S. Pat. No. 5,135,916. Antibodies are prepared using purified components of the complement C5 component as immunogens according to known methods. Complement component C5, C5a or C5b may be used as the immunogen. The immunogen may be the alpha chain of C5.

[0042] Particularly useful antibodies share the required functional properties discussed in the preceding paragraph and have any of the following characteristics:

(1) they compete for binding to portions of C5 that are specifically immunoreactive with 5G1.1;
(2) they specifically bind to the C5 alpha chain -- such specific binding, and competition for binding can be determined by various methods well known in the art, including the plasmon surface resonance method (Johne et al., J. Immunol. Meth. 1993, 160:191-198); and
(3) they block the binding of C5 to either C3 or C4 (which are components of the C5 convertases).

[0043] The compound that inhibits the production and/or activity of at least one complement component can be administered in a variety of unit dosage forms. The dose will vary according to the particular compound employed. For example, different antibodies may have different masses and/or affinities, and thus require different dosage levels. Antibodies prepared as fragments (e.g., Fab, $F(ab')_2$, scFv) will also require differing dosages than the equivalent intact immunoglobulins, as they are of considerably smaller mass than intact immunoglobulins, and thus require lower dosages to reach the same molar levels in the patient's blood.

[0044] The dose will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician.

[0045] Administration of the compound that inhibits the production and/or activity of at least one complement component will generally be in an aerosol form with a suitable pharmaceutical carrier, via intravenous infusion by injection, subcutaneous injection, orally, or sublingually. Other routes of administration may be used if desired.

[0046] It is further contemplated that a combination therapy can be used wherein a complement-inhibiting compound is administered in combination with a regimen of known therapy for hemolytic disease. Such regimens include administration of 1) one or more compounds known to increase hematopoiesis (for example, either by boosting production, eliminating stem cell destruction or eliminating stem cell inhibition) in combination with 2) a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. Suitable compounds known to increase hematopoiesis include, for example, steroids, immunosuppressants (such as, cyclosporin), anti-coagulants (such as, warfarin), folic acid, iron and the like, erythropoietin (EPO) and antithymocyte globulin (ATG), antilymphocyte globulin (ALG), EPO derivatives, and darbepoetin alfa (commercially available as Aranesp® from Amgen, Inc., Thousand Oaks, CA (Aranesp® is a man-made form of EPO produced in Chinese hamster ovary (CHO) cells by recombinant DNA technology)). Erythropoietin (EPO) (a compound known to increase hematopoiesis), EPO derivatives, or darbepoetin alfa may be administered in combination with an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1.

[0047] Formulations suitable for injection are found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, Pa., 17th ed. (1985). Such formulations must be sterile and non-pyrogenic, and generally will include a pharmaceutically effective carrier, such as saline, buffered (e.g., phosphate buffered) saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions. The formulations may contain pharmaceutically acceptable auxiliary substances as required, such as, tonicity adjusting agents, wetting agents, bactericidal agents, preservatives, stabilizers

[0048] Described are methods of reducing hemolysis in a patient afflicted with a hemolytic disease by administering one or more compounds which bind to or otherwise block the generation and/or activity of one or more complement components. Reducing hemolysis means that the duration of time a person suffers from hemolysis is reduced by about 25% or more. The effectiveness of the treatment can be evaluated in any of the various manners known to those skilled in the art for determining the level of hemolysis in a patient. One qualitative method for detecting hemolysis is to observe the occurrences of hemoglobinuria. Quite surprisingly, treatment in accordance with the described methods reduces hemolysis as determined by a rapid reduction in hemoglobinuria.

Samsung Bioepis Exhibit No. 1002
Page 1499

[0049] A more qualitative manner of measuring hemolysis is to measure lactate dehydrogenase (LDH) levels in the patient's bloodstream. LDH catalyzes the interconversion of pyruvate and lactate. Red blood cells metabolize glucose to lactate, which is released into the blood and is taken up by the liver. LDH levels are used as an objective indicator of hemolysis. As those skilled in the art will appreciate, measurements of "upper limit of normal" levels of LDH will vary from lab to lab depending on a number of factors including the particular assay employed and the precise manner in which the assay is conducted. Generally speaking, however, the described methods can reduce hemolysis in a patient afflicted with a hemolytic disease as reflected by a reduction of LDH levels in the patients to within 20% of the upper limit of normal LDH levels. Alternatively, the described methods can reduce hemolysis in a patient afflicted with a hemolytic disease as reflected by a reduction of LDH levels in the patients of greater than 50% of the patient's pre-treatment LDH level, such as greater than 65% of the patient's pre-treatment LDH level, or greater than 80% of the patients pre-treatment LDH level.

[0050] Another quantitative measurement of a reduction in hemolysis is the presence of GPI-deficient red blood cells (Type III red blood cells). As those skilled in the art will appreciate, Type III red blood cells have no GPI-anchor protein expression on the cell surface. The proportion of GPI-deficient cells can be determined by flow cytometry using, for example, the technique described in Richards, et al., Clin. Appl. Immunol. Rev., vol. 1, pages 315-330, 2001. The described methods can reduce hemolysis in a patient afflicted with a hemolytic disease as reflected by an increase in Type III red blood cells. An increase in Type III red blood cell levels in the patient of greater than 25% of the total red blood cell count may be achieved, more preferably an increase in Type III red blood cell levels in the patients greater than 50% of the total red blood cell count is achieved, most preferably an increase in Type III red blood cell levels in the patients greater than 75% of the total red blood cell count is achieved.

[0051] Methods of reducing one or more symptoms associated with PNH or other hemolytic diseases are also described. Such symptoms include, for example, abdominal pain, fatigue, dyspnea and insomnia. Symptoms can be the direct result of lysis of red blood cells (e.g., hemoglobinuria, anemia, fatigue, low red blood cell count, etc.) or the symptoms can result from low nitric oxide (NO) levels in the patient's bloodstream (e.g., abdominal pain, erectile dysfunction, dysphagia, thrombosis, etc.). It has recently been reported that almost all patients with greater than 40% PNH type III granulocyte clone have thrombosis, abdominal pain, erectile dysfunction and dysphagia, indicating a high hemolytic rate (see Moyo et al., British J. Haematol. 126:133-138 (2004)).

[0052] The described methods provide a reduction in one or more symptoms associated with PNH or other hemolytic diseases in a patient having a platelet count in excess of 40,000 per microliter (a hypoplastic patient), preferably in excess of 75,000 per microliter, most preferably in excess of 150,000 per microliter. The present methods may provide a reduction in one or more symptoms associated with PNH or other hemolytic diseases in a patient where the proportion of PNH type III red blood cells of the subject's total red blood cell content is greater than 10%, such as greater than 25%, or in excess of 50%. The present methods may provide a reduction in one or more symptoms associated with PNH or other hemolytic diseases in a patient having a reticulocyte count in excess of 80 x 10^9 per liter, such as in excess of 120 x 10^9 per liter, or in excess of 150 x 10^9 per liter. Patients in the latter ranges recited above have active bone marrow and will produce adequate numbers of red blood cells. While in a patient afflicted with PNH or other hemolytic disease the red blood cells may be defective in one or more ways (e.g., GPI deficient), the described methods are particularly useful in protecting such cells from lysis resulting from complement activation. Thus, patients within the latter ranges benefit most from the present methods.

[0053] Also described is a method of reducing fatigue, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing fatigue means the duration of time a person suffers from fatigue is reduced by about 25% or more. Fatigue is a symptom believed to be associated with intravascular hemolysis as the fatigue relents when hemoglobinuria resolves even in the presence of anemia. By reducing the lysis of red blood cells, the described methods reduce fatigue. Patients within the above-mentioned ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

[0054] Described is furthermore a method of reducing abdominal pain contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing abdominal pain means the duration of time a person suffers from abdominal pain is reduced by about 25% or more. Abdominal pain is a symptom resulting from the inability of a patient's natural levels of haptoglobin to process all the free hemoglobin released into the bloodstream as a result of intravascular hemolysis, resulting in the scavenging of NO and intestinal dystonia and spasms. By reducing the lysis of red blood cells, the described methods reduce the amount of free hemoglobin in the bloodstream, thereby reducing abdominal pain. Patients within the above-mentioned ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

[0055] Furthermore, a method of reducing dysphagia is described, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing dysphagia means the duration of time a person has

Samsung Bioepis Exhibit No. 1002
Page 1500

dysphagia attacks is reduced by about 25% or more. Dysphagia is a symptom resulting from the inability of a patient's natural levels of haptoglobin to process all the free hemoglobin released into the bloodstream as a result of intravascular hemolysis, resulting in the scavenging of NO and esophageal spasms. By reducing the lysis of red blood cells, the described methods reduce the amount of free hemoglobin in the bloodstream, thereby reducing dysphagia. Patients within the above-mentioned ranges of type III red blood cells, reticulocytes and platelets benefit most from the described methods.

[0056] Furthermore, a method of reducing erectile dysfunction is described, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing erectile dysfunction means the duration of time a person suffers from erectile dysfunction is reduced by about 25% or more. Erectile dysfunction is a symptom believed to be associated with scavenging of NO by free hemoglobin released into the bloodstream as a result of intravascular hemolysis. By reducing the lysis of red blood cells, the described methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of NO and reducing erectile dysfunction. Patients within the above-mentioned ranges of type III red blood cells, reticulocytes and platelets benefit most from the described methods.

[0057] Moreover, a method of reducing hemoglobinuria is described, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing hemoglobinuria means a reduction in the number of times a person has red, brown, or darker urine, wherein the reduction is typically about 25% or more. Hemoglobinuria is a symptom resulting from the inability of a patient's natural levels of haptoglobin to process all the free hemoglobin released into the bloodstream, as a result of intravascular hemolysis. By reducing the lysis of red blood cells, the described methods reduce the amount of free hemoglobin in the bloodstream and urine thereby reducing hemoglobinuria. Quite surprisingly, the reduction in hemoglobinuria occurs rapidly. Patients within the above-mentioned rangers of type III red blood cells, reticulocytes and platelets benefit most from the described methods.

[0058] In addition, a method of reducing thrombosis is described, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing thrombosis means the duration of time a person has thrombosis attacks is reduced by about 25% or more. Thrombosis is a symptom believed to be associated with scavenging of NO by free hemoglobin released into the bloodstream as a result of intravascular hemolysis and/or the lack of CD59 on the surface of platelets resulting in terminal complement mediated activation of the platelet. By reducing the lysis of red blood cells, the described methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of NO and reducing thrombosis. In addition, blockade of complement will prevent terminal complement-mediated activation of platelets and thrombosis. C5a will also be inhibited by this method which can induce platelet aggregation through C5a receptors on platelets and endothelial cells.

[0059] Thrombosis is thought to be multi-factorial in etiology including NO scavenging by free hemoglobin, the absence of terminal complement inhibition on the surface of circulating platelets and changes in the endothelium surface by cell free heme. The intravascular release of free hemoglobin may directly contribute to small vessel thrombosis. NO has been shown to inhibit platelet aggregation, induce disaggregation of aggregated platelets and inhibit platelet adhesion. Conversely, NO scavenging by hemoglobin or the reduction of NO generation by the inhibition of arginine metabolism results in an increase in platelet aggregation. PNH platelets also lack the terminal complement inhibitor CD59 and multiple studies have shown that deposition of terminal complement (C5b-9) on platelets causes membrane vesiculation and the generation of microvesicles. The microvesicles act as a site for the generation of the clotting components factor Va, Xa or the prothrombinase complex. It is thought that these particles may also contribute to the genesis of thrombosis in PNH. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of NO and reducing thrombosis. In addition, inhibiting complement at C5 will prevent C5b-9 and C5a mediated activation of platelets and/or endothelial cells.

[0060] The described methods may reduce thrombosis, especially patients having a platelet count in excess of 40,000 per microliter, preferably in excess of 75,000 per microliter, most preferably in excess of 150,000 per microliter. The described methods may reduce thrombosis in patients where the proportion of PNH type III red blood cells of the subject's total red blood cell content is greater than 10%, such as greater than 25%, or in excess of 50%, e.g. in excess of 75%. The described methods may reduce transfusion thrombosis in patients having a reticulocyte count in excess of 80×10^9 per liter, such as in excess of 120×10^9 per liter, or in excess of 150×10^9 per liter.

[0061] In addition, a method of reducing anemia is described, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing anemia means the duration of time a person has anemia is reduced by about 25% or more. Anemia in hemolytic diseases results from the blood's reduced capacity to carry oxygen due to the loss of red blood cell mass. By reducing the lysis of red blood cells, the described methods assist red blood cell levels to increase thereby reducing anemia.

Samsung Bioepis Exhibit No. 1002
Page 1501

[0062] Furthermore, a method of increasing the proportion of complement sensitive type III red blood cells and therefore the total red blood cell count in a patient afflicted with a hemolytic disease is described. By increasing the patient's RBC count, fatigue, anemia and the patient's need for blood transfusions is reduced. The reduction in transfusions can be in frequency of transfusions, amount of blood units transfused, or both.

[0063] The method of increasing red blood cell count in a patient afflicted with a hemolytic disease includes the step of administering a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components to a patient afflicted with a hemolytic disease. The present methods may increase red blood cell count in a patient afflicted with a hemolytic disease, especially patients having a platelet count in excess of 40,000 per microliter, such as in excess of 75,000 per microliter, or in excess of 150,000 per microliter. The methods may increase red blood cell count in a patient afflicted with a hemolytic disease where the proportion of PNH type III red blood cells of the subject's total red blood cell content is greater than 10%, such as greater than 25%, or in excess of 50%, e.g. in excess of 75%. The described methods may increase red blood cell count in a patient afflicted with a hemolytic disease having a reticulocyte count in excess of 80 x 10^9 per liter, such as in excess of 120 x 10^9 per liter, or in excess of 150 x 10^9 per liter. The methods of the present disclosure may result in a decrease in the frequency of transfusions by about 50%, typically a decrease in the frequency of transfusions by about 70%, more typically a decrease in the frequency of transfusions by about 90%.

[0064] Described is also a method of rendering a subject afflicted with a hemolytic disease less dependent on transfusions or transfusion-independent by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. As those skilled in the art will appreciate, the normal line cycle for a red blood cell is about 120 days. Treatment for six months or more is required for the evaluation of transfusion independence given the long half life of red blood cells. It has unexpectedly been found that in some patients transfusion-independence can be maintained for twelve months or more, in some cases more than two years, long beyond the 120 day life cycle of red blood cells. The described methods may provide decreased dependence on transfusions or transfusion-independence in a patient afflicted with a hemolytic disease, especially patients having a platelet count in excess of 40,000 per microliter, such as in excess of 75,000 per microliter, or in excess of 150,000 per microliter. The described methods may provide decreased dependence on transfusions or transfusion-independence in a patient afflicted with a hemolytic disease where the proportion of PNH type III red blood cells of the subject's total red blood cell content is greater than 10%, such as greater than 25%, or in excess of 50%, e.g. in excess of 75%. The escribed methods may provide decreased dependence on transfusions or transfusion-independence in a patient afflicted with a hemolytic disease having a reticulocyte count in excess of 80 x 10^9 per liter, such as in excess of 120 x 10^9 per liter, or in excess of 150 x 10^9 per liter.

[0065] Methods of increasing the nitric oxide (NO) levels in a patient having PNH or some other hemolytic disease are also described. These methods of increasing NO levels include the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Low NO levels arise in patients afflicted with PNH or other hemolytic diseases as a result of scavenging of NO by free hemoglobin released into the bloodstream as a result of intravascular hemolysis. By reducing the lysis of red blood cells, the described methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of NO. NO homeostasis may be restored as evidenced by a resolution of symptoms attributable to NO deficiencies.

EXAMPLES

[0066] Eleven patients participated in therapy trials to evaluate the effects of anti-C5 antibody on PNH and symptoms associated therewith. PNH patients were transfusion-dependent and hemolytic. Patients were defined as transfusion dependent with a history of four or more transfusions within twelve months. The median number of transfusions within the patient pool was nine in the previous twelve months. The median number of transfusion units used in the previous twelve months was twenty-two for the patient pool.

[0067] Over the course of four weeks, each of 11 patients received a weekly 600 mg intravenous infusion of anti-C5 antibody for approximately thirty minutes. The specific anti-C5 antibody used in the study was eculizumab. Patients received 900 mg of eculizumab 1 week later then 900 mg on a biweekly basis. The first twelve weeks of the study constituted the pilot study. Following completion of the initial acute phase twelve week study, all patients participated in an extension study conducted to a total of 64 weeks. Ten of the eleven patients participated in an extension study conducted to a total of two years.

[0068] The effect of anti-C5 antibody treatments on PNH type III red blood cells ("RBCs") was tested. "PNH Type" refers to the density of GPI-anchored proteins expressed on the cell surface. Type I is normal expression, Type II is intermediate expression, and Type III has no GPI-anchor protein expression on the cell surface. The proportion of GPI-deficient cells is determined by flow cytometry in the manner described in Richards, et al., Clin. Appl. Immunol. Rev.,

Samsung Bioepis Exhibit No. 1002
Page 1502

vol. 1, pages 315-330, 2001. As compared to pre-therapy conditions, PNH Type III red blood cells increased more than 50% during the extension study. The increase from a pre-study mean value of 36.7% of all red blood cells to a 64 week mean value of 58.4% of Type III red blood cells indicated that hemolysis had decreased sharply. See Table 1, below. Eculizumab therapy protected PNH type III RBCs from complement-mediated lysis, prolonging the cells survival. This protection of the PNH-affected cells reduced the need for transfusions, paroxysms and overall hemolysis in all patients in the trial.

Table 1

PNH Cell Populations Pre- and Post- Eculizumab Treatment in All Patients				
	Proport ion of PNH C ells (%)			
PNH Cell Type	baseline	12 weeks	64 weeks	p-value[a]
Type III RBCs	36.7 +/- 5.9	59.2 +/- 8.0	58.4 +/- 8.5	0.005
Type II RBCs	5.3 +/- 1.4	7.5 +/- 2.1	13.2 +/- 2.4	0.013
Type III WBCs	92.1 +/- 4.6	89.9 +/- 6.6	91.1 +/- 5.8	N.S.
Type III Platelets	92.4 +/- 2.4	93.3 +/- 2.8	92.8+/- 2.6	N.S.
[a]comparison of mean change from baseline to 64 weeks				

[0069] During the course of the two year extension study, it was found that PNH red cells with a complete deficiency of GPI-linked proteins (Type III red cells) progressively increased during the treatment period from a mean of 36.7% to 58.9% (p=0.001) while partially deficient PNH red cells (Type II) increased from 5.3% to 8.7% (p=0.01). There was no concomitant change in the proportion of PNH neutrophils in any of the patients during eculizumab therapy, indicating that the increase in the proportion of PNH red cells was due to a reduction in hemolysis and transfusions rather than a change in the PNH clone(s) themselves.

[0070] The effect of anti-C5 antibody treatments on lactate dehydrogenase levels ("LDH") was measured on all eleven patients. LDH catalyzes the interconversion of pyruvate and lactate. Red blood cells metabolize glucose to lactate, which is released into the blood and is taken up by the liver. LDH levels are used as an objective indicator of hemolysis. The LDH levels were decreased by greater than 80% as compared to pre-treatment levels. The LDH levels were lowered from a pre-study mean value of 3111 U/L to a mean value of 594 U/L during the pilot study and a mean value of 622 U/L after 64 weeks (p = 0.002 for 64 week comparison; see Figures 1A and 1 B).

[0071] Similarly, aspartate aminotransferase (AST) levels, another marker of red blood cell hemolysis, decreased from a mean baseline value of 76 IU/L to 26 IU/L and 30 IU/L during the 12 and 64 weeks of treatment, respectively (p=0.02 for 64 week comparison). Levels of haptoglobin, hemoglobin and bilirubin, and numbers of reticulocytes, did not change significantly from prestudy values during the 64 week treatment period.

[0072] Paroxysm rates were measured and compared to pre-treatment levels. Paroxysm as used in this disclosure is defined as incidences of dark-colored urine with a colorimetric level of 6 of more on a scale of 1-10. Figure 2 shows the urine color scale devised to monitor the incidence of paroxysm of hemoglobinuria in patients with PNH before and during treatment. As compared to pre-treatment levels, the paroxysm percentage rate was reduced by 93% (see, Figure 3) from 3.0 paroxysms per patient per month before eculizumab treatment to 0.1 paroxysms per patient per month during the initial 12 weeks and 0.2 paroxysms per patient per month during the 64 week treatment (Figure 3 ($p< 0.001$)).

[0073] Serum hemolytic activity in nine of the eleven patients was completely blocked throughout the 64 week treatment period with trough levels of eculizumab at equilibrium ranging from approximately 35 μg/ml to 350 μg/ml. During the extension study, 2 patients did not sustain levels of eculizumab necessary to consistently block complement. This breakthrough in serum hemolytic activity occurred in the last 2 days of the 14 day dosing interval, a pattern that was repeated between multiple doses. In one of the patients, as seen in Figure 4, break-through of complement blockade resulted in hemoglobinuria, dysphagia, and increased LDH and AST, which correlated with the return of serum hemolytic activity. At the next dose, symptoms resolved (Figure 5) and reduction in the dosing interval from 900 mg every 14 days to 900 mg every 12 days resulted in a regain of complement control which was maintained over the extension study to 64 weeks in both patients. This patient showed a 24 hour resolution of dysphagia and hemoglobinuria. A reduction in the dosing interval from 14 to 12 days was sufficient to maintain levels of eculizumab above 35 μg/ml and effectively and consistently blocked serum hemolytic activity for the remainder of the extension study for both patients.

[0074] The patients' need for transfusions was also reduced by the treatment with eculizumab. Figure 6a compares the number of transfusion units required per patient per month, prior to and during treatment with an anti-C5 antibody for cytopenic patients, while Figure 6b compares the number of transfusion units required per patient per month, prior to and during treatment with an anti-C5 antibody for non-cytopenic patients. A significant reduction in the need for

Samsung Bioepis Exhibit No. 1002
Page 1503

transfusion was also noted in the entire group (mean transfusion rates decreased from 2.1 units per patient per month during a 1 year period prior to treatment to 0.6 units per patient per month during the initial 12 weeks and 0.5 units per patient per month during the combined 64 week treatment period), with non-cytopenic patients benefiting the most. In fact, four of the non-thrombocytopenic patients with normal platelet counts (≥150,000 per microliter) became transfusion-independent during the 64 week treatment.

[0075] The effect of eculizumab administered in combination with erythropoietin (EPO) was also evaluated in a thrombocytopenic patient. EPO (NeoRecormon®, Roche Pharmaceuticals, Basel, Switzerland) was administered in an amount of 18,000 I.U. three times per week beginning in week 23 of the study. As shown in Figure 7, the frequency of transfusions required for this patient was significantly reduced, and soon halted.

[0076] For the two year extension study, 10 of the 11 patients from the initial 3 month study continued to receive 900 mg of eculizumab every other week. (One patient discontinued eculizumab therapy after 23 months.) Six of the 11 patients had normal platelets (no clinical evidence of marrow failure) whereas 5 of the 11 had low platelets. For the patient who discontinued eculizumab therapy after 23 months, intravascular hemolysis was successfully controlled by eculizumab, but the patient continued to be transfused even after erythropoietin therapy. This patient had the most severe hypoplasia at the start of eculizumab therapy with a platelet count below $30x10^9/l$, suggesting that the ongoing transfusions were likely a result of the underlying bone marrow failure.

[0077] Results of the two year extension study also demonstrated that there was a statistically significant decrease in transfusion requirements for the patients. Three patients remained transfusion independent during the entire two year treatment period, and four cytopenic patients became transfusion independent, three following treatment with EPO (NeoRecormon®). The reduction in transfusion requirements was found to be most pronounced in patients with a good marrow reserve.

[0078] Pharmacodynamic levels were measured and recorded according to eculizumab doses. The pharmacodynamic analysis of eculizumab was determined by measuring the capacity of patient serum samples to lyse chicken erythrocytes in a standard total human serum complement hemolytic assay. Briefly, patient samples or human control serum (Quidel, San Diego CA) was diluted to 40% vol/vol with gelatin veronal-buffered saline (GVB2+, Advanced Research technologies, San Diego, CA) and added in triplicate to a 96-well plate such that the final concentrations of serum in each well was 20%. The plate was then incubated at room temperature while chicken erythrocytes (Lampire Biologics, Malvern, PA) were washed. The chicken erythrocytes were sensitized by the addition of anti-chicken red blood cell polyclonal antibody (0.1% vol/vol). The cells were then washed and resuspended in GVB2+ buffer. Chicken erythrocytes ($2.5 x10^6$ cells/30 μL) were added to the plate containing human control serum or patient samples and incubated at 37°C for 30 min. Each plate contained six additional wells of identically prepared chicken erythrocytes of which four wells were incubated with 20% serum containing 2 mM EDTA as the blank and two wells were incubated with GVB2+ buffer alone as a negative control for spontaneous hemolysis. The plate was then centrifuged and the supernatant transferred to a new flat bottom 96-well plate. Hemoglobin release was determined at OD 415 nm using a microplate reader. The percent hemolysis was determined using the following formula:

$$\text{Percent Hemolysis} = 100 \times \frac{(\text{OD patient sample} - \text{OD blank})}{(\text{OD human serum control} - \text{OD blank})}$$

The graph of the pharmacodynamics (Figure 8), the study of the physiological effects, shows the percentage of serum hemolytic activity (i.e. the percentage of cell lysis) over time. Cell lysis was dramatically reduced in the majority of the patients to below 20% of normal serum complement activity while under eculizumab treatment. Two patients exhibited a breakthrough in complement activity, but complement blockade was permanently restored by reducing the dosing interval to 12 days (See, Figure 4).

[0079] Improvement of quality of life issues was also evaluated using the European Organization for Research and Treatment of Cancer Core (http://www.eortc.be) questionnaires ("EORTC QLC-C30"). Each of the participating patients completed the QLC-30 questionnaire before and during the eculizumab therapy. Overall improvements were observed in global health status, physical functioning, role functioning, emotional functioning, cognitive functioning, fatigue, pain, dyspnea and insomnia. (See Figure 9).

[0080] Patients in the two year study experienced a reduction in adverse symptoms associated with PNH. For example, as set forth in Figure 10, there was a demonstrated decrease of abdominal pain, dysphagia, and erectile dysfunction after administration of eculizumab in those patients reporting those symptoms before administration of eculizumab.

Samsung Bioepis Exhibit No. 1002
Page 1504

Claims

1. Eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered intravenously to the patient under the following dosing schedule:

   (i) 600 mg each week for four weeks;
   (ii) 900 mg 1 week later;
   (iii) 900 mg on a biweekly basis; and thereafter
   (iv) 900 mg every 12 days,

   wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii).

2. Eculizumab for use according to claim 1, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

3. Eculizumab for use according to claim 2, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin, antithymocyte globulin, and antilymphocyte globulin.

4. Eculizumab for use according to any one of claims 1 to 3, wherein the patient is **characterized by** one or more characteristics selected from the group consisting of:

   (a) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;
   (b) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 25%;
   (c) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 50%;
   (d) the patient's platelet count is greater than 40,000 per microliter;
   (e) the patient's platelet count is greater than 75,000 per microliter;
   (f) the patient's platelet count is greater than 150,000 per microliter;
   (g) the patient's reticulocyte count is greater than 80×10^9 per liter;
   (h) the patient's reticulocyte count is greater than 120×10^9 per liter;
   (i) the patient's reticulocyte count is greater than 150×10^9 per liter;
   (j) the patient has a greater than 40% type III granulocyte clone; and
   (k) the patient has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

5. Use of eculizumab in the preparation of a medicament for treating a patient with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered intravenously to the patient under the following dosing schedule:

   (i) 600 mg each week for four weeks;
   (ii) 900 mg 1 week later;
   (iii) 900 mg on a biweekly basis; and thereafter
   (iv) 900 mg every 12 days,

   wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii).

6. The use according to claim 5, wherein eculizumab is to be administered in combination with one or more compounds that increase hematopoiesis.

7. The use according to claim 6, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin, antithymocyte globulin, and antilymphocyte globulin.

8. The use according to any one of claims 5 to 7, wherein the patient is **characterized by** one or more characteristics selected from the group consisting of:

   (l) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;
   (m) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 25%;

Samsung Bioepis Exhibit No. 1002
Page 1505

(n) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 50%;
(o) the patient's platelet count is greater than 40,000 per microliter;
(p) the patient's platelet count is greater than 75,000 per microliter;
(q) the patient's platelet count is greater than 150,000 per microliter;
(r) the patient's reticulocyte count is greater than 80 x 10^9 per liter;
(s) the patient's reticulocyte count is greater than 120 x 10^9 per liter;
(t) the patient's reticulocyte count is greater than 150 x 10^9 per liter;
(u) the patient has a greater than 40% type III granulocyte clone; and
(v) the patient has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

Patentansprüche

1. Eculizumab zur Verwendung in der Behandlung eines Patienten, der unter paroxysmaler nächtlicher Hämoglobinurie (PNH) leidet, wobei Eculizumab dem Patienten intravenös gemäß dem folgenden Dosierungsschema zu verabreichen ist:

   (i) 600 mg jede Woche für eine Dauer von vier Wochen;
   (ii) 900 mg eine Woche später;
   (iii) 900 mg vierzehntäglich; und danach
   (iv) 900 mg alle zwölf Tage,

   wobei der Patient einen Durchbruch der Serum-hämolytischen Aktivität zeigt, wenn er gemäß einem Dosierungsschema entsprechend den Punkten (i) bis (iii) behandelt wird.

2. Eculizumab für die Verwendung gemäß Anspruch 1, wobei Eculizumab in Kombination mit einer oder mehreren Verbindungen, die die Hämatopoese verstärken, zu verabreichen ist.

3. Eculizumab zur Verwendung gemäß Anspruch 2, wobei die eine oder mehreren Verbindungen, die die Hämatopoese verstärken, ausgewählt sind aus der Gruppe bestehend aus: Steroiden, Immunsuppressiva, Antikoagulanzien, Folsäure, Eisen, Erythropoietin, Anti-Thymozytenglobulin und Anti-Lymphozytenglobulin.

4. Eculizumab zur Verwendung nach einem der Ansprüche 1 bis 3, wobei sich der Patient durch eine oder mehrere Charakteristika auszeichnet, die aus der folgenden Gruppe ausgewählt sind:

   (a) der Anteil roter Blutzellen vom Typ III an der Gesamtzahl der roten Blutzellen des Patienten ist größer als 10%;
   (b) der Anteil roter Blutzellen vom Typ III an der Gesamtzahl der roten Blutzellen des Patienten ist größer als 25%;
   (c) der Anteil roter Blutzellen vom Typ III an der Gesamtzahl der roten Blutzellen des Patienten ist größer als 50%;
   (d) die Blutplättchenzahl des Patienten ist größer als 40000 pro Mikroliter;
   (e) die Blutplättchenzahl des Patienten ist größer als 75000 pro Mikroliter;
   (f) die Blutplättchenzahl des Patienten ist größer als 150000 pro Mikroliter;
   (g) die Retikulozytenzahl des Patienten ist größer als 80 x 10^9 pro Liter;
   (h) die Retikulozytenzahl des Patienten ist größer als 120 x 10^9 pro Liter;
   (i) die Retikulozytenzahl des Patienten ist größer als 150 x 10^9 pro Liter;
   (j) der Patient hat einen Granulozytenklon vom Typ III, der mehr als 40% ausmacht; und
   (k) der Patient hat einen LDH-Spiegel, der größer als oder gleich dem 1,5fachen der Obergrenze für den normalen LDH-Spiegel in einer Person ist.

5. Verwendung von Eculizumab in der Herstellung eines Medikaments zur Behandlung eines Patienten, der paroxysmale nächtliche Hämoglobinurie (PNH) hat, wobei Eculizumab dem Patienten intravenös gemäß dem folgenden Dosierungsschema zu verabreichen ist:

   (i) 600 mg jede Woche für eine Dauer von vier Wochen;
   (ii) 900 mg eine Woche später;
   (iii) 900 mg vierzehntäglich; und danach
   (iv) 900 mg alle zwölf Tage,

   wobei der Patient einen Durchbruch der Serum-hämolytischen Aktivität zeigt, wenn er gemäß einem Dosierungs-

Samsung Bioepis Exhibit No. 1002
Page 1506

schema entsprechend den Punkten (i) bis (iii) behandelt wird.

6. Verwendung nach Anspruch 5, wobei Eculizumab in Kombination mit einer oder mehreren Verbindungen, die die Hämatopoese verstärken, zu verabreichen ist.

7. Verwendung nach Anspruch 6, wobei die eine oder mehreren Verbindungen, die die Hämatopoese verstärken, ausgewählt sind aus der Gruppe bestehend aus: Steroiden, Immunsuppressiva, Antikoagulanzien, Folsäure, Eisen, Erythropoietin, Anti-Thymozytenglobulin, und Anti-Lymphozytenglobulin.

8. Verwendung nach einem der Ansprüche 5 bis 7, wobei sich der Patient durch eine oder mehrere Charakteristika auszeichnet, die aus der folgenden Gruppe ausgewählt sind:

(l) der Anteil roter Blutzellen vom Typ III an der Gesamtzahl der roten Blutzellen des Patienten ist größer als 10%;
(m) der Anteil roter Blutzellen vom Typ III an der Gesamtzahl der roten Blutzellen des Patienten ist größer als 25%;
(n) der Anteil roter Blutzellen vom Typ III an der Gesamtzahl der roten Blutzellen des Patienten ist größer als 50%;
(o) die Blutplättchenzahl des Patienten ist größer als 40000 pro Mikroliter;
(p) die Blutplättchenzahl des Patienten ist größer als 75000 pro Mikroliter;
(q) die Blutplättchenzahl des Patienten ist größer als 150000 pro Mikroliter;
(r) die Retikulozytenzahl des Patienten ist größer als 80 x 10^9 pro Liter;
(s) die Retikulozytenzahl des Patienten ist größer als 120 x 10^9 pro Liter;
(t) die Retikulozytenzahl des Patienten ist größer als 150 x 10^9 pro Liter;
(u) der Patient hat einen Granulozytenklon vom Typ III, der mehr als 40% ausmacht; und
(v) der Patient hat einen LDH-Spiegel, der größer als oder gleich dem 1,5fachen der Obergrenze für den normalen LDH-Spiegel in einer Person ist.

Revendications

1. Eculizumab destiné à l'utilisation dans le traitement d'un patient souffrant d'hémoglobinurie nocturne paroxysmique (HNP), dans lequel l'éculizumab doit être administré en intraveineuse au patient en respectant la posologie suivante :

(i) 600 mg chaque semaine pendant quatre semaines ;
(ii) 900 mg 1 semaine plus tard ;
(iii) 900 mg une fois toute les deux semaines ; et par la suite
(iv) 900 mg tous les 12 jours,

dans lequel ledit patient présente une chute de l'activité hémolytique sérique une fois traité en respectant la posologie selon les points (i) à (iii).

2. Eculizumab destiné à l'utilisation selon la revendication 1, dans lequel l'éculizumab doit être administré combiné à un ou plusieurs composés qui augmentent l'hématopoïèse.

3. Eculizumab destiné à l'utilisation selon la revendication 2, dans lequel lesdits un ou plusieurs composés qui augmentent l'hématopoïèse sont choisis dans le groupe constitué par : les stéroïdes, les immunosuppresseurs, les anticoagulants, l'acide folique, le fer, l'érythropoïétine, la globuline antithymocyte, et la globuline antilymphocyte.

4. Eculizumab destiné à l'utilisation selon l'une quelconque des revendications 1 à 3, dans lequel le patient est **caractérisé par** une ou plusieurs caractéristiques choisies dans le groupe constitué par :

(a) la proportion de globules rouges de type III de la numération globulaire des hématies du patient est supérieure à 10 % ;
(b) la proportion de globules rouges de type III de la numération globulaire des hématies du patient est supérieure à 25 % ;
(c) la proportion de globules rouges de type III de la numération globulaire des hématies du patient est supérieure à 50 % ;
(d) la numération plaquettaire du patient est supérieure à 40 000 par microlitre ;
(e) la numération plaquettaire du patient est supérieure à 75 000 par microlitre ;
(f) la numération plaquettaire du patient est supérieure à 150 000 par microlitre ;

Samsung Bioepis Exhibit No. 1002
Page 1507

(g) la numération réticulocytaire du patient est supérieure à 80 x 10^9 par litre ;
(h) la numération réticulocytaire du patient est supérieure à 120 x 10^9 par litre ;
(i) la numération réticulocytaire du patient est supérieure à 150 x 10^9 par litre ;
(j) le patient a un taux supérieur à 40 % de clones granulocytaires de type III ; et
(k) le patient a un taux de LDH supérieur ou égal à 1,5 fois la limite supérieure du taux de LDH normal chez une personne.

5. Utilisation de l'éculizumab dans la préparation d'un médicament destiné à traiter un patient atteint d'hémoglobinurie nocturne paroxysmique (HNP), dans laquelle l'éculizumab doit être administré en intraveineuse au patient en respectant la posologie suivante :

(i) 600 mg chaque semaine pendant quatre semaines ;
(ii) 900 mg 1 semaine plus tard ;
(iii) 900 mg une fois toute les deux semaines ; et par la suite
(iv) 900 mg tous les 12 jours,

dans lequel ledit patient présente une chute de l'activité hémolytique sérique une fois traité en respectant la posologie selon les points (i) à (iii).

6. Utilisation selon la revendication 5, dans laquelle l'éculizumab doit être administré combiné à un ou plusieurs composés qui augmentent l'hématopoïèse.

7. Utilisation selon la revendication 6, dans laquelle un ou plusieurs composés qui augmentent l'hématopoïèse sont choisis dans le groupe constitué par : les stéroïdes, les immunosuppresseurs, les anticoagulants, l'acide folique, le fer, l'érythropoïétine, la globuline antithymocyte, et la globuline antilymphocyte.

8. Utilisation selon l'une quelconque des revendications 5 à 7, dans laquelle le patient est **caractérisé par** une ou plusieurs caractéristiques choisies dans le groupe constitué par :

(l) la proportion de globules rouges de type III de la numération globulaire des hématies du patient est supérieure à 10 % ;
(m) la proportion de globules rouges de type III de la numération globulaire des hématies du patient est supérieure à 25 % ;
(n) la proportion de globules rouges de type III de la numération globulaire des hématies du patient est supérieure à 50 % ;
(o) la numération plaquettaire du patient est supérieure à 40 000 par microlitre ;
(p) la numération plaquettaire du patient est supérieure à 75 000 par microlitre ;
(q) la numération plaquettaire du patient est supérieure à 150 000 par microlitre ;
(r) la numération réticulocytaire du patient est supérieure à 80 x 10^9 par litre ;
(s) la numération réticulocytaire du patient est supérieure à 120 x 10^9 par litre ;
(t) la numération réticulocytaire du patient est supérieure à 150 x 10^9 par litre ;
(u) le patient a un taux supérieur à 40 % de clones granulocytaires de type III ; et
(v) le patient a un taux de LDH supérieur ou égal à 1,5 fois la limite supérieure du taux de LDH normal chez une personne.

Samsung Bioepis Exhibit No. 1002
Page 1508

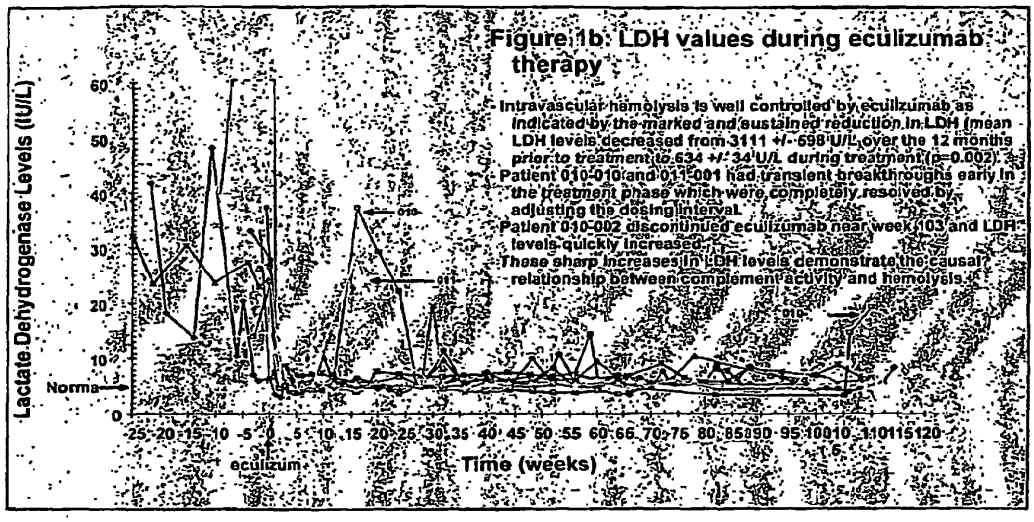
Figure 1A.
Biochemical Parameters of Hemolysis During Eculizumab Treatment

Biochemical Marker	Normal range	Time of Analysis			p-value[a]
		Pre-study[b]	12 weeks	64 weeks	
LDH (IU/L)	150 - 480	3110.7 +/- 598.4	594.0 +/- 31.7	622.4 +/-41.1	0.002
AST (IU/L)	10 - 40	76.2 +/- 16.0	26.2 +/- 2.3	30.1 +/- 3.2	0.02
Haptoglobin (g/L)	0.5 - 2	<0.06	<0.06	0.14 +/- 0.07[c]	N.S.[d]
Hemoglobin (g/dL)	11.5 - 18	10.0 +/- 0.4	10.3 +/- 0.4	10.4 +/- 0.4	N.S.
Bilirubin	3 - 15	25.9 +/- 4.3	28.2 +/- 4.4	28.7 +/- 4.0	N.S.
Reticulocytes ($x10^{-3}/mm^3$)	20 - 80	161.4 +/- 25.9	191.2 +/- 23.6	189.6 +/- 21.8	N.S.

[a]from comparisons of mean change from pre-study to 64 weeks
[b]values represent means during 52 week period prior to treatment except for AST which represents the baseline mean
[c]10 of 11 patients were below the detectable limit of haptoglobin (<0.06 g/L); 1 patient had a value of 0.69 g/L.
[d]not significant

Samsung Bioepis Exhibit No. 1002
Page 1509

Figure 1b



Samsung Bioepis Exhibit No. 1002
Page 1510

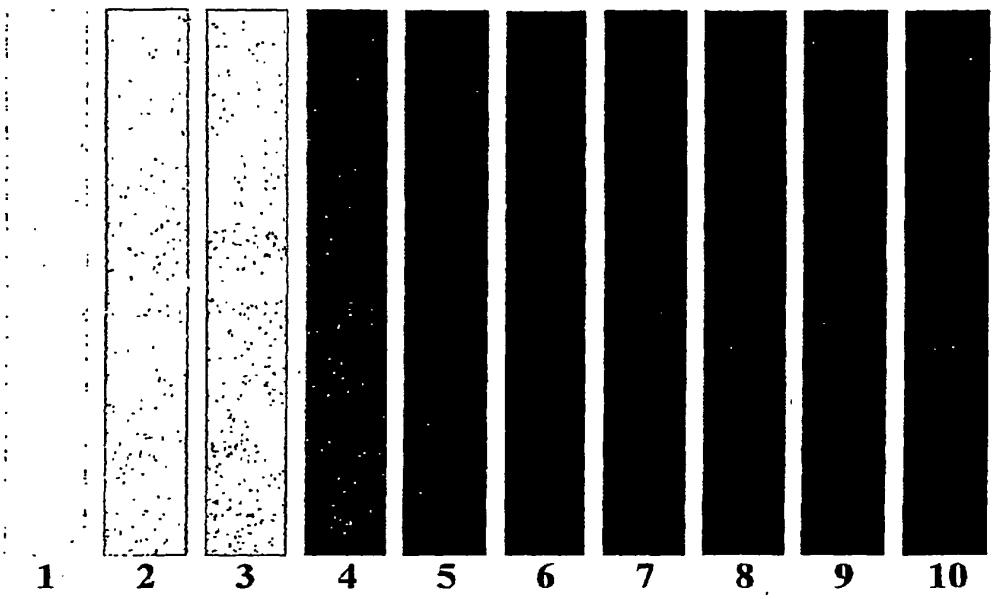
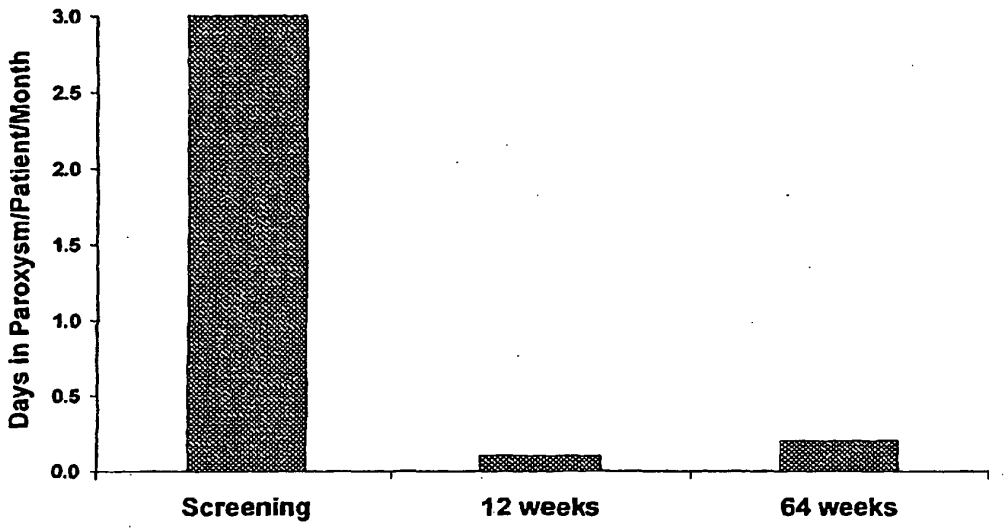


Figure 2

Samsung Bioepis Exhibit No. 1002
Page 1511

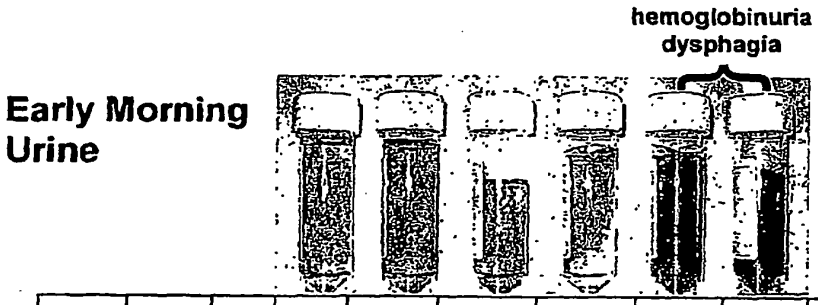
Figure 3.
Effect of Eculizumab on Paroxysm Rate (n=8)



Bars represent the paroxysm rates (number of paroxysms per patient per month) during the screening period (pre-eculizumab treatment), and during the first 12 weeks and the entire 64 weeks of eculizumab treatment. Three patients were not included in the analysis as either pretreatment urine scores were inadvertently not collected (2 patients) or an iron chelating agent that resulted in artificially colored urine was administered during the extension study (1 patient).

Samsung Bioepis Exhibit No. 1002
Page 1512

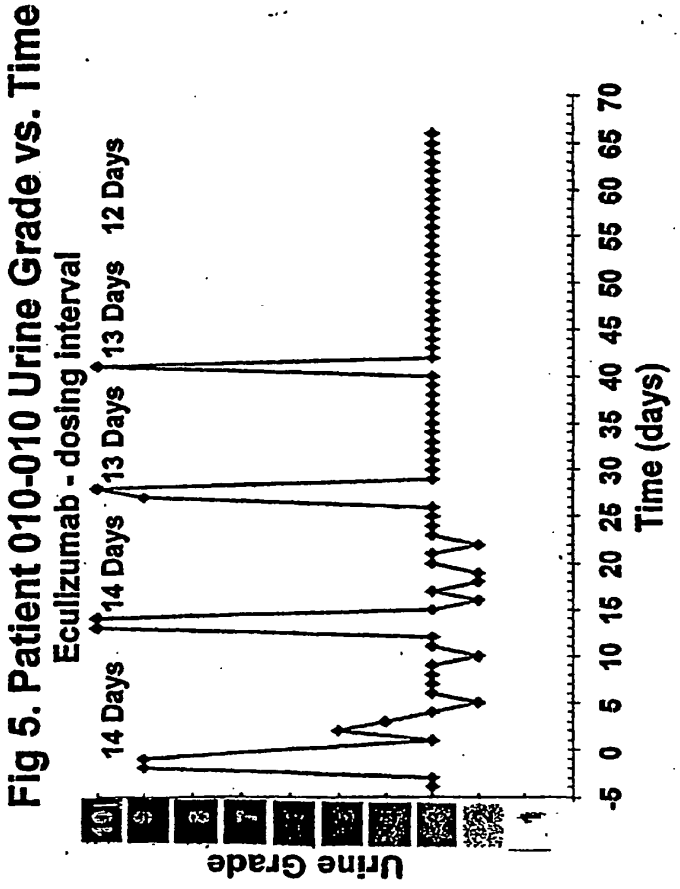
Figure 4.
Analysis of Complement Breakthrough



Visit	4							5			9
Day	0*	1-8	9	10	11	12	13	0*	1-2		0*
Urine	10	2-3	3	3	3	3	9	10	3		2
LDH	2624	–	784	–	–	697	1687	2917	–		495
AST	119	–	38	–	–	31	87	–	–		–
PK	28	–	51	–	–	35	31	23	–		51
PD	72	–	1	–	–	2	56	56	–		2

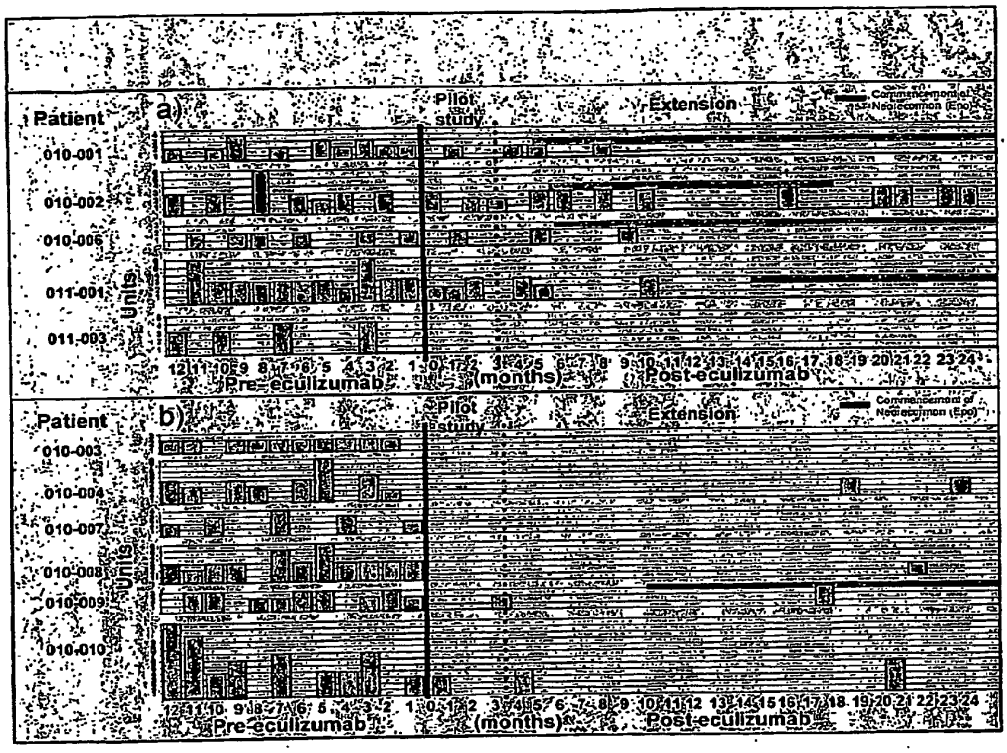
*Dose of eculizumab

Samsung Bioepis Exhibit No. 1002
Page 1513



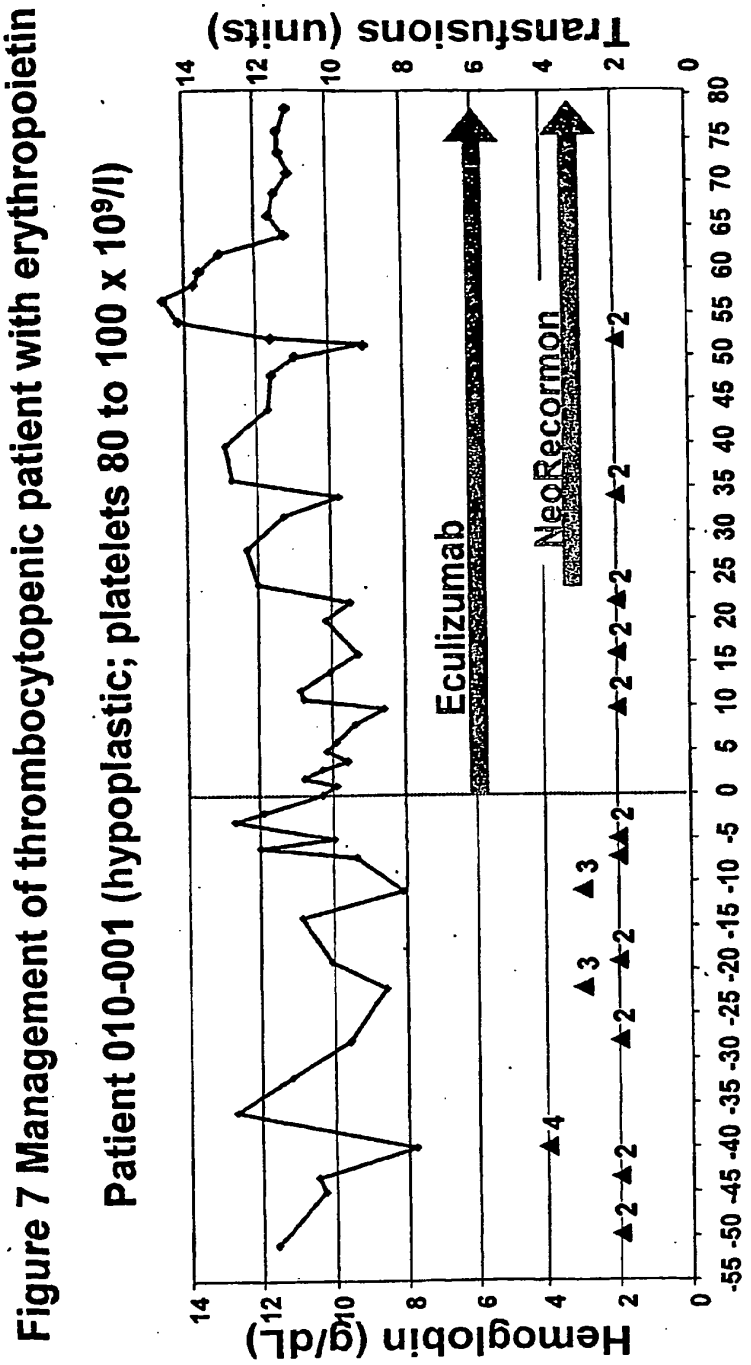
Samsung Bioepis Exhibit No. 1002
Page 1514

Figure 6.
Units Transfused Pre- and Post-Eculizumab in (a) Cytopenic Patients and (b) Non-Cytopenic Patients



Samsung Bioepis Exhibit No. 1002
Page 1515

Figure 7 Management of thrombocytopenic patient with erythropoietin



Transfusions reduced with eculizumab and transfusion independence with combined eculizumab and erythropoietin (NeoRecormon 18,000U 3x/week)

Samsung Bioepis Exhibit No. 1002
Page 1516

Figure 8.
Eculizumab Pharmacodynamics Pre- and Post-dose

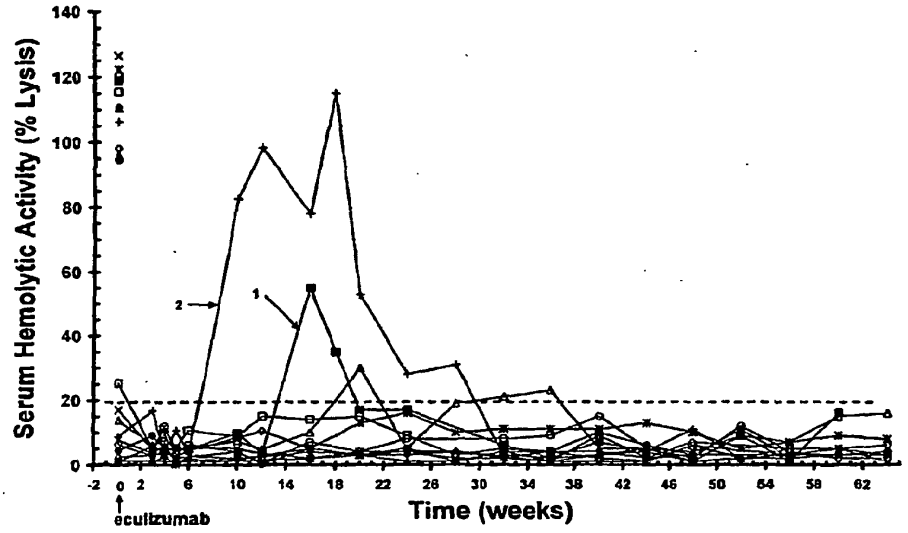


Figure 8 shows serum hemolytic activity (PD) during the 64 week treatment period as determined by the ability of serum to lyse antibody-presensitized chicken erythrocytes. The percentage of hemolytic activity at which complement is considered to be effectively inhibited (≤ 20 %) is indicated by dashed line. Two patients that demonstrated trough serum hemolytic activity values less than 20% are identified (patients 1 and 2).

Samsung Bioepis Exhibit No. 1002
Page 1517

Figure 9.
Eculizumab Pharmacodynamics Pre- and Post-dose

Domain (a)	Mean Base-Line Score (b)	64 Week Change from Base-line (c)	p-value (d)
Global Health Status	56.1	13.8	0.009
Physical Functioning	70.9	14.3	<0.001
Emotional Functioning	70.5	12.5	<0.001
Role Functioning	66.7	14.5	0.003
Cognitive Functioning	77.3	10.3	0.001
Fatigue	47.5	-17.8	<0.001
Dyspnea	39.4	-16.6	0.002
Insomnia	30.3	-8.2	0.031
Pain	21.2	-8.2	0.023
Constipation	3.0	4.1	<0.001

a) the quality of life was assessed using the European Organization for Research and Treatment of Cancer QLQ-C30 instrument
b) numbers represent mean values of linearly transformed scores
c) values represent least-square means; positive change indicates improvement for Global Health Status and Functional Scales while a negative change indicates improvement for Symptom Scales
d) values are from a mixed analysis-of-covariance model with visit as a fixed effect, patient as a random effect, and baseline as a covariate

Samsung Bioepis Exhibit No. 1002
Page 1518

Figure 10.
Symptoms Pre- and 2 Years Post-Eculizumab

Patient	Pre-Eculizumab			Post-Eculizumab		
	Abdominal Pain	Dysphagia	Erectile Dysfunction	Abdominal Pain	Dysphagia	Erectile Dysfunction
010-001	Every 4 – 8 weeks	Every 4 – 8 weeks	Every 4 – 8 weeks	None	None	None
010-002	None	None	–	None	None	–
010-003	Every week	Every week	Every week	None	None	None
010-004	Every 4 – 6 weeks	None	–	None	None	–
010-006	None	None	None	None	None	None
010-007	None	At least every 4 weeks	–	None	None	–
010-008	None	Every 10 weeks	Every 10 weeks	None	None	Intermittent
010-009	None	None	None	None	None	None
010-010	Every 4 weeks	Every 4 weeks	None	None	2 episodes in 2 years*	None
011-001	None	None	–	None	None	–
011-003	None	None	–	None	None	–

*This patient experienced a transient breakthrough in complement blockade with a return of hemolysis and symptoms. Increasing the dosing frequency to 12 days re-established complete complement blockade and prevented further symptoms.

Samsung Bioepis Exhibit No. 1002
Page 1519

REFERENCES CITED IN THE DESCRIPTION

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Samsung Bioepis Exhibit No. 1002
Page 1520

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(54) **PROLONGATION OF SURVIVAL OF AN ALLOGRAFT BY INHIBITING COMPLEMENT ACTIVITY**

VERLÄNGERTE LEBENSDAUER EINES ALLOTRANSPLANTATS MITTELS HEMMUNG VON KOMPLEMENTAKTVITÄT

PROLONGATION DE LA SURVIE D'UNE ALLOGREFFE PAR INHIBITION DE L'ACTIVITE DU COMPLEMENT

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IS IT LI LT LU MC NL PL PT RO SE SI SK TR

(30) Priority: **14.05.2004 US 571444 P**

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(73) Proprietor: **ALEXION PHARMACEUTICALS, INC.**
Cheshire, CT 06410 (US)

(72) Inventors:
- **ROTHER, Russell, P.**
  Oklahoma City
  Oklahoma 73151 (US)
- **WANG, Hao**
  London, Ontario N5X 4H2 (CA)
- **ZHONG, Zhen**
  deceased (CA)

(74) Representative: **Vossius & Partner**
Siebertstrasse 4
81675 München (DE)

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(Cont. next page)

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Samsung Bioepis Exhibit No. 1002
Page 1522

Description

TECHNICAL FIELD

[0001] The invention relates to the embodiments as characterized in the claims.

[0002] The present disclosure relates to methods for prolonging survival of an allograft in a mammal. In particular, the present disclosure relates to prolonging survival of an allograft by administering an inhibitor of complement or terminal complement formation, especially an inhibitor of complement C5 cleavage, in addition to one or more drugs that are immunosuppressant.

BACKGROUND

[0003] Organ transplantation is the preferred treatment for most patients with chronic organ failure. Although transplantation of kidney, liver, lung, and heart offers an excellent opportunity for rehabilitation as recipients return to a more normal lifestyle, it is limited by the medical/surgical suitability of potential recipients, an increasing shortage of donors, and premature failure of transplanted organ function.

[0004] Transplantation of cells, tissues and organs has become very common and is often a life-saving procedure. Organ transplantation is the preferred treatment for most patients with chronic organ failure. Despite great improvement in treatments to inhibit rejection, rejection continues to be the single largest impediment to successful organ transplantation. Rejection includes not only acute rejection but also chronic rejection. One-year survival rates for transplanted kidneys average 88.3% with kidneys from deceased donors and 94.4% with kidneys received from living donors. The corresponding five year survival rates for the transplanted kidneys are 63.3% and 76.5% (OPTN/SRTR Annual Report, 2002). For livers the one year survival rates are 80.2% and 76.5% for livers from deceased and living donors, respectively. The corresponding five year liver graft survival rates are 63.5% and 73.0% (OPTN/SRTR Annual Report, 2002). The use of immunosuppressant drugs, especially cyclosporin A and more recently tacrolimus, has dramatically improved the success rate of organ transplantation especially by preventing acute rejection. But as the numbers above show, there is still a need to improve the success rates, both short-term and especially long-term. As seen from the above numbers for kidney and liver transplants, the five year failure rates for these transplanted organs are on the order of 25-35%. In the year 2001 alone there were more than 23,000 patients who received an organ transplant of which approximately 19,000 were kidney or liver (OPTN/SRTR Annual Report, 2002). For this one year of transplants alone, with present techniques it can be expected that approximately 5,000-6,000 of these transplanted kidneys and livers will fail within 5 years. These numbers do not even include other transplanted organs or transplanted tissues or cells such as bone marrow.

[0005] There are multiple types of transplants. These are described in Abbas et al., 2000. A graft transplanted from one individual to the same individual is called an autologous graft or autograft. A graft transplanted between two genetically identical or syngeneic individual is called a syngeneic graft. A graft transplanted between two genetically different individuals of the same species is called an allogeneic graft or allograft. A graft transplanted between individuals of different species is called a xenogeneic graft or xenograft. The molecules that are recognized as foreign on allografts are called alloantigens and those on xenografts are called xenoantigens. The lymphocytes or antibodies that react with alloantigens or xenoantigens are described as being alloreactive or xenoreactive, respectively.

[0006] Currently more than 40,000 kidney, heart, lung, liver and pancreas transplants are performed in the United States each year (Abbas et al., 2000). Other possible transplants include, but are not limited to, vascular tissue, eye, cornea, lens, skin, bone marrow, muscle, connective tissue, gastrointestinal tissue, nervous tissue, bone, stem cells, islets, cartilage, hepatocytes, and hematopoietic cells. Unfortunately, there are many more candidates for a transplant than there are donors. To overcome this shortage, a major effort is being made to learn how to use xenografts. While progress is being made in this field, the fact is that at present most transplants are allografts. An allogeneic transplant, while presently being more likely to be successful than a xenogeneic transplant, must surmount numerous obstacles to be successful. There are several types of immunological attacks made by the recipient against the donor organ which can lead to rejection of the allograft. These include hyperacute rejection, acute vascular rejection (including accelerated humoral rejection and de novo acute humoral rejection), and chronic rejection. Rejection is normally a result of T-cell mediated or humoral antibody attack, but may include additional secondary factors such as the effects of complement and cytokines.

[0007] An ever growing gap between the number of patients requiring organ transplantation and the number of donor organs available has become a major problem throughout the world. Park et al., 2003. Individuals who have developed anti-HLA antibodies are said to be immunized or sensitized. Gloor, 2005. HLA sensitization is the major barrier to optimal utilization of organs from living donors in clinical transplantation (Warren et al., 2004) due to the development of severe antibody-mediated rejection (ABMR). For example, more than 50% of all individuals awaiting kidney transplantation are presensitized patients (Glotz et al., 2002) who have elevated levels of broadly reactive alloantibodies, resulting from

Samsung Bioepis Exhibit No. 1002
Page 1523

multiple transfusions, prior failed allografts, or pregnancy (Kupiec-Weglinski, 1996). The role of ABMR is currently one of the most dynamic areas of study in transplantation, due to recognition that this type of rejection can lead to either acute or chronic loss of allograft function. Mehra et al., 2003. Numerous cases of ABMR, including hyperacute rejection (HAR) or accelerated humoral rejection (ACHR), have been reported that are characterized by acute allograft injury that is resistant to potent anti-T cell therapy, the detection of circulating donor specific antibodies, and the deposition of complement components in the graft. ABMR with elevated circulating alloantibodies and complement activation that occurs in 20-30% of acute rejection cases has a poorer prognosis than cellular rejection. Mauiyyedi et al., 2002.

[0008] Highly presensitized patients, who exhibit high levels of alloantibodies, usually suffer an immediate and aggressive HAR. In clinical practice, with great efforts and significant advances in technology, HAR is avoided by obtaining a pretransplant lymphocytotoxic crossmatch to identify sensitized patients with antibodies specific for donor HLA antigens. However, circulating antibodies against donor HLA or other non-MHC endothelial antigens may also be responsible for a delayed form of acute humoral rejection, which is associated with an increased incidence of graft loss. Collins et al., 1999. Therefore, development of a novel presensitized animal model to mimic ABMR in clinical settings would be beneficial to studies on the mechanism, and to the much needed progress in the management of allograft rejection in presensitized hosts.

[0009] Some highly presensitized patients can benefit from intervention programs such as immunoadsorption (Palmer et al.,1989; Ross et al., 1993; Kriaa et al., 1995), plasmapheresis and intravenous immunoglobulin (Sonnenday et al., 2002; Rocha et al., 2003), that have been designed and implemented to temporarily eliminate anti-donor antibodies. However, in addition to their benefits, the aforementioned therapies carry with them numerous drawbacks as some individuals are less susceptible to their effects (Kriaa et al., 1995; Hakim et al., 1990; Glotz et al., 1993; Tyan et al., 1994); they are extremely expensive, time-consuming, and risky (Salama et al., 2001). Moreover, the transient and variable effect of these protocols has limited their impact. Glotz et al., 2002; Kupin et al., 1991; Schweitzer et al., 2000. Therefore, developing novel strategies to reduce the risk and cost in prevention of ABMR would be beneficial to presensitized recipients receiving an allograft.

SUMMARY

[0010] Accordingly, means of prolonging survival of transplanted cells, tissues or organs are provided. In particular, means of prolonging survival of allotransplanted cells, tissues or organs are provided. These means involve using one or more immunosuppressants in addition to an inhibitor of complement activity as defined in the claims. Use of one or more immunosuppressants and an inhibitor of complement activity as defined in the claims in the manufacture of one or more medicaments or medicament packages is also provided. Such medicaments or medicament packages are useful in prolonging survival of an allograft in a subject mammal.

[0011] In certain embodiments, the inhibition of complement activity is effected by chronic administration of a drug directed against complement C5 as defined in the claims. A drug that inhibits complement activity is an antibody specific to one or more components of complement, for example, C5. In certain preferred embodiments, the antibody inhibits the cleavage of C5 and thereby inhibits the formation of both C5a and C5b-9. The antibody may be, e.g., a monoclonal antibody, a chimeric antibody (e.g., a humanized antibody), an antibody fragment (e.g., Fab), a single chain antibody, an Fv, or a domain antibody. The recipient is also treated with one or more immunosuppressive drugs, for example, cyclosporin A.

[0012] In certain embodiments, either an MHC mismatched recipient (i.e., a mammalian recipient of an MHC mismatched allograft), a presensitized recipient or an ABO mismatched recipient (i.e., a mammalian recipient of an AMB mismatched allograft) is treated. In this model, the recipient is again chronically treated with a complement inhibitor, preferably an anti-C5 monoclonal antibody, together with immunosuppressive drugs, preferably a chronic administration of cyclosporin A and a short-term administration of cyclophosphamide. This triple therapy results in extended graft survival in the presensitized allotransplant recipient.

[0013] The present disclosure also describes methods of prolonging survival of an allograft in a mammalian recipient by administering to the recipient agents that modulate the level and/or ratio of subclasses and/or isotypes of anti-donor immunoglobulins (Ig) in the recipient. In certain embodiments, an agent that reduces the level of anti-donor IgG1 in the recipient is preferred. In certain embodiments, an agent that increases the level of anti-donor IgG2a and/or IgG2b in the recipient is preferred. In certain embodiments, an agent that reduces the ratio of anti-donor IgG1/anti-donor IgG2a or IgG2b in the recipient is preferred.

[0014] The present disclosure also describes a method of prolonging survival of an allograft in a second mammalian recipient using an allograft that has been accommodated in a first mammalian recipient (i.e., the allograft has prolonged survival in the first recipient). The present disclosure further describes an allograft that is resistant to anti-donor antibodies in a mammalian recipient, and the allograft is prepared from a first recipient that has accommodated the allograft. In preferred embodiments, the first recipient has accommodated the allograft by receiving a treatment as described herein, such a triple therapy treatment involving administering to the first recipient a drug that inhibits complement activity and

Samsung Bioepis Exhibit No. 1002
Page 1524

two immunosuppressive agents.

[0015] Further provided are pharmaceutical packages. A pharmaceutical package of the present disclosure may comprise a drug that inhibits complement activity as defined in the claims and at least one immunosuppressive agent. The pharmaceutical package may further comprise a label for chronic administration. The pharmaceutical package may also comprise a label for self-administration by a patient, for example, a recipient of a transplant graft, or instructions for a caretaker of a recipient of a transplant graft. In certain embodiments, the drug and the agent in the pharmaceutical package are in a formulation or separate formulations that are suitable for chronic administration and/or self-administration.

[0016] The present disclosure also provides lyophilized formulations and formulations suitable for injection. Certain embodiments provide a lyophilized antibody formulation comprising an antibody that inhibits complement activity and a lyoprotectant. In preferred embodiments, the antibody formulation is suitable for chronic administration, for example, the antibody formulation is stable. Alternative embodiments provide an injection system comprising a syringe; the syringe comprises a cartridge containing an antibody that inhibits complement activity and is in a formulation suitable for injection.

[0017] An antibody as defined in the claims and employed in various embodiments of the present disclosure preferably inhibits the formation of terminal complement or C5a. In certain embodiments, antibody inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment. The whole antibody or antibody fragment may be a human, humanized, chimerized or deimmunized antibody or antibody fragment. In certain embodiments, the whole antibody or antibody fragment may inhibit cleavage of complement C5. In certain embodiments, the antibody fragment is a Fab, an F(ab')2, an Fv, a domain antibody, or a single-chain antibody. In preferred embodiments, the antibody fragment is pexelizumab. In alternative preferred embodiments, the whole antibody is eculizumab.

[0018] In certain embodiments, the antibody as defined in the claims, that inhibits complement activity is present in unit dosage form, which can be particularly suitable for self-administration. Similarly, an immunosuppressive agent of the present disclosure may also be present in unit dosage form.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019]

Figures 1A-1D show anti-donor antibody levels in presensitized versus unsensitized recipients under different treatments.

Figures 2A and 2B show comparison between triple therapy using anti-C5 antibody, CsA and CyP in presensitized allograft recipients and combination therapy using only anti-C5 antibody and CsA in presensitized allograft recipients. Figure 2A compares heart-allograft survival in various recipients under different treatments as indicated. Figure 2B shows histology and immunohistology, for example, for lymphocyte infiltration in heart allografts of recipients in different groups.

Figure 3 shows blocked terminal complement activity by anti-C5 antibody as compared to immunosuppressive agents.

Figures 4A-4D compare levels of anti-donor antibodies in presensitized recipients of allografts under monotherapy with anti-C5 antibody alone, double combination therapy with anti-C5 antibody and CsA, and triple combination therapy with anti-C5 antibody, CsA and CyP.

Figures 5A and 5B show change of ratios of IgG isotypes in allograft recipients that were untreated or under different treatments.

Figure 6 shows high-level expression of Bcl-2 and Bcl-xl proteins in long-term surviving heart grafts as compared to heart grafts of untreated animals.

Figure 7 shows improved second transplantation (re-transplantation) of an accommodated graft from a first transplantation recipient.

Figure 8 shows results from re-transplantation experiments.

Figure 9 shows results from re-transplantation experiments.

Samsung Bioepis Exhibit No. 1002
Page 1525

DETAILED DESCRIPTION

Overview: Rejection of Transplants or Grafts

[0020] Hyperacute rejection occurs within minutes to hours after transplant and is due to preformed antibodies to the transplanted tissue antigens. It is characterized by hemorrhage and thrombotic occlusion of the graft vasculature. The binding of antibody to endothelium activates complement, and antibody and complement induce a number of changes in the graft endothelium that promote intravascular thrombosis and lead to vascular occlusion, the result being that the grafted organ suffers irreversible ischemic damage (Abbas et al., 2000). Hyperacute rejection is often mediated by preexisting IgM alloantibodies, e.g., those directed against the ABO blood group antigens expressed on red blood cells. This type of rejection, mediated by natural antibodies, is the main reason for rejection of xenotransplants. Hyperacute rejection due to natural IgM antibodies is no longer a major problem with allografts because allografts are usually selected to match the donor and recipient ABO type. Hyperacute rejection of an ABO matched allograft may still occur, usually mediated by IgG antibodies directed against protein alloantigens, such as foreign MHC molecules, or against less well defined alloantigens expressed on vascular endothelial cells. Such antibodies may arise as a result of prior exposure to alloantigens through blood transfusion, prior transplantation, or multiple pregnancies (this prior exposure being referred to as "presensitization"). Abbas et al., 2000.

[0021] Acute rejection is a process of vascular and parenchymal injury mediated by T cells, macrophages, and antibodies that usually begins after the first week of transplantation. Abbas et al., 2001. T lymphocytes play a central role in acute rejection by responding to alloantigens, including MHC molecules, present on vascular endothelial and parenchymal cells. The activated T cells cause direct lysis of graft cells or produce cytokines that recruit and activate inflammatory cells, which cause necrosis. Both $CD4^+$ and $CD8^+$ cells may contribute to acute rejection. The destruction of allogeneic cells in a graft is highly specific and a hallmark of $CD8^+$ cytotoxic T lymphocyte killing. Abbas et al., 2000. $CD4^+$ T cells may be important in mediating acute graft rejection by secreting cytokines and inducing delayed-type hypersensitivity-like reactions in grafts, with some evidence available that indicates that $CD4^+$ T cells are sufficient to mediate acute rejection. Abbas et al., 2000. Antibodies can also mediate acute rejection after a graft recipient mounts a humoral immune response to vessel wall antigens and the antibodies that are produced bind to the vessel wall and activate complement. Abbas et al., 2000.

[0022] Chronic rejection is characterized by fibrosis with loss of normal organ structures occurring over a prolonged period. The pathogenesis of chronic rejection is less well understood than that of acute rejection. Graft arterial occlusion may occur as a result of the proliferation of intimal smooth muscle cells (Abbas et al., 2000). This process is called accelerated or graft arteriosclerosis and can develop in any vascularized organ transplant within 6 months to a year after transplantation.

[0023] For a transplant to be successful, the several modes of rejection must be overcome. Multiple approaches are utilized in preventing rejection. This may require administration of immunosuppressants, often several types to prevent the various modes of attack, e.g., inhibition of T-cell attack, antibodies, and cytokine and complement effects. Prescreening of donors to match them with recipients is also a major factor in preventing rejection, especially in preventing hyperacute rejection. Immunoadsorption of anti-HLA antibodies prior to grafting may reduce hyperacute rejection. Prior to transplantation the recipient or host may be administered anti-T cell reagents, e.g., the monoclonal antibody OKT3, Anti-Thymocyte Globulin (ATG), cyclosporin A, or tacrolimus (FK 506). Additionally, glucocorticoids and/or azathioprine may be administered to the host prior to transplant. Drugs used to aid in preventing transplant rejection include, but are not limited to, ATG or ALG, OKT3, daclizumab, basiliximab, corticosteroids, 15-deoxyspergualin, cyclosporins, tacrolimus, azathioprine, methotrexate, mycophenolate mofetil, 6-mercaptopurine, bredinin, brequinar, leflunamide, cyclophosphamide, sirolimus, anti-CD4 monoclonal antibodies, CTLA4-Ig, anti-CD154 monoclonal antibodies, anti-LFA1 monoclonal antibodies, anti-LFA-3 monoclonal antibodies, anti-CD2 monoclonal antibodies, and anti-CD45.

[0024] Allografts are rejected in part by the activation of T cells. The transplant recipient mounts a rejection response following $CD4^+$ T cell recognition of foreign antigens in the allograft. These antigens are encoded by the major histocompatibility complex (MHC). There are both Class I and Class II MHC molecules. In humans the class I MHC molecules are HLA-A, HLA-B, and HLA-C. The class II MHC molecules in humans are called HLA-DR, HLA-DQ and HLA-DP. In mice the class I MHC molecules are H-2K, H-2D and H-2L and the class II MHC molecules are I-A and I-E. When $CD4^+$ T cells bind the foreign MHC antigens they are activated and undergo clonal proliferation. The activated T cells secrete cytokines which aid in activating monocytes/macrophages, B cells and cytotoxic $CD8^+$ T cells. The activated monocytes/macrophages release agents which result in tissue damage, the B cells produce alloantibodies which lead to complement mediated tissue destruction, and the $CD8^+$ T cells kill graft cells in an antigen-specific manner through induction of apoptosis and cell lysis.

Samsung Bioepis Exhibit No. 1002
Page 1526

Immunosuppressive Agents

[0025] The numerous drugs utilized to delay graft rejection (i.e., to prolong their survival) work in a variety of ways. Immunosuppressive agents are widely used. See Stepkowski, 2000, for a review of the mechanism of action of several immunosuppressive drugs. Cyclosporin A is one of the most widely used immunosuppressive drugs for inhibiting graft rejection. It is an inhibitor of interleukin-2 or IL-2 (it prevents mRNA transcription of interleukin-2). More directly, cyclosporin inhibits calcineurin activation that normally occurs upon T cell receptor stimulation. Calcineurin dephosphorylates NFAT (nuclear factor of activated T cells) enabling it to enter the nucleus and bind to interleukin-2 promoter. By blocking this process, cyclosporin A inhibits the activation of the $CD4^+$ T cells and the resulting cascade of events which would otherwise occur. Tacrolimus is another immunosuppressant that acts by inhibiting the production of interleukin-2.

[0026] Rapamycin (Sirolimus), SDZ RAD, and interleukin-2 receptor blockers are drugs that inhibit the action of interleukin-2 and therefore prevent the cascade of events described above.

[0027] Inhibitors of purine or pyrimidine biosynthesis are also used to inhibit graft rejection. These prevent DNA synthesis and thereby inhibit cell division including the ability of T cells to divide. The result is the inhibition of T cell activity by preventing the formation of new T cells. Inhibitors of purine synthesis include azathioprine, methotrexate, mycophenolate mofetil (MMF) and mizoribine (bredinin). Inhibitors of pyrimidine synthesis include brequinar sodium and leflunomide. Cyclophosphamide is an inhibitor of both purine and pyrimidine synthesis.

[0028] Yet another method for inhibiting T cell activation is to treat the recipient with antibodies to T cells. OKT3 is a murine monoclonal antibody against CD3 which is part of the T cell receptor. This antibody inhibits the T cell receptor and suppresses T cell activation.

[0029] Numerous other drugs and methods for delaying allotransplant rejection are known to and used by those of skill in the art. One approach has been to deplete T cells, e.g., by irradiation. This has often been used in bone marrow transplants, especially if there is a partial mismatch of major HLA. Administration to the recipient of an inhibitor (blocker) of the CD40 ligand-CD40 interaction and/or a blocker of the CD28-B7 interaction has been used (U.S. Patent 6,280,957). Published PCT patent application WO 01/37860 teaches the administration of an anti-CD3 monoclonal antibody and IL-5 to inhibit the Th1 immune response. Published PCT patent application WO 00/27421 teaches a method for prophylaxis or treatment of corneal transplant rejection by administering a tumor necrosis factor-a antagonist. Glotz et al. (2002) show that administration of intravenous immunoglobulins (IVIg) can induce a profound and sustained decrease in the titers of anti-HLA antibodies thereby allowing a transplant of an HLA-mismatched organ. Similar protocols have included plasma exchanges (Taube et al., 1984) or immunoadsorption techniques coupled to immunosuppressive agents (Hiesse et al., 1992) or a combination of these (Montgomery et al., 2000). Changelian et al. (2003) teach a model in which immunosuppression is caused by an oral inhibitor of Janus kinase 3 (JAK3) which is an enzyme necessary for the proper signaling of cytokine receptors which use the common gamma chain (yc) (Interleukins-2, -4, -7, -9, -15, -21), the result being an inhibition of T cell activation. Antisense nucleic acids against ICAM-1 have been used alone or in combination with a monoclonal antibody specific for leukocyte-function associated antigen 1 (LFA-1) in a study of heart allograft transplantation (Stepkowski, 2000). Similarly, an anti-ICAM-1 antibody has been used in combination with anti-LFA-1 antibody to treat heart allografts (Stepkowski, 2000). Antisense oligonucleotides have additionally been used in conjunction with cyclosporin in rat heart or kidney allograft models, resulting in a synergistic effect to prolong the survival of the grafts (Stepkowski, 2000). Chronic transplant rejection has been treated by administering an antagonist of TGF-β which is a cytokine involved in differentiation, proliferation and apoptosis (U.S. Patent Application Publication US 2003/0180301).

Complement and Transplant/Graft Rejection

[0030] The role of complement in transplant rejection is well known. This is especially true in the case of xenotransplantation, but complement also plays a role in allotransplant rejection. For review, see Platt and Saadi, 1999. One aspect of complement's role is that ischemia-reperfusion injury may occur at the time that an organ graft is reperfused with the blood of the recipient. Complement may also cause some manifestations of allograft rejection.

[0031] The complement system is described in detail in U.S. Patent 6,355,245. The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of cellular and viral pathogens. There are at least 25 complement proteins, which are found as a complex collection of plasma proteins and membrane cofactors. The plasma proteins make up about 10% of the globulins in vertebrate serum. Complement components achieve their immune defensive functions by interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions.

[0032] The complement cascade progresses via the classical pathway or the alternative pathway. These pathways share many components and, while they differ in their initial steps, they converge and share the same "terminal complement" components (C5 through C9) responsible for the activation and destruction of target cells.

Samsung Bioepis Exhibit No. 1002
Page 1527

[0033] The classical complement pathway is typically initiated by antibody recognition of and binding to an antigenic site on a target cell. The alternative pathway is usually antibody independent and can be initiated by certain molecules on pathogen surfaces. Both pathways converge at the point where complement component C3 is cleaved by an active protease (which is different in each pathway) to yield C3a and C3b. Other pathways activating complement attack can act later in the sequence of events leading to various aspects of complement function.

[0034] C3a is an anaphylatoxin. C3b binds to bacterial and other cells, as well as to certain viruses and immune complexes, and tags them for removal from the circulation. C3b in this role is known as opsonin. The opsonic function of C3b is considered to be the most important anti-infective action of the complement system. Patients with genetic lesions that block C3b function are prone to infection by a broad variety of pathogenic organisms, while patients with lesions later in the complement cascade sequence, i.e., patients with lesions that block C5 functions, are found to be more prone only to Neisseria infection, and then only somewhat more prone (Fearon, 1983).

[0035] C3b also forms a complex with other components unique to each pathway to form classical or alternative C5 convertase, which cleaves C5 into C5a and C5b. C3 is thus regarded as the central protein in the complement reaction sequence since it is essential to both the alternative and classical pathways (Wurzner et al., 1991). This property of C3b is regulated by the serum protease Factor I, which acts on C3b to produce iC3b. While still functional as opsonin, iC3b cannot form an active C5 convertase.

[0036] C5 is a 190 kDa beta globulin found in normal serum at approximately 75 μg/mL (0.4 μM). C5 is glycosylated, with about 1.5-3 percent of its mass attributed to carbohydrate. Mature C5 is a heterodimer of a 999 amino acid 115 kDa alpha chain that is disulfide linked to a 656 amino acid 75 kDa beta chain. C5 is synthesized as a single chain precursor protein product of a single copy gene (Haviland et al., 1991). The cDNA sequence of the transcript of this gene predicts a secreted pro-C5 precursor of 1659 amino acids along with an 18 amino acid leader sequence.

[0037] The pro-C5 precursor is cleaved after amino acid 655 and 659, to yield the beta chain as an amino terminal fragment (amino acid residues +1 to 655) and the alpha chain as a carboxyl terminal fragment (amino acid residues 660 to 1658), with four amino acids deleted between the two.

[0038] C5a is cleaved from the alpha chain of C5 by either alternative or classical C5 convertase as an amino terminal fragment comprising the first 74 amino acids of the alpha chain (i.e., amino acid residues 660-733). Approximately 20 percent of the 11 kDa mass of C5a is attributed to carbohydrate. The cleavage site for convertase action is at or immediately adjacent to amino acid residue 733. A compound that would bind at or adjacent to this cleavage site would have the potential to block access of the C5 convertase enzymes to the cleavage site and thereby act as a complement inhibitor.

[0039] C5 can also be activated by means other than C5 convertase activity. Limited trypsin digestion (Minta and Man, 1977; Wetsel and Kolb, 1982) and acid treatment (Yamamoto and Gewurz, 1978; Vogt et al., 1989) can also cleave C5 and produce active C5b.

[0040] C5a is another anaphylatoxin. C5b combines with C6, C7, and C8 to form the C5b-8 complex at the surface of the target cell. Upon binding of several C9 molecules, the membrane attack complex (MAC, C5b-9, terminal complement complex-TCC) is formed. When sufficient numbers of MACs insert into target cell membranes the openings they create (MAC pores) mediate rapid osmotic lysis of the target cells. Lower, non-lytic concentrations of MACs can produce other effects. In particular, membrane insertion of small numbers of the C5b-9 complexes into endothelial cells and platelets can cause deleterious cell activation. In some cases activation may precede cell lysis.

[0041] As mentioned above, C3a and C5a are anaphylatoxins. These activated complement components can trigger mast cell degranulation, which releases histamine and other mediators of inflammation, resulting in smooth muscle contraction, increased vascular permeability, leukocyte activation, and other inflammatory phenomena including cellular proliferation resulting in hypercellularity. C5a also functions as a chemotactic peptide that serves to attract pro-inflammatory granulocytes to the site of complement activation.

[0042] Complement-binding recipient antibodies to donor alloantigens are considered to be the main cause of hyperacute graft rejection. Owing to pretransplant crossmatch testing, this prototype of humoral rejection is now rarely observed (Regele et al., 2001). Data are now showing that humoral immune mechanisms might contribute to other types of allograft rejection (Regele et al., 2001). High levels of panel reactive antibodies indicating humoral presensitization were found to be associated with inferior kidney graft survival (Opelz, 1992), the appearance of alloantibodies during the post-transplant period has been reported to predict poor graft outcome (Jeannet et al., 1970; Halloran et al., 1992), and selective removal of recipient IgG by immunoadsorption reversed some rejection episodes indicating the contribution of humoral immune mechanisms to rejection (Persson et al., 1995; Böhmig et al., 2000). Complement activation within a graft might indicate antibody-mediated graft injury. The complement cleavage product C4d is a marker for activation of the antibody-dependent classical pathway. Capillary C4d deposits in kidney allograft biopsies were associated with poor graft outcome.

[0043] Recently increasing evidence indicates that complement activation significantly contributes to the sensitization of allograft recipients and the development of tissue injury in allografts (Platt et al., 1999). Antibodies are the most thoroughly investigated mediators of activating the classical complement pathway. Clinically, alloantibodies are known

Samsung Bioepis Exhibit No. 1002
Page 1528

to activate complement (Baldwin et al., 2001). Halloran and Collins indicate that C4d deposition in peritubular capillaries of renal allografts is a sensitive and diagnostic marker of acute humoral rejection that correlates strongly with the presence of circulating donor-specific antibodies (Collins et al., 1999; Halloran, 2003). Further supporting evidence is seen in animals with complement inhibition (Pratt et al., 1996; Pruitt et al., 1991; Forbes et al., 1978) or deficiency (Pratt et al., 2000; Baurer et al., 1995) which exhibit significantly reduced inflammatory injury and lowered anti-donor immune responses. In ABMR, complement is suggested to be activated by the classical pathway and to play a key role in the pathogenesis (Collard et al., 1997). Although the role of complement in HAR or acute vascular rejection (AVR) following xenotransplantation has been well documented (Platt et al., 1999), precise mechanisms of complement in the pathogenesis of ABMR following allotransplantation has not yet been elucidated.

[0044] The C5 component of complement is cleaved to form products with multiple proinflammatory effects and thus represents an attractive target for complement inhibition within the immune-mediated inflammatory response. As described above, C5a is a powerful anaphylatoxin and chemotactic factor. Cellular activation by C5a induces the release of multiple additional inflammatory mediators (Jose et al., 1983). The complement activation pathways (classical, alternative, or mannan-binding lectin pathway) ultimately lead to the formation of the cytolytic membrane attack complex C5b-9 (Kirschfunk, 2001), which can mediate both direct tissue injury by cell lysis, and proinflammatory cell activation at sublytic doses (Saadi et al., 1995; Papadimitriou et al., 1991). Therefore, blocking both C5a and C5b-9 generation may be required for the optimal inhibition of complement-mediated inflammatory response following transplantation. At the same time, inhibition of the complement cascade at C5 does not impair the generation of C3b, preserving C3b-mediated opsonization of pathogenic microorganisms as well as solubilization and clearance of immune complexes (Liszewski, 1993).

[0045] The beneficial effect of anti-C5 mAb has previously been reported in several experimental models including myocardial reperfusion (Vakeva et al., 1998), systemic lupus erythematosus (Wang et al., 1996) and rheumatoid arthritis (Wang et al., 1995); as well as in human clinical trials (Kirschfink, 2001) of autoimmune disease, cardiopulmonary bypass and acute myocardial infarction. In addition, complement inactivation by a functionally blocking anti-C5 monoclonal Ab (mAb) prevented HAR in xenotransplantation models (Kroshus et al., 1995; Wang et al., 1999).

[0046] Methods of delaying allotransplant rejection by administration of complement inhibitors have been tested. Published PCT patent application WO 92/10205 discloses the use of a combination of cyclosporin and a soluble complement receptor (sCR1) to inhibit rejection of a cardiac allotransplant in a presensitized rat model. Complement receptor 1 binds complements C3b and C4b. Soluble forms of complement receptor 1 occur naturally or can be generated via recombinant DNA procedures. These soluble complement receptors have inhibited in vitro the consequences of complement activation (U.S. Patent 6,057,131). In WO 92/10205, rats, which had been presensitized to the cardiac allograft they were receiving, were administered cyclosporin A intramuscularly at 10 mg/kg/day beginning two days prior to transplant and continued until the time of graft rejection. Additionally, soluble complement receptor 1 (sCR1) was administered as a single intravenous bolus at 15 mg/kg immediately prior to reperfusion of the graft. Control animals with no drug treatment had the graft rejected at an average of 3.8 days. Those administered cyclosporin A alone rejected the grafts at an average of 57 days (this was quite variable with two rats rejecting quickly at 2 and 4 days and a third rat rejecting at 166 days). Rats administered sCR1 alone rejected the grafts at an average of 44 days. Those rats administered the combination of cyclosporin A and sCR1 rejected the grafts at an average of 147 days. The combination of chronic cyclosporin A and single bolus sCR1 was seen to result in a synergistic effect greatly prolonging the time until graft rejection. Earlier studies by Pruitt and Bollinger (1991) used a similar model of a presensitized rat allograft to show that administration of sCR1 alone to inactivate complement resulted in increased time before graft rejection.

[0047] Sims et al. (U.S. Patent 5,135,916) suggest using inhibitors of complement, e.g., CD59 or antibodies against C7 or C9 to block the formation of the C5b-9 complex, to treat the vascular endothelium of organs and tissues to be transplanted. This would prevent the C5b-9 initiated cell necrosis. The C5b-9 inactivators would be added to the perfusate or storage medium to protect the vascular lining cells from ongoing complement activation during in vitro storage. Additionally the organ or tissue would be protected from the cytolytic and thrombotic effects arising from complement activation initiated upon transplantation, thereby circumventing complement mediated acute rejection. Sims et al. (U.S. Patent 5,573,940 and U.S. Patent 6,100,443) also teach a method of expressing CD59 in the transplanted tissue or organ to protect the transplanted organ from rejection. This can be accomplished by transfecting the cells being transplanted.

[0048] Although the several drugs developed to date in combination with methods of prescreening donors and recipients to match the donor allograft to the recipient have over time increased the average length of time of survival of allografts, many allografts are nonetheless rejected during the life-time of the recipient. In general, the prior art advances have mainly been directed to overcoming acute graft rejection. Further, the role of activated terminal complement components in antibody-mediated allograft rejection has not been examined using inhibitors that specifically target the complement cascade at the C5 protein level. The methods described herein and as exemplified in the Examples advance the allotransplant art by inhibiting chronic rejection of allografts, in particular, allografts in a presensitized recipient. New methods are presented for further prolonging allograft survival by using a proper combination of immunosuppressive

Samsung Bioepis Exhibit No. 1002
Page 1529

drugs in combination with a chronic administration of a complement inhibitor.

Methods and Uses

[0049] The means disclosed herein are used to prolong allograft survival. The means generally include using an inhibitor of complement activity as defined in the claims in combination with one or more immunosuppressants.

[0050] Further suitable complement inhibitors are known to those of skill in the art. Antibodies can be made to individual components of activated complement, e.g., antibodies to C5a, C7, C9, etc. (see, e.g., U.S. Patent 6,534,058; published U.S. patent application US 2003/0129187; and U.S. Patent 5,660,825). Proteins are known which inhibit complement-mediated lysis, including CD59, CD55, CD46 and other inhibitors of C8 and C9 (see, e.g., U.S. Patent 6,100,443). U.S. Patent 6,355,245 teaches an antibody which binds to C5 and prevents it from being cleaved into C5a and C5b thereby preventing the formation not only of C5a but also the C5b-9 complex. Proteins known as complement receptors and which bind complement are also known (see, Published PCT Patent Application WO 92/10205 and U.S. Patent 6,057,131). Use of soluble forms of complement receptors, e.g., soluble CR1, can inhibit the consequences of complement activation such as neutrophil oxidative burst, complement mediated hemolysis, and C3a and C5a production. Those of skill in the art recognize the above as some, but not all, of the known methods of inhibiting complement and its activation.

[0051] Suitable immunosuppressants include, but are not limited to, ATG or ALG, OKT3, daclizumab, basiliximab, corticosteroids, 15-deoxyspergualin, cyclosporins, tacrolimus, azathioprine, methotrexate, mycophenolate mofetil, 6-mercaptopurine, bredinin, brequinar, leflunamide, cyclophosphamide, sirolimus, anti-CD4 monoclonal antibodies, CTLA4-Ig, anti-CD154 monoclonal antibodies, anti-LFA1 monoclonal antibodies, anti-LFA-3 monoclonal antibodies, anti-CD2 monoclonal antibodies, and anti-CD45.

[0052] An allograft can include a transplanted organ, part of an organ, tissue or cell. These include, but are not limited to, heart, kidney, lung, pancreas, liver, vascular tissue, eye, cornea, lens, skin, bone marrow, muscle, connective tissue, gastrointestinal tissue, nervous tissue, bone, stem cells, islets, cartilage, hepatocytes, and hematopoietic cells.

[0053] At least part of the reason for the failure of allografts is that one response by the recipient of an allograft is the activation of complement. This results in the formation of C5a and C5b-9 which are potent proinflammatory molecules which aid in causing graft failure. Without wishing to be bound by any proposed theory, Applicants theorized that inhibiting the formation of C5a and C5b-9 or inhibiting C5a and C5b-9 which was present would aid in preventing graft failure. Furthermore, it was theorized that so long as the allograft is present, the recipient will continue to attempt to mount an immune response against the graft, and this response will include attempts to produce C5a and C5b-9. If not prevented, this complement response will lead to acute vascular rejection in the short term and could contribute to chronic graft rejection in the long term. Prior art methods of using inhibitors of complement activity were limited to administering these inhibitors only at the time of transplant. This was helpful in preventing acute rejection, but as the results disclosed herein illustrate, improved results are obtained by administration of such inhibitors for a longer term. This long-term administration aids in preventing a chronic rejection of the allograft as opposed to only aiding in preventing an acute rejection. The result is a longer term survival of the allograft as compared to either not administering an inhibitor of complement activity or administering such an inhibitor only at the time of transplant of the allograft. Although very commonly it is desirable that the allograft will survive for the remaining lifetime of the recipient, there are times when the allograft is needed only for a shorter length of time, e.g., a bridge organ to bridge the time until the recipient's own organ can recover on its own, at which time the allograft will no longer be needed. The length of time such a graft will be needed will vary, but will usually be longer than the time at which acute rejection would occur and may be long enough for chronic rejection to occur. This period of desired survival for a bridge graft may be several months, e.g., six months.

[0054] To prove that long-term inhibition of complement activity will prolong allograft survival, experiments were performed in which complement activation was inhibited in a chronic fashion and not merely at the time of transplant. Chronic treatment means treatment during an extended period up to the lifetime of the allograft. This can be daily treatment but is not limited to daily treatment. Chronic treatment will maintain an effective amount of the drug in the allograft recipient. For example, a preferred method is to include the anti-C5 monoclonal antibody eculizumab in the treatment. In studies of persons suffering from paroxysmal nocturnal hemoglobinuria (PNH), eculizumab has been administered at a dose of 900 mg/patient once every 12-14 days. This dosing has been found to completely and consistently block terminal complement activity and has greatly inhibited the symptoms of PNH (Hillmen et al., 2004). The administered dose is able to block the effects of complement for approximately two weeks before the eculizumab is inactivated or removed from the body. Therefore, a chronic treatment of eculizumab may be, e.g., the administration of 900 mg to the allograft recipient once every two weeks for the remaining life-time of the patient. Similarly, other drugs can be delivered chronically as needed, whether this is on a daily basis or another schedule is required to maintain an effective amount of the drug in the allograft recipient. Because it is well known that graft rejection can be caused by more than just complement activation, e.g., by T cell activity, the experiments included immunosuppressants such as cyclosporin to further aid in preventing graft rejection.

[0055] A preferred method of inhibiting complement activity is to use a monoclonal antibody which binds to complement

Samsung Bioepis Exhibit No. 1002
Page 1530

C5 and prevents C5 from being cleaved. This prevents the formation of both C5a and C5b-9 while at the same time allowing the formation of C3a and C3b which are beneficial to the recipient. Such antibodies that are specific to human complement are known (U.S. Patent 6,355,245). These antibodies disclosed in U.S. Patent 6,355,245 include both a whole or full-length antibody (now named eculizumab) and a single-chain antibody (now named pexelizumab). A similar antibody against mouse C5 is called BB5.1 (Frei et al., 1987). BB5.1 was utilized in the experiments set forth below. Antibodies to inhibit complement activity need not be monoclonal antibodies. They can be, e.g., polyclonal antibodies. They may additionally be antibody fragments. An antibody fragment includes, but is not limited to, an Fab, F(ab'), $F(ab')_2$, a single-chain antibody, a domain antibody and an Fv. Furthermore, it is well known by those of skill in the art that antibodies can be humanized (Jones et al., 1986), chimerized, or deimmunized. An antibody may also comprise a mutated Fc portion, such that the mutant Fc does not activate complement. The antibodies to be used in the present disclosure may be any of these. It is preferable to use humanized antibodies when the recipient of the allograft is a human.

Administration and Formulations

[0056] Administration of the inhibitor of complement activity as defined in the claims is performed according to methods known to those of skill in the art. These inhibitors are administered preferably before the time of allograft transplantation or at the time of transplantation with administration continuing in a chronic fashion. These inhibitors can additionally be administered during a rejection episode in the event such an episode does occur.

[0057] The present disclosure also provides uses of a drug that inhibits complement activity as defined in the claims and an immunosuppressive agent in the manufacture of a medicament or medicament package. Such medicament or medicament package is useful in prolonging allograft survival in a recipient, in particular, chronic survival of the allograft. In preferred embodiments, the medicament or medicament package is formulated and prepared such that it is suitable for chronic administration to the recipient of the allograft, for example, stable formulations are employed. In certain embodiments, the medicament or medicament package is formulated and prepared such that it is suitable for concurrent administration of the drug that inhibits complement activity as defined in the claims and the immunosuppressive drug to the recipient of the allograft. In certain embodiments, the medicament or medicament package is formulated and prepared such that it is suitable for sequential (in either order) administration of the drug that inhibits complement activity as defined in the claims and the immunosuppressive drug to the recipient of the allograft.

[0058] A pharmaceutical package of the present disclosure may comprise a drug that inhibits complement activity as defined in the claims and at least one immunosuppressive agent. The pharmaceutical package may further comprise a label for chronic administration. The pharmaceutical package may also comprise a label for self-administration by a patient, for example, a recipient of a transplant graft, or instructions for a caretaker of a recipient of a transplant graft. In certain embodiments, the drug and the agent in the pharmaceutical package are in a formulation or separate formulations that are suitable for chronic administration and/or self-administration.

[0059] The present disclosure also provides lyophilized formulations and formulations suitable for injection. Certain embodiments provide a lyophilized antibody formulation comprising an antibody that inhibits complement activity and a lyoprotectant. In preferred embodiments, the antibody formulation is suitable for chronic administration, for example, the antibody formulation stable. Alternative embodiments provide an injection system comprising a syringe; the syringe comprises a cartridge containing an antibody that inhibits complement activity and is in a formulation suitable for injection.

[0060] An antibody as defined in the claims and employed in various embodiments of the present disclosure preferably inhibits the formation of terminal complement or C5a. In certain embodiments, antibody inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment. The whole antibody or antibody fragment may be a human, humanized, chimerized or deimmunized antibody or antibody fragment. In certain embodiments, the whole antibody or antibody fragment may inhibit cleavage of complement C5. In certain embodiments, the antibody fragment is a Fab, an F(ab')2, an Fv, a domain antibody, or a single-chain antibody. In preferred embodiments, the antibody fragment is pexelizumab. In alternative preferred embodiments, the whole antibody is eculizumab.

[0061] In certain embodiments, the antibody as defined in the claims, that inhibits complement activity is present in unit dosage form, which can be particularly suitable for self-administration. Similarly, an immunosuppressive agent of the present disclosure may also be present in unit dosage form. A formulated product of the present disclosure can be included within a container, typically, for example, a vial, cartridge, prefilled syringe or disposable pen. A doser such as the doser device described in U.S. Patent 6,302,855 may also be used, for example, with an injection system of the present disclosure.

[0062] A "stable" formulation is one in which the drug (e.g., an antibody) or agent therein essentially retains its physical and chemical stability and integrity upon storage. Various analytical techniques for measuring protein stability are available in the art and are reviewed in Peptide and Protein Drug Delivery, 247-301, Vincent Lee Ed., Marcel Dekker, Inc., New York, N.Y., Pubs. (1991) and Jones, A. Adv. Drug Delivery Rev. 10: 29-90 (1993). Stability can be measured at a selected temperature for a selected time period. For example, the extent of aggregation following lyophilization and storage can be used as an indicator of protein stability. For example, a "stable" formulation may be one wherein less

Samsung Bioepis Exhibit No. 1002
Page 1531

than about 10% and preferably less than about 5% of the protein is present as an aggregate in the formulation. In other embodiments, any increase in aggregate formation following lyophilization and storage of the lyophilized formulation can be determined. For example, a "stable" lyophilized formulation may be one wherein the increase in aggregate in the lyophilized formulation is less than about 5% and preferably less than about 3%, when the lyophilized formulation is stored at 2-8 °C for at least one year. In other embodiments, stability of the protein formulation may be measured using a biological activity assay.

[0063] A "reconstituted" formulation is one which has been prepared by dissolving a lyophilized protein formulation in a diluent such that the protein is dispersed in the reconstituted formulation. The reconstituted formulation in suitable for administration (e.g. parenteral administration) to a patient to be treated with the protein of interest and, in certain embodiments of the invention, may be one which is suitable for subcutaneous administration.

[0064] An isotonic reconstituted formulation is preferable in certain embodiments. By "isotonic" is meant that the formulation of interest has essentially the same osmotic pressure as human blood. Isotonic formulations will generally have an osmotic pressure from about 250 to 350 mOsm. Isotonicity can be measured using a vapor pressure or ice-freezing type osmometer, for example.

[0065] A "lyoprotectant" is a molecule which, when combined with a drug (e.g., antibody) of interest, significantly prevents or reduces chemical and/or physical instability of the drug (e.g., antibody) upon lyophilization and subsequent storage. Exemplary lyoprotectants include sugars such as sucrose or trehalose; an amino acid such as monosodium glutamate or histidine; a methylamine such as betaine; a lyotropic salt such as magnesium sulfate; a polyol such as trihydric or higher sugar alcohols, e.g. glycerin, erythritol, glycerol, arabitol, xylitol, sorbitol, and mannitol; propylene glycol; polyethylene glycol; Pluronics; and combinations thereof. The preferred lyoprotectant is a non-reducing sugar, such as trehalose or sucrose.

[0066] The lyoprotectant is added to the pre-lyophilized formulation in a "lyoprotecting amount" which means that, following lyophilization of the drug (e.g., antibody) in the presence of the lyoprotecting amount of the lyoprotectant, the drug (e.g., antibody) essentially retains its physical and chemical stability and integrity upon lyophilization and storage.

[0067] The "diluent" of interest herein is one which is pharmaceutically acceptable (safe and non-toxic for administration to a human) and is useful for the preparation of a reconstituted formulation. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (e.g. phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution.

[0068] A "preservative" is a compound which can be added to the diluent to essentially reduce bacterial action in the reconstituted formulation, thus facilitating the production of a multi-use reconstituted formulation, for example. Examples of potential preservatives include octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride (a mixture of alkylbenzyldimethylammonium chlorides in which the alkyl groups are long-chain compounds), and benzethonium chloride. Other types of preservatives include aromatic alcohols such as phenol, butyl and benzyl alcohol, alkyl parabens such as methyl or propyl parahen, catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol.

[0069] A "bulking agent" is a compound which adds mass to the lyophilized mixture and contributes to the physical structure of the lyophilized cake (e.g. facilitates the production of an essentially uniform lyophilized cake which maintains an open pore structure). Exemplary bulking agents include mannitol, glycine, polyethylene glycol and xorbitol.

[0070] Accordingly, a stable lyophilized antibody formulation can be prepared using a lyoprotectant (preferably a sugar such as sucrose or trehalose), which lyophilized formulation can be reconstituted to generate a stable reconstituted formulation having an antibody concentration which is significantly higher (e.g. from about 2-40 times higher, preferably 3-10 times higher and most preferably 3-6 times higher) than the antibody concentration in the pre-lyophilized formulation. Such high protein concentrations in the reconstituted formulation are considered to be particularly useful where the formulation is intended for subcutaneous administration. Despite the very high protein concentration in the reconstituted formulation, the reconstituted formulation can be stable (i.e. fails to display significant or unacceptable levels of chemical or physical instability of the protein) at 2-8 °C. for at least about 30 days. See U.S. Patent 6,821,515. In certain embodiments, the reconstituted formulation is isotonic.

[0071] When reconstituted with a diluent comprising a preservative (such as bacteriostatic water for injection, BWFI), the reconstituted formulation may be used as a multi-use formulation. Such a formulation is useful, for example, where a subject patient requires frequent administrations of the drug or antibody and/or agent to treat a chronic medical condition. The advantage of a multi-use formulation is that it facilitates ease of use for the patient, reduces waste by allowing complete use of vial contents, and results in a significant cost savings for the manufacturer since several doses are packaged in a single vial (lower filling and shipping costs).

[0072] The present disclosure also describes a method for preparing a formulation comprising the steps of: (a) lyophilizing a mixture of an antibody and a lyoprotectant; and (b) reconstituting the lyophilized mixture of step (a) in a diluent such that the reconstituted formulation is isotonic and stable.

[0073] An article of manufacture is also described herein which comprises: (a) a container which holds a lyophilized mixture of an antibody and a lyoprotectant; and (b) instructions for reconstituting the lyophilized mixture with a diluent to a desirable antibody concentration in the reconstituted formulation. The article of manufacture may further comprise

Samsung Bioepis Exhibit No. 1002
Page 1532

a second container which holds a diluent (e.g. bacteriostatic water for injection (BWFI) comprising an aromatic alcohol).

[0074] An injection system of the present disclosure may employ a medication delivery pen as described in U.S. Patent 5,308,341. Medication delivery pens have been developed to facilitate the self-administration of medication. A medication of the present disclosure can be a drug that inhibits complement activity, for example an antibody specific to complement C5, and/or an immunosuppressive agent. One medication delivery pen includes a vial holder into which a vial of insulin or other medication may be received. The vial holder is an elongate generally tubular structure with proximal and distal ends. The distal end of the vial holder includes mounting means for engaging a double-ended needle cannula. The proximal end also includes mounting means for engaging a pen body which includes a driver and dose setting apparatus. A disposable medication containing vial for use with the prior art vial holder includes a distal end having a pierceable elastomeric septum that can be pierced by one end of a double-ended needle cannula. The proximal end of this vial includes a stopper slidably disposed in fluid tight engagement with the cylindrical wall of the vial. This medication delivery pen is used by inserting the vial of medication into the vial holder. A pen body then is connected to the proximal end of the vial holder. The pen body includes a dose setting apparatus for designating a dose of medication to be delivery by the pen and a driving apparatus for urging the stopper of the vial distally for a distance corresponding to the selected dose.

[0075] The user of the pen mounts a double-ended needle cannula to the distal end of the vial holder such that the proximal point of the needle cannula pierces the septum on the vial. The patient then selects a dose and operates the pen to urge the stopper distally to deliver the selected dose. The dose selecting apparatus returns to zero upon injection of the selected dose. The patient then removes and discards the needle cannula, and keeps the prior art medication delivery pen in a convenient location for the next required medication administration. The medication in the vial will become exhausted after several such administrations of medication. The patient then separates the vial holder from the pen body. The empty vial may then be removed and discarded. A new vial can be inserted into the vial holder, and the vial holder and pen body can be reassembled and used as explained above.

[0076] Accordingly, a medication delivery pen generally has a drive mechanism for accurate dosing and ease of use. A dosage mechanism such as a rotatable knob allows the user to accurately adjust the amount of medication that will be injected by the pen from a prepackaged vial of medication. To inject the dose of medication, the user inserts the needle under the skin and depresses the knob once as far as it will depress. The pen may be an entirely mechanical device or it may be combined with electronic circuitry to accurately set and/or indicate the dosage of medication that is injected into the user. See U.S. Patent 6,192,891.

[0077] The present disclosure also presents controlled-release or extended-release formulations suitable for chronic and/or self-administration of a medication.

[0078] The various formulations can be administered to a patient in need of treatment (e.g., a recipient of an allograft) with the medication (e.g., an antibody of the present disclosure and at least one immunosuppressive agent) by intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes.

[0079] In certain embodiments, a formulation is administered to the patient by subcutaneous (i.e. beneath the skin) administration. For such purposes, the formulation may be injected using a syringe. However, other devices for administration of the formulation are available such as injection devices (e.g. the Inject-ease® and Genject® devices); injector pens (such as the GenPen®); needleless devices (e.g. MediJector® and BioJector®); and subcutaneous patch delivery systems.

[0080] The present methods and uses are described with reference to the following Examples, which are offered by way of illustration and are not intended to limit the disclosure in any manner. Standard techniques well known in the art or the techniques specifically described below are utilized. The following abbreviations are used herein: ABMR, antibody-mediated rejection; ACHR, accelerated humoral rejection; ACR, acute cellular rejection; AVR, acute vascular rejection; CsA, cyclosporin; CyP, cyclophosphamide; HAR, hyperacute rejection; MCP-1, monocyte chemotactic protein 1; MST, mean survival time; POD, postoperative day.

Example 1

Methods

[0081] *Animals and Immunosuppressive Drugs* Male adult C3H (H-2^k) mice and BALB/c (H-2^d) mice (Jackson Labs, Bar Harbor, Maine) weighing 25-30 g were chosen as donors and recipients, respectively. In the groups receiving immunosuppression, the recipients were injected with CsA (15 mg/kg/day, s.c., daily from day 0 to endpoint rejection or until day 100), or with CyP (40 mg/kg/day, i.v., on day 0 and 1), or with anti-C5 mAb (clone BB5.1, Alexion Pharmaceuticals Inc., 40 mg/kg/day, i.p., day 0-2, followed by twice a week, day 0-60). Animals were housed under conventional conditions at the Animal Care Facility, University of Western Ontario, and were cared for in accordance with the guidelines established by the Canadian Council on Animal Care. Olfert et al., 1993.

[0082] *Skin Presensitization* Full-thickness skin grafts taken from C3H donors were cut into square pieces of 1×1 cm^2

Samsung Bioepis Exhibit No. 1002
Page 1533

and transplanted onto the back of the BALB/c recipients' thorax one week prior to heart transplantation from the same donors. Rejection was defined as complete necrosis of the skin grafts.

[0083] *Abdominal and Cervical Cardiac Transplantation* Seven days after skin presensitization, C3H mouse hearts were transplanted into the abdomen of presensitized BALB/c recipients by anastomosing the donor aorta and recipient aorta, and the donor pulmonary artery and recipient inferior vena cava. In the groups with re-transplantation, second heart grafts harvested from either naive C3H mice or long-term surviving presensitized BALB/c recipients were transplanted into the cervical area of the recipients carrying a long-term surviving first abdominal heart graft by anastomosing the donor aorta and recipient carotid artery, and the donor pulmonary artery and recipient external jugular vein (end-to-side). The heart grafts were monitored daily until rejection unless otherwise indicated and rejection was defined as complete cessation of pulsation.

[0084] *Experimental Groups* Presensitized recipients were randomly assigned to eight groups, each consisting of eight animals: Group 1, mice with no treatment; Group 2, mice treated with CsA; Group 3, mice treated with CyP; Group 4, mice treated with CsA plus CyP; Group 5, mice treated with anti-C5 mAb; Group 6, mice treated with anti-C5 mAb plus CsA; Group 7, mice treated with anti-C5 mAb plus CyP; Group 8, mice treated with anti-C5 mAb in combination of CsA and CyP. When cardiac impulses were no longer palpable or at POD100, the grafts were removed for routine histology, immunohistochemistry and western blot analysis, serum samples were collected for flow cytometric analysis and complement hemolytic assay. Five additional animals were placed and sacrificed in groups 6 and 8 on POD3 (MST for groups 1-5, 7) to allow for comparisons at a uniform time point. Serum samples were also collected on POD 11, 21, 28 and 60 in Group 8 for detecting the sequential changes of anti-donor antibody levels and complement activity. In addition, when triple therapy treated presensitized recipients carried a first heart graft for 100 days, they were re-transplanted with a second heart. A naive C3H heart or a 100-day surviving C3H heart from another presensitized BALB/c recipient was used as the second heart. Eight animals were included in each re-transplant group.

[0085] *Graft Histology* Tissue samples were fixed in 10% buffered formaldehyde. Specimens were then embedded in paraffin, and sectioned for H&E staining. The microscopic sections were examined in a blinded fashion for severity of rejection by a pathologist. Criteria for graft rejection included the presence of vasculitis, thrombosis, hemorrhage and lymphocyte infiltration. These changes were scored as: 0, no change; 1, minimum change; 2, mild change; 3, moderate change; or 4, marked change.

[0086] *Immunohistochemistry* Four micrometer sections were cut from tissue samples embedded in Tissue-Tek O.C.T gel (Optimum Cutting Temperature, Skura Finetek, Torrance, CA) mounted on gelatin-coated glass microscope slides and stained by a standard indirect avidin-biotin immunoperoxidase staining method using an Elite Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA). Specimens were stained for $CD4^+$ and $CD8^+$ cells with biotin-conjugated rat anti-mouse CD4 mAb (clone YTS 191.1.2, Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) and biotin-conjugated rat anti-mouse CD8 mAb (clone 53-6.7, Pharmingen, Franklin Lakes, NJ), respectively. Intragraft monocyte/macrophage infiltration was detected by staining with biotin-conjugated rat anti-mouse Mac-1 mAb (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada). Mouse IgG and IgM deposition in grafts was detected using biotin-conjugated goat anti-mouse-IgG and goat anti-mouse-IgM (Cedarlane). For identification of complement deposition, sections were serially incubated with goat anti-C3 or anti-C5 polyclonal Abs (Quidel, San Diego, CA), biotinylated rabbit anti-goat IgG (Vector Laboratories), and HRP-conjugated-streptavidin (Zymed Laboratories, South San Francisco, CA). Slides were washed with phosphate-buffered saline between steps, and examined under light microscopy. Negative controls were performed by omitting the primary antibodies. The immunostaining was scored in five high-power fields of each section, and five independent experiments were performed. The sections of immunoperoxidase staining were graded from 0 to 4+ according to the staining intensity: 0, negative; 1+, equivocal; 2+, weak staining; 3+, moderate staining; and 4+, very intensive staining.

[0087] *Flow Cytometry* The circulating anti-donor specific IgG and IgM antibodies were evaluated in the recipient serum by FACScan flow cytometry (Becton Dickinson, Mountain View, CA). Glotz et al., (1993); Tyan et al. (1994). Briefly, C3H mouse splenocytes were isolated and incubated at 37 °C for 30 minutes with serum from naive control and experimental groups. To stain for total IgG, IgG1, IgG2a, IgG2b and IgM, the cells were washed and incubated with FITC-conjugated goat antibody specific for the Fc portion of mouse IgG or with phycoerythrin-conjugated goat antibody specific for the μ-chain of mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA), or with FITC-conjugated goat anti-mouse IgG1 (CALTAG Laboratories, Burlingame, CA), or with FITC-conjugated goat anti-mouse IgG2a (CALTAG), or with FITC-conjugated goat anti-mouse IgG2b (CALTAG). After 1 hour of staining at 4 °C, the cells were washed with PBS, resuspended at 5×10^6/mL, and analyzed by flow cytometry for mean channel fluorescence intensity, which represents the antibody-binding reactivity.

[0088] *Complement Hemolytic Assay* The purified anti-C5 mAb was serially diluted twofold (175-0.1 μg/ml) in GVB^{2+} buffer (gelatin Veronal-buffered saline: 0.1% gelatin, 141 mM NaCl, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, and 1.8 mM sodium barbital) and added in triplicate (50 μl/well) to a 96-well plate. BALB/c mouse serum was diluted to 40% v/v with GVB^{2+} buffer and added (50 μl/ml) to the rows of the same 96-well plate such that the final concentration of BALB/c mouse serum in each well was 20%. The plate was then incubated at room temperature for approximately 30 min while chicken

Samsung Bioepis Exhibit No. 1002
Page 1534

erythrocytes were prepared. Chicken erythrocytes were washed 5×1 ml with GVB^{2+} buffer and resuspended to a final concentration of 5×10^7/ml in GVB^{2+}. Four milliliters of the chicken erythrocytes were sensitized by adding anti-chicken RBC polyclonal antibody (Intercell Technologies, Hopewell, NJ, 0.1% v/v) and the cells were incubated at 4 °C for 15 min with frequent vortexing. The cells were then washed 2×1 ml with GVB^{2+} and resuspended to a final volume of 2.4 ml in GVB^{2+}. The chicken erythrocytes (30 μl/well, 2.5×10^6 cells) were added to the plate containing serum and anti-C5 mAb as described above, mixed well, and incubated at 37 °C for 30 min. The plate was then centrifuged at 1000xg for 2 min, and 85 μl of the supernatant was transferred to a new 96-well microtiter plate. The plate was read at OD 415 nm using a microplate reader and the percentage of hemolysis was determined using this formula:

$$\%\ \text{hemolysis} = 100 \times \frac{(\text{OD sample}) - (\text{OD } GVB^{2+} \text{ control})}{(\text{OD 100\% lysed control}) - (\text{OD } GVB^{2+} \text{ control})}$$

with 100% lysed control obtained by the addition of 100 μl GVB^{2+} containing 0.1% NP-40 to the 30 μg/ml of chicken erythrocytes as prepared above.

[0089] *Western Blot Analysis* Sonication of frozen heart samples was performed in RIPA lysis buffer (Santa Cruz Biotechnology, Inc.) at 4 °C for 1 minute at 10-second intervals, followed by microcentrifugation at 13,000 rpm for 10 minutes at 4 °C. Clarified supernatants were immediately quantitated in triplicate for protein content using Detergent-compatible protein assay kit (BIO-RAD). Heart lysates (10μg protein/well) were separated on NuPAGE, 4-12% gradient Bis-Tris gels and MES buffer system (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membrane (0.45 μm pore size; Invitrogen) using a semi-dry transfer apparatus (BIO-RAD). Membranes were cut appropriately at the correct molecular weights to allow the development of the blots with two different primary antibodies per blot such that each blot was exposed to a test antibody and an internal control antibody to insure equal sample loading. The test primary antibodies including anti-Bcl-2 (N-19) rabbit polyclonal sera (Santa Cruz Biotechnology, Inc.) and anti-Bcl-XS/L (M-125) rabbit polyclonal sera (Santa Cruz Biotechnology, Inc.) were used to detect intragraft expression of Bcl-2 and Bcl-xl proteins. Anti-calsequestrin rabbit polyclonal sera (Calbiochem) were used as internal control primary antibody (Kobayashi et al., 1999). Detection of primary antibody binding was performed as previously described (Arp et al., 1996) by exposing washed incubated blots to a polyclonal goat anti-rabbit IgG fraction conjugated to horseradish peroxidase (HRP; Roche Laboratories) and then appropriately developed by exposure to enhance chemiluminescence for HRP-conjugated antibodies (Roche Laboratories).

[0090] *Statistical Analysis* The data were reported as the mean ± SD. Allograft survival among experimental groups was compared using the rank-log test. Histological and immunohistological findings were analyzed using the Mann-Whitney U test. Flow cytometric data and western blot data were analyzed using one-way ANOVA. Differences with p values less than 0.05 were considered significant.

Example 2

Presensitization with C3H donor skin graft induces antibody-mediated ACHR in heart allografts of BALB/c recipients.

[0091] To develop a suitable small animal model that mimics presensitized patients in the clinic and to study ABMR, a novel, fully MHC-mismatched mouse ABMR model has been developed through presensitization of mouse recipients. In this model, BALB/c recipients were presensitized with C3H donor skin grafts one week prior to heart transplantation from the same donor. Seven days after donor skin presensitization, serum level of anti-donor IgG, but not IgM antibody was markedly elevated and reached to a peak level in the presensitized BALB/c recipients (Figure 1A). Heart transplantation from same donor was then performed in these highly sensitized recipients. Without immunosuppression, C3H heart grafts were rapidly rejected in 3.1 ± 0.4 days by ACHR, characterized by severe thrombosis, hemorrhage and infarction (Figure 1B-a). In contrast, same heart grafts in unsensitized BALB/c recipients (with mean survival time, MST of 8.2 ± 0.8 days) show the normal histology on post-operative day (POD) 3 (Figure 1B-b). When compared to unsensitized BALB/c recipients at the same day, heart grafts in presensitized animals revealed massive IgG antibody and complement (C3 and C5) deposition, but minimal $CD4^+$ and $CD8^+$ cell infiltration (Table 1). Furthermore, circulating anti-donor IgG levels in presensitized recipients were significantly higher than those of unsensitized same recipients receiving a heart graft on POD3 ($P< 0.01$, Figure 1C). However, anti-donor IgM remained at very low levels both in circulation

Samsung Bioepis Exhibit No. 1002
Page 1535

(Figure 1C) and in heart grafts (Table 1) and it showed no significant difference between unsensitized and presensitized recipients. In addition, normal levels of complement hemolytic activity were shown in both presensitized and unsensitized heart recipients without treatment (Figure 1D). These data indicate that this is an ideal transplant model to study ABMR in presensitized recipients in which complement plays an important role in the pathogenesis.

Table 1.
Comparison of immunohistological changes of C3H heart allografts in unsensitized and Dresensitized BALB/c recipients on POD3

Groups	Unsensitized	Presensitized
IgG	1+	4+
IgM	1+	1+
C3	2+	3+
C5	2+	3+
CD4	0	1+
CD8	0	1+

Grades for immunoperoxidase staining: 0, negative; 1+, equivocal; 2+, weak; 3+, moderate; 4+, intense.

Anti-C5 mAb in combination with CsA and CyP prevents ABMR and achieves indefinite heart allograft survival in presensitized mouse recipients.

[0092] Complement has been shown to play an important role in ABMR. However, the inhibitory effect of functionally blocking terminal complement cascade at the C5 level in highly sensitized recipients is unknown. In the study presented herein, the presensitized model was used to study the efficacy of anti-C5 mAb either alone or combined with CsA and /or CyP in prevention of ABMR. As presented in Figure 2A, treatment with either CsA or CyP or the two drugs in combination did not prevent ABMR and grafts were rejected in 3.0 $\pm$ 0.0 days, 3.3 $\pm$ 0.5 days and 3.5 $\pm$ 0.6 days, respectively with typical pathological features of ACHR including intravascular thrombosis and interstitial hemorrhage (Figure 2B-b, c, d), which were indistinguishable from heart grafts in untreated presensitized BALB/c recipients (Figure 2B-a). Anti-C5 monotherapy or combined with CyP was not able to improve graft survival and heart grafts were rejected by ACHR (Figure 2B-e, f) in 3.5 $\pm$ 0.6 days and 3.2 $\pm$ 0.4 days, respectively (Figure 2A). Although the combination therapy of anti-C5 mAb and CsA, the protocol capable of inducing long-term heart allograft survival in unsensitized animals, marginally prolonged graft survival in this presensitized model, heart grafts were also rejected by severe humoral rejection with vasculitis, thrombosis, hemorrhage and minimal cell infiltration (Figure 2B-g) in 11.9 $\pm$ 1.8 days (Figure 2A). In contrast, triple therapy of anti-C5 mAb in combination of CsA and CyP achieved indefinite heart graft survival over 100 days (Figure 2A) in presensitized animals ($P<0.01$ vs. the animals without treatment or treated with either monotherapy or two drugs in combination) with no evidence of rejection (Figure 2B-h). In this presensitized mouse model, as shown in Table 2, only minor intragraft $CD4^+$ and $CD8^+$ cell infiltration was observed in the recipients that rejected their heart grafts within 3 days. However, the number of these T cells was slightly increased if heart grafts survived longer in anti-C5 mAb plus CsA-treated recipients at the time of rejection (POD11) and in triple therapy-treated recipients at early stages of graft survival (e.g. POD11). Furthermore, with continuous treatment of CsA in the triple therapy group, $CD4^+$ and $CD8^+$ cell infiltration was inhibited in long-term surviving heart grafts on POD60 and 100. In addition, moderate intragraft Mac-1^+ cell infiltration, including monocytes and macrophages, was found in untreated and CsA-, CyP- or CsA plus CyP-treated animals, while the infiltration of these cells was significantly reduced in anti-C5 mAb treated animals (Table 2). These results indicate that functionally blocking anti-C5 mAb enables the use and efficacy of conventional immunosuppressive agents, thereby preventing ABMR and achieving indefinite heart graft survival in presensitized recipients.

Table 2.
Grades for immunoperoxidase staining of heart allografts in presensitized mouse recipients at necropsy

Groups	Date for sample collection (POD)	C3	C5	**CD4**	CD8	Mac-1	IgG	IgM
Untreated	3	3+	3+	1+	1+	3+	4+	1+
CsA	3	3+	3+	1+	1+	3+	4+	1+
CyP	3	3+	3+	1+	1+	3+	3+	1+
CsA+CyP	3	3+	3+	1+	1+	3+	3+	1+
Anti-C5mAb	3	3+	0	1+	1+	2+	4+	1+

Samsung Bioepis Exhibit No. 1002
Page 1536

(continued)

Grades for immunoperoxidase staining of heart allografts in presensitized mouse recipients at necropsy

Groups	Date for sample collection (POD)	C3	C5	**CD4**	CD8	Mac-1	IgG	IgM
Anti-C5mAb+CsA	11	3+	0	2+	2+	2+	4+	1+
Anti-C5mAb+CyP	3	3+	0	1+	1+	2+	3+	1+
Anti-C5mAb+CsA+CyP	3	3+	0	1+	1+	2+	3+	1+
Anti-C5mAb+CsA+CyP	11	3+	0	2+	2+	1+	3+	1+
Anti-C5mAb+CsA+CyP	60	3+	0	1+	1+	0	2+	1+
Anti-C5mAb+CsA+CyP	100	3+	2+	0	0	0	2+	1+

Grades: 0, negative; 1+, equivocal; 2+, weak; 3+, moderate; 4+, intense.

Anti-C5 mAb completely inhibits total complement hemolytic activity and local C5 deposition in presensitized recipients receiving a heart allograft.

[0093] Anti-C5 mAb was previously shown to block the cleavage of complement protein C5 into the proinflammatory molecules C5a and C5b-9 (Kroshus et al., 1995), and to completely and consistently block terminal complement activity in mice (Wang et al., 1999). In the current study, terminal complement activity was measured by assessing the ability of recipient mouse sera to lyse antibody presensitized chicken erythrocytes and was compared at the same time-point (POD3). Treatment of mice with either CsA or CyP or the two drugs in combination had no effect on terminal complement activity, while treatment with anti-C5 mAb either alone or combined with CsA or/and CyP completely inhibited this activity (Figure 3; $P<0.01$, vs. naive and untreated animals, as well as CsA-, CyP-, or CsA plus CyP-treated animals). In addition, sera obtained from anti-C5 mAb treated animals at several earlier time points showed similarly diminished hemolytic activity, suggesting that serum terminal complement was inhibited throughout the treatment period. Furthermore, local C5 deposition in heart grafts was completely prevented in the anti-C5 mAb treated presensitized recipients, but not in untreated, or CsA-, CyP- and CsA plus CyP-treated presensitized animals (Table 2). As predicted, treatment with anti-C5 mAb did not prevent C3 deposition in the grafts (Table 2). These results suggest anti-C5 therapy completely blocks total complement activity after cardiac allografting in highly sensitized recipients.

Long-term surviving heart grafts in presensitized animals are resistant to humoral injury in the presence of low level of anti-donor antibodies and complement- a situation of accommodation.

[0094] To further investigate the role of anti-C5 mAb in humoral rejection, anti-donor alloantibody levels were measured in recipient sera by flow cytometry and intragraft antibody deposition by using immunostaining techniques in different groups. Figure 4A shows that on POD3 untreated presensitized BALB/c recipients had high levels of circulating anti-donor IgG antibodies. When presensitized recipients receiving either monotherapy or two drugs in combination, CsA and/or CyP partially down-regulated circulating anti-donor IgG levels, while treatment with anti-C5 mAb either alone or combined with CsA or CyP did not further affect anti-donor antibody levels at the same day. In contrast, with triple therapy of anti-C5 mAb, CsA and CyP, a high level of circulating anti-donor IgG was gradually downregulated and reached a low level on POD60, thereafter remaining at this level until day 100 (Figure 4B). Similar to levels of circulating antibodies in the different treatment groups, Table 2 shows that strong deposition of anti-mouse IgG was present in the rapidly rejected heart grafts of presensitized animals with no treatment or treated with monotherapy or two drugs in combination therapy. Interestingly, with triple therapy, IgG antibody deposition was gradually attenuated to a mild level in the long-term surviving heart grafts on POD 100 (Figure 4C-a, Table 2). In this model, IgM remained at very low levels in either circulation (Figure 4A, B) or transplanted heart grafts (Figure 4C-b, Table 2) in presensitized recipients with or without treatment. In addition, treatment with anti-C5 mAb eliminated complement activity to an undetectable level until day 60, followed by a progressive recovery to predepletion levels on POD 100 after discontinuation of anti-C5 therapy in presensitized mouse recipients receiving triple therapy (Figure 4D). Furthermore, intragraft C5 deposition was also detected in 100-day surviving presensitized animals (Table 2). These data demonstrate that ongoing transplant accommodation occurs in triple therapy treated presensitized recipients despite the presence of anti-graft antibodies and complement activation.

Samsung Bioepis Exhibit No. 1002
Page 1537

Anti-C5 mAb in combination with CsA and CyP reduces the IgG1/IgG2a ratio and leads to a shift in IgG subclass to IgG2b in recipients with accommodated grafts.

[0095] To determine whether anti-C5 mAb-based triple therapy would induce a shift in IgG subclass, which may be associated with accommodation, serum levels of anti-donor IgG subclasses of IgG1, IgG2a and IgG2b were compared between untreated recipients and the recipients with accommodated heart graft. Sera from untreated recipients contained predominant IgG1 isotype, indicated by a high ratio of IgG1/IgG2a (Figure 5A). In contrast, a significant reduction in the ratio of IgG1/IgG2a was observed in the recipients carrying accommodated grafts (Figure 5A, $P<0.01$). Furthermore, presensitized recipients with the accommodated heart grafts displayed an increased level of anti-donor IgG2b as compared to the same recipients with rejected grafts (Figure 5B, $P<0.01$). In addition, the pattern of IgG isotypes in the recipients treated with either monotherapy or two drugs in combination is indistinguishable from that of untreated animals. These data indicate that anti-donor IgG1 isotype may be associated with graft rejection, while production of anti-donor IgG2b subclass may function as a protective antibody and plays an important role in the induction of accommodation.

Anti-C5 mAb in combination with CsA and CyP induces intragraft Bcl-2 and Bcl-xl expression in highly sensitized mouse recipients.

[0096] To determine whether a causal relationship exists between intragraft expression of protective proteins and graft resistance to humoral injury in this model, western blot analysis was employed to detect proteins of interest in heart graft tissues from highly sensitized mouse recipients. Long-term surviving heart grafts were found to express high levels of Bcl-2 and Bcl-x1 proteins on POD100, and these proteins were detected as early as 12 days after heart transplantation in highly sensitized recipients receiving anti-C5 mAb-based triple therapy (Figure 6). In contrast, there were no Bcl-2 and Bcl-x1 proteins expressed on heart grafts of untreated animals (Figure 6) or animals treated with either monotherapy or two drugs in combination therapy. This result suggests that graft resistance to humoral injury in indefinite surviving animals is associated with the protection provided by Bcl-2 and Bcl-xl proteins in this presensitized model.

Presensitized recipients with an accommodating first heart graft accept a second accommodated heart graft but reject a second naive heart graft from the same donors.

[0097] The ability of accommodated grafts to resist rejection has not been tested directly under pathophysiological conditions where naive grafts undergo rejection following allotransplantation. In this model, to determine whether presensitized recipients with an accommodating first heart graft will accept a second accommodated graft but reject a second naive graft, we performed re-transplantation scenarios. After the accommodated C3H heart grafts have survived to the 100-day point, the time at which low levels of alloantibodies were detected (Figure 4B) and complement activity has returned to pretreatment levels (Figure 4D), in presensitized BALB/c recipient treated with anti-C5 mAb-based triple therapy, these recipients received a second heart graft. Specifically, either a naive (Figure 7A) or a 100-day accommodated C3H heart (Figure 7B) from another presensitized BALB/c recipient was transplanted into the neck of the presensitized recipients carrying an accorrimodating first C3H heart. These recipients rejected a second naive heart at 6.6 ± 1.1 days (Figure 8A) with severe AVR (Figure 8B-a) while the first heart continued to survive. In contrast, when the accommodated hearts that had been already surviving for 100 days in different presensitized mice were used as second grafts, these grafts were accepted by the presensitized recipients carrying an accommodating first heart graft (Figure 8A). There was no sign of rejection in those accommodated second heart grafts 90 days after second transplantation (Figure 8B-b). These data indicate that accommodated grafts become resistant to the effects of anti-donor antibodies and complement that normally mediate allograft rejection in these presensitized recipients. Furthermore, the fact that the host of the accommodated graft rejected a new graft suggests that accommodation involves changes to the graft.

Presensitized recipients being treated with CsA reject accommodated heart grafts.

[0098] Another re-transplantation was performed to determine whether accommodation in this presensitized model would be caused by the changes in the grafts and/or the recipients. Specifically, after C3H heart grafts have been accommodated in presensitized BALB/c mice for 100 days, the accommodated heart graft will then be re-transplanted into a second presensitized BALB/c recipient being treated with CsA alone (Figure 7C), a therapy that can prevent cellular rejection but cannot prevent accelerated humoral rejection of a fresh C3H heart in presensitized recipients. The accommodated C3H heart grafts were rapidly rejected in CsA treated presensitized BALB/c recipients. After re-transplantation, the pathology in accommodated heart grafts was changed from normal (Figure 9A) to severe ACHR with massive interstitial hemorrhage but few cell infiltrates (Figure 9B). In addition, high levels of anti-donor IgG and normal levels of complement hemolytic activity in these recipients receiving an accommodated C3H heart were similar to those of CsA treated presensitized recipients receiving a naive C3H heart. This result further indicates that accommodation

Samsung Bioepis Exhibit No. 1002
Page 1538

induced by anti-C5 mAb-based triple therapy can originate from mechanisms involving changes not only to the graft, but also to the recipient.

Example 3

Acute Vascular Rejection in a Heart Transplantation Model

[0099] Experiments were performed to determine whether inclusion of an inhibitor of formation of terminal complement would attenuate acute vascular rejection and whether the use of such an inhibitor in conjunction with an immunosuppressant would achieve long-term allograft survival. In this set of experiments an anti-C5 monoclonal antibody was used in conjunction with cyclosporin. The model used was an allograft heterotopic heart transplant from C3H mice into BALB/c mice. This model is a stringent acute vascular rejection model with the C3H and BALB/c mice being strongly MHC mismatched. The transplantations and other methods were performed as described in Wang et al. (2003).

Heterotopic Cardiac Transplantation

[0100] Intra-abdominal heterotopic cardiac transplantation was performed as previously described by Wang et al. (2003). Briefly, a median sternotomy was performed on the donor, and the heart graft was slowly perfused in situ with 1.0 ml of cold heparinized Ringer's lactate solution through the inferior vena cava and aorta before the superior vena cava and pulmonary veins were ligated and divided. The ascending aorta and pulmonary artery were transected, and the graft was removed from the donor. The graft was then revascularized with end-to-side anastomoses between the donor's pulmonary artery and the recipient's inferior vena cava as well as the donor's aorta and the recipient's abdominal aorta using 11-0 nylon suture. The beating of the grafted heart was monitored daily by direct abdominal palpation. The degree of pulsation was scored as: A, beating strongly; B, noticeable decline in the intensity of pulsation; or C, complete cessation of cardiac impulses. When cardiac impulses were no longer palpable, the graft was removed for routine histology. In certain instances, mice in which the graft was still functioning were sacrificed to perform histology.

Results

[0101] Mice (male 8-12 week old mice weighing 25 - 30 g) were split into six experimental groups with six to eight mice per group. Transplant occurred on day 0. Histological changes were checked at the endpoint (the endpoint being graft failure) or in some cases a mouse was sacrificed prior to graft failure. The dosage of BB5.1 which was administered (40 mg/kg body weight three times per week) was known from prior studies to completely inhibit terminal complement activity.

[0102] Group 1' (control) - mice were administered 0.75 mL of saline intraperitoneally on days -1,0, 1 and 2. Subsequently these mice were treated with 0.75 mL of saline intraperitoneally three times per week (Monday, Wednesday, Friday) until the endpoint.

[0103] Group 2' (cyclosporin A alone) - mice were administered 15 mg/kg body weight of cyclosporin A subcutaneously on a daily basis beginning at day 0 (day of transplant) until the endpoint.

[0104] Group 3' (anti-complement antibody alone) - mice were administered the anti-mouse C5 antibody BB5.1 (Frei et al., 1987) at 40 mg/kg body weight intraperitoneally on days -1, 0, 1 and 2 followed by 40 mg/kg body weight administered three times per week (Monday, Wednesday, Friday) until the endpoint.

[0105] Group 4' (anti-complement antibody until day 14 post-transplant plus cyclosporin A) - mice were administered the anti-mouse C5 antibody BB5.1 at 40 mg/kg body weight intravenously on days -1 through day 14 and were also administered cyclosporin A at 15 mg/kg of body weight on a daily basis beginning at day 0 until the endpoint. Note that this differs from the other groups in that the BB5.1 was administered intravenously and on a daily basis.

[0106] Group 5' (anti-complement antibody until day 28 post-transplant plus cyclosporin A) - mice were administered the anti-mouse C5 antibody BB5.1 at 40 mg/kg body weight intraperitoneally on days -1, 0, 1 and 2 followed by 40 mg/kg body weight administered three times per week (Monday, Wednesday, Friday) until day 28 and were also administered cyclosporin A at 15 mg/kg of body weight on a daily basis beginning at day 0 until the endpoint.

[0107] Group 6' (anti-complement antibody chronically until 100 days plus cyclosporin)-mice were administered the anti-mouse C5 antibody BB5.1 at 40 mg/kg body weight intraperitoneally on days -1, 0, 1 and 2 followed by 40 mg/kg body weight administered three times per week (Monday, Wednesday, Friday) until 100 days and were also administered cyclosporin A at 15 mg/kg of body weight on a daily basis beginning at day 0 until 100 days.

[0108] The results of this experiment are shown in Tables 3 and 4. Table 3 shows the survival time for the grafts. Table 4 sets forth the histological scores.

Samsung Bioepis Exhibit No. 1002
Page 1539

Table 3

Allograft Survival		
Group (Treatment)	Individual Survival (days)	Mean Survival Time (days)
1'. Saline	8, 8, 8, 8, 8, 9	8.3 ± 0.5
2'. Cyclosporin A	14, 15, 15, 16, 16,16, 17	15.5 ± 1.1
3'. BB5.1	7, 8, 8, 8, 8, 9	8.0 ± 0.6
4'. BB5.1 until day 14 + cyclosporin A	35, 38, 43, 45, 46, 47	42.3 ± 4.8
5'. BB5.1 until day 28 + cyclosporin A	77, 80, 80, 81, 82	80 ± 1.9
6'. BB5.1 until day 100 + cyclosporin A	>100 days (7 mice; one sacrificed for histology)	> 100 days

Table 4

Median Scores of Histological Changes of Heart Allografts at Necropsy							
Groups	Vasc*	Infarc	Lymph	Throm	Hemo	Fibrin	PMN
1'. Saline (endpoint)	3.0	3.0	1.0	4.0	3.0	3.0	3.0
2'. Cyclosporin A (endpoint)	2.0	1.0	2.0	3.0	2.0	2.0	3.0
3'. BB5.1 (endpoint)	2.0	1.0	2.0	2.0	1.0	0.0	0.0
4'. BB5.1 until day 14 + cyclosporin A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
5'. BB5.1 28 days + Cyclosporin A (post-operative day 8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5'. BB5.1 28 days + Cyclosporin A (endpoint)	0.0	0.0	1.0	1.0	2.0	1.0	0.0
6'. BB5.1 until day 100 + Cyclosporin A (post-operative day 100)	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Median scores: 0 - normal; 1- minimum change; 2 - mild change; 3 - moderate change; 4 - marked change. N/A - not available. *Vasc - vasculitis; Infar - infarction; Lymph - lymphocyte infiltration; Throm - thrombosis; Hemo - hemorrhage; Fibrin - fibrin deposition; PMN - polymorphonuclear cell infiltrate							

[0109] The results indicate the synergistic effects of using a complement inhibiting drug in addition to an immunosuppressant. In untreated mice the grafts were rejected in about 8 davs. Use of the immunosuppressant cyclosporin A alone on a daily, chronic basis resulted in an increase in graft survival until approximately 15 days post-transplant. The use of the anti-C5 antibody BB5.1 to inhibit formation of terminal complement had no effect on its own, graft rejection occurring at 8 days post-transplant as in the control group (Group 1'). The combination of BB5.1 through day 28 post-transplant plus cyclosporin A showed a synergistic effect with graft survival being extended until approximately day 80. A more surprising result is that of Group 5' in which BB5.1 and cyclosporin A were each administered chronically post-transplant. In this case the graft survival was for more than 100 days (as much data as presently available). Additionally, the histological results shown in Table 4 indicate that the administration of both BB5.1 and cyclosporin A protected the graft from changes much better than either BB5.1 or cyclosporin A alone, and that the chronic administration of BB5.1 and cyclosporin A protected the graft to such an extent that even at 100 days post-transplant there were no histological changes seen in the engrafted hearts. A survival time of 100 days in these models is considered to be the gold standard. A survival of 100 days in the model is believed to indicate that there will be an indefinite survival of the allograft. When BB5.1 administration was stopped after 28 days, the grafts were protected but they did begin to show some minimal to mild histological changes by about day 80 which was the time at which graft failure occurred.

[0110] The Group 4' mice were treated differently in that they were administered BB5.1 on a daily basis by an intravenous administration. These animals became ill, showing weight loss and urine retention and were sacrificed at a time at which the grafted hearts were still beating although their function had declined. This was the first group of mice studied and it is unknown why these ill effects were seen. These ill effects were not seen when the BB5.1 was administered intraperitoneally with a schedule of three times per week. As seen below in Example 4, daily administration of BB5.1 via an

Samsung Bioepis Exhibit No. 1002
Page 1540

intraperitoneal route did not cause ill effects. Also, intravenous administration was not necessarily the cause of the illness in these animals. Intravenous administration of eculizumab (a human equivalent antibody to BB5.1 in that it binds to human C5) has been successfully administered intravenously without ill effects to humans in a study of PNH (Hillmen et al., 2004). Complement inhibitors may be administered by other routes in addition to intravenous and intraperitoneal, with all such routes being well known by those skilled in the art.

Example 4

Accelerated Rejection in a Presensitized Heart Transplantation Model

[0111] A second set of experiments similar to those of Example 3 was performed but the recipient mouse was presensitized to the donor organ. In these experiments, the presensitization was brought about by prior transplantation of a skin graft. In general, presensitization can occur not only as a result of having received an earlier allograft, but can also be caused by having received multiple blood transfusions or in women who have been pregnant. Besides such presensitization methods, allografts with an ABO mismatch will be rapidly attacked and rejected because of preformed antibodies to the ABO antigens unless steps are taken to prevent such an attack.

[0112] Some mice in these studies were administered cyclophosphamide in addition to BB5.1 and/or cyclosporin A. For these experiments BALB/c recipient mice were presensitized with C3H skin grafts one week prior to heart transplantation from the same donor (using the method of Pruitt and Bollinger, 1991). This model is designed to mimic presensitized transplantation in humans, especially in relation to accelerated humoral rejection. Recipient mice were split into eight groups of six to eight mice each. The treatments were as follow.

[0113] Group 1" (control) - mice (male 8-12 week old mice weighing 25 - 30 g) were administered 0.75 mL saline intraperitoneally on a daily basis beginning at day -1 and continuing until the endpoint (graft rejection).

[0114] Group 2" (cyclosporin A alone) - mice were administered cyclosporin A subcutaneously at a dose of 15 mg/kg body weight beginning on day 0 (day of transplant) until the endpoint.

[0115] Group 3" (BB5.1 alone) - mice were administered the anti-mouse complement monoclonal antibody BB5.1 at a dose of 40 mg/kg body weight delivered intraperitoneally on a daily basis beginning at day -1 and continuing until the endpoint.

[0116] Group 4" (cyclophosphamide alone) - mice were administered cyclophosphamide intravenously at a dose of 40 mg/kg body weight on each of days 0 and 1.

[0117] Group 5" (BB5.1 plus cyclosporin A) - mice were administered BB5.1 intraperitoneally at a dose of 40 mg/kg body weight on a daily basis beginning at day -1 and continuing until the endpoint. These mice were additionally administered cyclosporin A subcutaneously at a dose of 15 mg/kg body weight on a daily basis from day 0 until the endpoint.

[0118] Group 6" (BB5.1 plus cyclophosphamide) - mice were administered BB5.1 intraperitoneally at a dose of 40 mg/kg body weight on a daily basis beginning at day -1 and continuing until the endpoint. These mice were additionally administered cyclophosphamide intravenously at a dose of 40 mg/kg body weight on each of days 0 and 1.

[0119] Group 7" (cyclosporin A plus cyclophosphamide) - mice were administered cyclosporin A subcutaneously at a dose of 15 mg/kg body weight on a daily basis from day 0 until the endpoint. These mice were additionally administered cyclophosphamide intravenously at a dose of 40 mg/kg body weight on each of days 0 and 1.

[0120] Group 8" (BB5.1 plus cyclosporin A plus cyclophosphamide) - mice were administered BB5.1 intraperitoneally at a dose of 40 mg/kg body weight on a daily basis beginning at day -1 and continuing until 100 days. These mice were also administered cyclosporin A subcutaneously at a dose of 15 mg/kg body weight on a daily basis from day 0 until 100 days. These mice were additionally administered cyclophosphamide intravenously at a dose of 40 mg/kg body weight on each of days 0 and 1. Two mice in this group were sacrificed at day 60 for histological studies (no rejection had yet occurred) and the four remaining mice still had not rejected their grafts by day 100.

[0121] Additionally a control group of mice which was not presensitized and received only the saline treatment as for Group 1" was tested.

[0122] The results of these experiments are shown in Tables 5 and 6. Table 5 lists survival times for the grafts and Table 6 summarizes the histological results.

Table 5

Allograft Survival		
Groups (Treatment)	**Individual survival (days)**	**Mean survival time (days)**
No presensitization	8, 8, 8, 8, 8, 9	8.3 ± 0.5*
1". One skin presensitization	3, 3, 3, 3, 3, 3, 3, 4	3.1 ± 0.4

Samsung Bioepis Exhibit No. 1002
Page 1541

(continued)

Allograft Survival		
Groups (Treatment)	**Individual survival (days)**	**Mean survival time (days)**
2". Cyclosporin A	3,3,3,3	3.0 ± 0.0
3". BB5.1	3, 3, 4, 4	3.5 ± 0.6
4". Cyclophosphamide	3,3,3,4	3.3 ± 0.5
5". BB5.1 + Cyclosporin A	10, 10, 11, 11, 12, 12, 14, 15	11.9 ± 1.8**
6". BB5.1 + Cyclophosphamide	3, 3, 3, 3, 3, 4	3.2 ± 0.4
7". Cyclosporin A + Cyclophosphamide	3, 3,3,4,4,4	3.5 ± 0.6
8". BB5.1 + Cyclosporin A + Cyclophosphamide	> 100 days (4 mice)	> 100***
*P < 0.01 group 1" vs. no presensitization **P < 0.01 group 5" vs. groups 1 "-4" and 6"-7". ***P<0.01 group 8" vs. groups 1"-7".		

Table 6

Median Scores of Histological Changes of Heart Allografts at Necropsy							
Groups	Vasc*	Infar	Lymph	Throm	Hemo	Fibrin	PMN
No presensitization (endpoint)	3.0	3.0	1.0	4.0	3.0	3.0	3.0
1". One skin presensitization (endpoint)	0.0	4.0	1.0	4.0	3.0	0.0	0.0
2". Cyclosporin A (endpoint)	0.0	4.0	0.0	3.0	3.0	N/A	N/A
3". BB5.1 (endpoint)	2.0	2.0	2.0	2.0	3.0	N/A	N/A
4". Cyclophosphamide (endpoint)	2.0	4.0	0.0	3.0	2.0	N/A	N/A
5". BB5.1 + Cyclosporin A (post-operative day 3)	0.0	1.0	1.0	0.0	0.0	0.0	0.0
5". BB5.1 + Cyclosporin A (endpoint)	2.0	2.0	2.0	2.0	2.0	0.0	0.0
6". BB5.1 + Cyclophosphamide (endpoint)	1.0	3.0	0.0	3.0	2.0	N/A	N/A
7". Cyclosporin A + Cyclophosphamide (endpoint)	0.0	1.0	1.0	2.0	3.0	N/A	N/A
8". BB5.1 + Cyclosporin A + Cyclophosphamide (post-operative day 3)	0.0	0.0	1.0	0.0	0.0	N/A	N/A
8. BB5.1 + Cyclosporin A + Cyclophosphamide (post-operative day 12)	0.0	0.0	1.0	0.0	0.0	N/A	N/A
8". BB5.1 + Cyclosporin A + Cyclophosphamide (post-operative day 60)	0.0	0.0	1.0	0.0	0.0	N/A	N/A
8". BB5.1 + Cyclosporin A + Cyclophosphamide (post-operative day 100)	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Median scores: 0 - normal; 1- minimum change; 2 - mild change; 3 - moderate change; 4 - marked change. N/A - not available. *Vasc - vasculitis; Infar - infarction; Lymph - lymphocyte infiltration; Throm - thrombosis; Hemo - hemorrhage; Fibrin - fibrin deposition; PMN - polymorphonuclear cell infiltrate							

[0123] The results shown in Table 5 indicate a difference between the presensitized mouse model and the nonpresensitized mouse model as used in Example 3. The results indicate that in the absence of presensitization, grafts are rejected in approximately 8 days in the absence of treatment with any drugs. Presensitizing the animals causes a more rapid rejection, the rejection of the graft in the presensitized animals being in approximately 3 days in the absence of

Samsung Bioepis Exhibit No. 1002
Page 1542

any drug treatment. Treatment with either BB5.1, cyclosporin A or cyclophosphamide had no effect upon graft survival, with the grafts being rejected in approximately 3-4 days in each of these groups of animals. The combination of BB5.1 and cyclosporin A showed some effect with rejection occurring about day 12. The combination of BB5.1 and cyclophosphamide had no protective effect with rejection occurring about day 3. Similarly the combination of cyclosporin A and cyclophosphamide had essentially no protective effect with rejection occurring at 3-4 days. Very surprisingly, the combination of all three drugs (chronic administration of BB5.1 and cyclosporin plus administration of cyclophosphamide at the time of transplant) showed a highly synergistic effect with all of the mice surviving for more than 100 days. Again, a survival of 100 days in this model is considered to be the gold standard and assumes an indefinite survival.

[0124] These results as well as the histological results as shown in Table 6 indicate that the combination of chronic treatment with a complement inhibitor and an immunosuppressant such as cyclosporin A in treating a presensitized mouse results in some attenuation of accelerated rejection. Treatment of these animals additionally with cyclophosphamide at the time of transplant and on the first day after transplant results in a much greater time of survival, no rejection having been seen by at least day 100.

[0125] It will be appreciated that the methods and compositions of the instant disclosure can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent to the artisan that other embodiments exist and do not depart from the spirit of the disclosure. Thus, the described embodiments are illustrative and should not be construed as restrictive.

LIST OF REFERENCES

[0126] The publications and other materials used herein to illuminate the background of the disclosure, and in particular, cases to provide additional details respecting the practice, are for convenience referenced by author and date in the text and respectively grouped in the following List of References.

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Samsung Bioepis Exhibit No. 1002
Page 1545

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U.S. Patent 6,534,058

U.S. Patent 6,821,515

[0127] The contents of all references cited herein are incorporated by reference in their entirety.

Claims

1. An antibody to C5 which inhibits complement activity for use in prolonging survival of an allograft in a recipient mammal, wherein said antibody to C5 is to be administered in conjunction with at least one immunosuppressive drug and chronically.

2. Use of an antibody to C5 which inhibits complement activity for the manufacture of a medicament or medicament package, wherein said medicament is to be administered with at least one immunosuppressive drug to prolong survival of an allograft in a recipient mammal, wherein said medicament is to be administered chronically.

3. An antibody to C5 which inhibits complement activity and at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal, wherein said antibody to C5 and said at least one immunosuppressive drug are to be administered chronically.

4. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to claim 1, the use according to claim 2 or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 3, wherein said mammal is a human.

5. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to claim 1 or 4, the use of claim 2 or 4, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 3 or 4, wherein said allograft is an MHC mismatched allograft.

6. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal, the use or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 5, wherein said MHC mismatched allograft is an HLA mismatched allograft.

7. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to claim 1 or 4, the use of claim 2 or 4, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 3 or 4, wherein said mammal is an ABO mismatch for said allograft.

8. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to claim 1 or 4, the use of claim 2 or 4, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claims 3 or 4, wherein said mammal is presensitized to said allograft, and wherein said antibody to C5 is to be administered with at least two immunosuppressive drugs or wherein said medicament is to be administered with at least two immunosuppressive drugs.

9. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 1 or 4 to 8, the use of any one of claims 2 or 4 to 8, or the antibody to C5 and the at least one immunosuppressive

Samsung Bioepis Exhibit No. 1002
Page 1546

drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 3 or 4 to 8, wherein said antibody to C5 which inhibits complement activity inhibits the formation of terminal complement or C5a.

10. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal, the use or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 9, wherein said antibody to C5 which
inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment.

11. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal, the use, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 10, wherein said whole antibody or antibody fragment is a human, humanized, chimerized or deimmunized antibody or antibody fragment.

12. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal, the use, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 10 or 11, wherein said whole antibody or antibody fragment inhibits cleavage of complement C5.

13. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal, the use, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 10 to 12, wherein said antibody fragment is selected from the group consisting of an Fab, an $F(ab')_2$, Fv, a domain antibody, and a single-chain antibody.

14. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal, the use, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 10, wherein said antibody fragment is pexelizumab.

15. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal, the use, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 10, wherein said whole antibody is eculizumab.

16. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal, the use, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 15, wherein said eculizumab is to be administered once every 2 weeks.

17. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 1 or 4 to 16, the use of any one of claims 2 or 4 to 16, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 3 to 16, wherein said immunosuppressive drug inhibits T-cell activity or B-cell activity.

18. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 1 or 4 to 16, the use of any one of claims 2 or 4 to 16, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 3 to 16, wherein said immunosuppressive drug inhibits T-cell activity and B-cell activity.

19. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 1 or 4 to 16, the use of any one of claims 2 or 4 to 16, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 3 to 16, wherein said immunosuppressive drug is selected from the group consisting of cyclosporine A, tacrolimus, sirolimus, OKT3, a corticosteroid, daclizumab, basiliximab, azathioprene, mycophenolate mofetil, methotrexate, 6-mercaptopurine, anti-T-cell antibodies, cyclophosphamide, leflunamide, brequinar, ATG, ALG, 15-deoxyspergualin, and bredinin.

20. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 1 or 4 to 19, the use of any one of claims 2 or 4 to 19, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 3 to 19, wherein more than one immunosuppressive drug is to be administered.

21. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal of claim 1 or the use of claim

Samsung Bioepis Exhibit No. 1002
Page 1547

2, wherein said medicament is to be administered in conjunction with cyclosporine A.

22. The antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 3 wherein said immunosuppressive drug is cyclosporine A.

23. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal, the use, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 20, wherein said medicament is to be administered in conjunction with cyclosporine A and cyclophosphamide.

24. The antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 3 wherein said immunosuppressive drug includes both cyclosporine A and cyclophosphamide.

25. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal or the use according to claim 21, wherein said antibody to C5 is an antibody which inhibits cleavage of complement C5.

26. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal, according to any one of claims 1 or 4 to 25, the use of any one of claims 2 or 4 to 25, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 3 to 25, wherein said allograft is selected from the group consisting of i) heart, ii) kidney, iii) lung, iv) pancreas, v) liver, vi) vascular tissue, vii) eye, viii) cornea, ix) lens, x) skin, xi) bone marrow, xii) muscle, xiii) connective tissue, xiv) gastrointestinal tissue, xv) nervous tissue, xvi) bone, xvii) stem cells, xviii) islets, xix) cartilage, xx) hepatocytes, and xxi) hematopoietic cells.

27. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 1 or 4 to 25, the use of any one of claims 2 or 4 to 25, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 3 to 25, wherein said allograft survives for a time at least 20% longer than would occur if said use were to be without said antibody to C5.

28. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 1 or 4 to 26, the use of any one of claims 2 or 4 to 26, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 3 to 26, wherein said allograft survives for a time at least 40% longer than would occur if said use were to be without said antibody to C5.

29. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 1 or 4 to 26, the use of any one of claims 2 or 4 to 26, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 3 to 26, wherein said allograft survives for the remaining life-time of said mammal.

30. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 1 or 4 to 26, the use of any one of claims 2 or 4 to 26, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 3 to 26, wherein said allograft survives for at least six months.

31. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 1 or 4 to 26, the use of any one of claims 2 or 4 to 26, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 30 to 26, wherein said allograft survives for at least one year.

32. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 1 or 4 to 26, the use of any one of claims 2 or 4 to 26, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 3 to 26, wherein said allograft survives for at least five years.

33. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal, the use, or the antibody to

Samsung Bioepis Exhibit No. 1002
Page 1548

C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 4, wherein said allograft survives for the remaining life-time of said human.

34. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 1 or 4 to 33, the use of any one of claims 2 or 4 to 33, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 3 to 33, wherein said antibody to C5 is to be administered chronically for at least 14 days.

35. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 1 or 4 to 33, the use of any one of claims 2 or 4 to 33, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 3 to 33, wherein said antibody to C5 is to be administered chronically for at least 28 days.

36. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 1 or 4 to 33, the use of any one of claims 2 or 4 to 33, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 3 to 33, wherein said antibody to C5 is to be administered chronically for at least 3 months.

37. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 1 or 4 to 33, the use of any one of claims 2 or 4 to 33, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 3 to 33, wherein said antibody to C5 is to be administered chronically for at least 6 months.

38. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 1 or 4 to 33, the use of any one of claims 2 or 4 to 33, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 3 to 33, wherein said antibody to C5 is to be administered chronically for at least 1 year.

39. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 1 or 4 to 33, the use of any one of claims 2 or 4 to 33, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 3 to 33, wherein said antibody to C5 is to be administered chronically for at least 5 years.

40. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 1 or 4 to 33, the use of any one of claims 2 or 4 to 33, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 3 to 33, wherein said antibody to C5 is to be administered chronically for the remaining life-time of said mammal.

41. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 1 or 4 to 33, the use of any one of claims 2 or 4 to 33, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any one of claims 3 to 33, wherein at least one immunosuppressive drug is to be administered chronically for the remaining life-time of said mammal.

42. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal, the use, or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 41, wherein at least cyclosporine A is to be administered chronically for the remaining life-time of said mammal.

43. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to claim 1 or the use of claim 2, wherein said antibody to C5 and said immunosuppressive drug are in a formulation suitable for concurrent administration to said mammal.

44. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to claim 1 or the use of claim 2, wherein said antibody to C5 and said immunosuppressive drug are in a formulation or formulations suitable for sequential administration to said mammal.

45. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to claim 1 or the

Samsung Bioepis Exhibit No. 1002
Page 1549

use of claim 2, wherein said antibody to C5 is in a formulation suitable for chronic administration to said mammal.

46. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to claim 1 or the use of claim 2, wherein said immunosuppressive drug is in a formulation suitable for chronic administration to said mammal.

47. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to claim 1, the use of claim 2 or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 3, wherein said medicament includes a lyophilized formulation comprising the antibody and a lyoprotectant.

48. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to claim 1, the use of claim 2 or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 3, wherein said immunosuppressive drug is in a lyophilized formulation comprising the immunosuppressive drug and a lyoprotectant.

49. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to claim 1, the use of claim 2 or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 3, wherein said antibody and said immunosuppressive drug are in the same lyophilized formulation comprising said antibody, said immunosuppressive drug, and a lyoprotectant.

50. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to claim 1, the use of claim 2 or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 3, wherein said antibody is in an injection system comprising a syringe that comprises a cartridge, wherein said cartridge contains said antibody in a formulation suitable for injection.

51. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to claim 1, the use of claim 2 or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 3, wherein said immunosuppressive drug is in an injection system comprising a syringe that comprises a cartridge, wherein said cartridge contains said immunosuppressive drug in a formulation suitable for injection.

52. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to claim 1, the use of claim 2 or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 3, wherein said antibody and said immunosuppressive drug are in an injection system comprising a syringe that comprises a cartridge, wherein said cartridge contains said antibody and said immunosuppressive drug in a formulation suitable for injection.

53. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to claim 1, the use of claim 2 or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 3, wherein said antibody is present in unit dosage form.

54. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to claim 1, the use of claim 2 or the antibody to C5 and the at least one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to claim 3, wherein said immunosuppressive drug is present in unit dosage form.

Patentansprüche

1. Antikörper gegen C5, der die Komplementaktivität hemmt, zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger, wobei der Antikörper gegen C5 in Verbindung mit wenigstens einem Immunsuppressivum und chronisch zu verabreichen ist.

2. Verwendung eines Antikörpers gegen C5, der die Komplementaktivität hemmt, zur Herstellung eines Arzneimittels oder einer Arzneimittelpackung, wobei das Arzneimittel mit wenigstens einem Immunsuppressivum zu verabreichen ist, um die Lebensdauer eines Allotransplantats in einem Empfänger-Säuger zu verlängern, wobei das Arzneimittel chronisch zu verabreichen ist.

Samsung Bioepis Exhibit No. 1002
Page 1550

3. Antikörper gegen C5, der die Komplementaktivität hemmt, und wenigstens ein Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger, wobei der Antikörper gegen C5 und das wenigstens eine Immunsuppressivum chronisch zu verabreichen sind.

4. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 1, Verwendung nach Anspruch 2 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 3, wobei es sich bei dem Säuger um einen Menschen handelt.

5. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 1 oder 4, Verwendung gemäß Anspruch 2 oder 4 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 3 oder 4, wobei es sich bei dem Allotransplantat um ein MHC-fehlgepaartes Allotransplantat handelt.

6. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger, Verwendung oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 5, wobei es sich bei dem MHC-fehlgepaarten Allotransplantat um ein HLA-fehlgepaartes Allotransplantat handelt.

7. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 1 oder 4, Verwendung gemäß Anspruch 2 oder 4 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 3 oder 4, wobei es sich bei dem Säuger um eine AB0-Fehlpaarung für das Allotransplantat handelt.

8. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 1 oder 4, Verwendung gemäß Anspruch 2 oder 4 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 3 oder 4, wobei der Säuger gegenüber dem Allotransplantat vorsensibilisiert wird und wobei der Antikörper gegen C5 mit wenigstens zwei Immunsuppressiva zu verabreichen ist oder wobei das Arzneimittel mit wenigstens zwei Immunsuppressiva zu verabreichen ist.

9. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 1 oder 4 bis 8, Verwendung gemäß einem der Ansprüche 2 oder 4 bis 8 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 3 oder 4 bis 8, wobei der Antikörper gegen C5, der die Komplementaktivität hemmt, die Ausbildung des terminalen Komplements oder C5a hemmt.

10. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger, Verwendung oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 9, wobei es sich bei dem Antikörper gegen C5, der die Ausbildung des terminalen Komplements oder C5a hemmt, um einen intakten Antikörper oder ein Antikörperfragment handelt.

11. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger, Verwendung oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 10, wobei es sich bei dem intakten Antikörper oder dem Antikörperfragment um einen menschlichen, humanisierten, chimärisierten oder deimmunisierten Antikörper bzw. ein menschliches, humanisiertes, chimärisiertes oder deimmunisiertes Antikörperfragment handelt.

12. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger, Verwendung oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 10 oder 11, wobei der intakte Antikörper oder das Antikörperfragment die Spaltung von Komplement C5

Samsung Bioepis Exhibit No. 1002
Page 1551

hemmt.

13. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger, Verwendung oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 10 bis 12, wobei das Antikörperfragment aus der aus einem Fab, einem $F(ab')_2$, Fv, einem Domäne-Antikörper und einem Einzelkette-Antikörper bestehenden Gruppe ausgewählt ist.

14. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger, Verwendung oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 10, wobei es sich bei dem Antikörperfragment um Pexelizumab handelt.

15. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger, Verwendung oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 10, wobei es sich bei dem intakten Antikörper um Eculizumab handelt.

16. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger, Verwendung oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 15, wobei das Eculizumab einmal alle 2 Wochen zu verabreichen ist.

17. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 1 oder 4 bis 16, Verwendung gemäß einem der Ansprüche 2 oder 4 bis 16 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 3 bis 16, wobei das Immunsuppressivum die Aktivität von T-Zellen oder B-Zellen hemmt.

18. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 1 oder 4 bis 16, Verwendung gemäß einem der Ansprüche 2 oder 4 bis 16 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 3 bis 16, wobei das Immunsuppressivum die Aktivität von T-Zellen und B-Zellen hemmt.

19. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 1 oder 4 bis 16, Verwendung gemäß einem der Ansprüche 2 oder 4 bis 16 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 3 bis 16, wobei das Immunsuppressivum aus der aus Cyclosporin A, Tacrolimus, Sirolimus, OKT3, einem Corticosteroid, Daclizumab, Basiliximab, Azathiopren, Mycophenolat-Mofetil, Methotrexat, 6-Mercaptopurin, Anti-T-Zellen-Antikörpern, Cyclophosphamid, Leflunamid, Brequinar, ATG, ALG, 15-Desoxyspergualin und Bredinin bestehenden Gruppe ausgewählt ist.

20. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 1 oder 4 bis 19, Verwendung gemäß einem der Ansprüche 2 oder 4 bis 19 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 3 bis 19, wobei mehr als ein Immunsuppressivum zu verabreichen ist.

21. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger gemäß Anspruch 1 oder Verwendung gemäß Anspruch 2, wobei das Arzneimittel in Verbindung mit Cyclosporin A zu verabreichen ist.

22. Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 3, wobei es sich bei dem Immunsuppressivum um Cyclosporin A handelt.

Samsung Bioepis Exhibit No. 1002
Page 1552

23. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger, Verwendung oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 20, wobei das Arzneimittel in Verbindung mit Cyclosporin A und Cyclophosphamid zu verabreichen ist.

24. Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 3, wobei das Immunsuppressivum sowohl Cyclosporin A als auch Cyclophosphamid enthält.

25. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger oder Verwendung nach Anspruch 21, wobei wobei es sich bei dem Antikörper gegen C5 um einen Antikörper handelt, der die Spaltung von Komplement C5 hemmt.

26. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 1 oder 4 bis 25, Verwendung gemäß einem der Ansprüche 2 oder 4 bis 25 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 3 bis 25, wobei das Allotransplantat aus der aus i) Herz, ii) Niere, iii) Lunge, iv) Pankreas, v) Leber, vi) Gefäßgewebe, vii) Auge, viii) Hornhaut, ix) Linse, x) Haut, xi) Knochenmark, xii) Muskel, xiii) Bindegewebe, xiv) Magen-Darm-Gewebe, xv) Nervengewebe, xvi) Knochen, xvii) Stammzellen, xviii) Inselzellen, xix) Knorpel, xx) Hepatozyten und xxi) hämatopoietischen Zellen bestehenden Gruppe ausgewählt ist.

27. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 1 oder 4 bis 25, Verwendung gemäß einem der Ansprüche 2 oder 4 bis 25 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 3 bis 25, wobei das Allotransplantat eine um wenigstens 20% längere Zeit überlebt als wenn die Verwendung ohne den Antikörper gegen C5 erfolgen würde.

28. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 1 oder 4 bis 26, Verwendung gemäß einem der Ansprüche 2 oder 4 bis 26 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 3 bis 26, wobei das Allotransplantat eine um wenigstens 40% längere Zeit überlebt als wenn die Verwendung ohne den Antikörper gegen C5 erfolgen würde.

29. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 1 oder 4 bis 26, Verwendung gemäß einem der Ansprüche 2 oder 4 bis 26 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 3 bis 26, wobei das Allotransplantat über die verbleibende Lebenszeit des Säugers überlebt.

30. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 1 oder 4 bis 26, Verwendung gemäß einem der Ansprüche 2 oder 4 bis 26 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 3 bis 26, wobei das Allotransplantat wenigstens sechs Monate überlebt.

31. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 1 oder 4 bis 26, Verwendung gemäß einem der Ansprüche 2 oder 4 bis 26 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 3 bis 26, wobei das Allotransplantat wenigstens ein Jahr überlebt.

32. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 1 oder 4 bis 26, Verwendung gemäß einem der Ansprüche 2 oder 4 bis 26 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 3 bis 26,

Samsung Bioepis Exhibit No. 1002
Page 1553

wobei das Allotransplantat wenigstens fünf Jahre überlebt.

33. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger, Verwendung oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 4, wobei das Allotransplantat über die verbleibende Lebenszeit des Menschen überlebt.

34. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 1 oder 4 bis 33, Verwendung gemäß einem der Ansprüche 2 oder 4 bis 33 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 3 bis 33, wobei der Antikörper gegen C5 wenigstens 14 Tage chronisch zu verabreichen ist.

35. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 1 oder 4 bis 33, Verwendung gemäß einem der Ansprüche 2 oder 4 bis 33 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 3 bis 33, wobei der Antikörper gegen C5 wenigstens 28 Tage chronisch zu verabreichen ist.

36. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 1 oder 4 bis 33, Verwendung gemäß einem der Ansprüche 2 oder 4 bis 33 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 3 bis 33, wobei der Antikörper gegen C5 wenigstens 3 Monate chronisch zu verabreichen ist.

37. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 1 oder 4 bis 33, Verwendung gemäß einem der Ansprüche 2 oder 4 bis 33 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 3 bis 33, wobei der Antikörper gegen C5 wenigstens 6 Monate chronisch zu verabreichen ist.

38. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 1 oder 4 bis 33, Verwendung gemäß einem der Ansprüche 2 oder 4 bis 33 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 3 bis 33, wobei der Antikörper gegen C5 wenigstens 1 Jahr chronisch zu verabreichen ist.

39. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 1 oder 4 bis 33, Verwendung gemäß einem der Ansprüche 2 oder 4 bis 33 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 3 bis 33, wobei der Antikörper gegen C5 wenigstens 5 Jahre chronisch zu verabreichen ist.

40. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 1 oder 4 bis 33, Verwendung gemäß einem der Ansprüche 2 oder 4 bis 33 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 3 bis 33, wobei der Antikörper gegen C5 über die verbleibende Lebenszeit des Säugers chronisch zu verabreichen ist.

41. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 1 oder 4 bis 33, Verwendung gemäß einem der Ansprüche 2 oder 4 bis 33 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach einem der Ansprüche 3 bis 33, wobei wenigstens ein Immunsuppressivum über die verbleibende Lebenszeit des Säugers chronisch zu verabreichen ist.

42. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger, Verwendung oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur

Samsung Bioepis Exhibit No. 1002
Page 1554

Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 41, wobei wenigstens Cyclosporin A über die verbleibende Lebenszeit des Säugers chronisch zu verabreichen ist.

43. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 1 oder Verwendung gemäß Anspruch 2, wobei der Antikörper gegen C5 und das Immunsuppressivum in einer für die gleichzeitige Verabreichung an den Säuger geeigneten Formulierung vorliegen.

44. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 1 oder Verwendung gemäß Anspruch 2, wobei der Antikörper gegen C5 und das Immunsuppressivum in einer Formulierung oder Formulierungen vorliegen, die für die nacheinander erfolgende Verabreichung an den Säuger geeignet ist bzw. sind.

45. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 1 oder Verwendung gemäß Anspruch 2, wobei der Antikörper gegen C5 in einer für die chronische Verabreichung an den Säuger geeigneten Formulierung vorliegt.

46. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 1 oder Verwendung gemäß Anspruch 2, wobei das Immunsuppressivum in einer für die chronische Verabreichung an den Säuger geeigneten Formulierung vorliegt.

47. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 1, Verwendung gemäß Anspruch 2 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 3, wobei das Arzneimittel eine den Antikörper und ein Lyoprotektivum umfassende lyophilisierte Formulierung enthält.

48. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 1, Verwendung gemäß Anspruch 2 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 3, wobei das Immunsuppressivum in einer das Immunsuppressivum und ein Lyoprotektivum umfassenden lyophilisierten Formulierung vorliegt.

49. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 1, Verwendung gemäß Anspruch 2 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 3, wobei der Antikörper und das Immunsuppressivum in der gleichen den Antikörper, das Immunsuppressivum und ein Lyoprotektivum umfassenden lyophilisierten Formulierung vorliegen.

50. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 1, Verwendung gemäß Anspruch 2 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 3, wobei der Antikörper in einem eine Spritze, die eine Patrone umfasst, umfassenden Injektionssystem vorliegt, wobei die Patrone den Antikörper in einer zur Injektion geeigneten Formulierung enthält.

51. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 1, Verwendung gemäß Anspruch 2 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 3, wobei das Immunsuppressivum in einem eine Spritze, die eine Patrone umfasst, umfassenden Injektionssystem vorliegt, wobei die Patrone das Immunsuppressivum in einer zur Injektion geeigneten Formulierung enthält.

52. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 1, Verwendung gemäß Anspruch 2 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 3, wobei der Antikörper und das Immunsuppressivum in einem eine Spritze, die

Samsung Bioepis Exhibit No. 1002
Page 1555

eine Patrone umfasst, umfassenden Injektionssystem vorliegen, wobei die Patrone den Antikörper und das Immunsuppressivum in einer zur Injektion geeigneten Formulierung enthält.

53. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 1, Verwendung gemäß Anspruch 2 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 3, wobei der Antikörper in Einheitsdosierungsform vorliegt.

54. Antikörper gegen C5 zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 1, Verwendung gemäß Anspruch 2 oder Antikörper gegen C5 und das wenigstens eine Immunsuppressivum zur Verwendung bei der Verlängerung der Lebensdauer eines Allotransplantats in einem Empfänger-Säuger nach Anspruch 3, wobei das Immunsuppressivum in Einheitsdosierungsform vorliegt.

Revendications

1. Anticorps contre C5 qui inhibe l'activité du complément pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur, ledit anticorps contre C5 devant être administré conjointement avec au moins un médicament immunosuppresseur et chroniquement.

2. Utilisation d'un anticorps contre C5 qui inhibe l'activité du complément pour la fabrication d'un médicament ou ensemble de médicaments, ledit médicament devant être administré avec au moins un médicament immunosuppresseur pour prolonger la survie d'une allogreffe chez un mammifère receveur, ledit médicament devant être administré chroniquement.

3. Anticorps contre C5 qui inhibe l'activité du complément et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur, ledit anticorps contre C5 et ledit au moins un médicament immunosuppresseur devant être administrés chroniquement.

4. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 1, utilisation selon la revendication 2 ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 3, ledit mammifère étant un humain.

5. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 1 ou 4, utilisation de la revendication 2 ou 4, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 3 ou 4, ladite allogreffe étant une allogreffe à mismatch CMH.

6. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur, utilisation ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 5, ladite allogreffe à mismatch CMH étant une allogreffe à mismatch HLA.

7. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 1 ou 4, utilisation de la revendication 2 ou 4, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 3 ou 4, ledit mammifère étant un mismatch ABO pour ladite allogreffe.

8. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 1 ou 4, utilisation de la revendication 2 ou 4, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon les revendications 3 ou 4, ledit mammifère étant présensibilisé à ladite allogreffe, et ledit anticorps contre C5 devant être administré avec au moins deux médicaments immunosuppresseurs ou ledit médicament devant être administré avec au moins deux médicaments immunosuppresseurs.

9. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 1 ou 4 à 8, utilisation de l'une quelconque des revendications 2 ou 4 à

Samsung Bioepis Exhibit No. 1002
Page 1556

8, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 3 ou 4 à 8, ledit anticorps contre C5 qui inhibe l'activité du complément inhibant la formation de complément terminal ou C5a.

10. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur, utilisation ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 9, ledit anticorps contre C5 qui inhibe la formation de complément terminal ou C5a étant un anticorps total ou un fragment d'anticorps.

11. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur, utilisation, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 10, ledit anticorps total ou fragment d'anticorps étant un anticorps ou fragment d'anticorps humain, humanisé, chimérique ou désimmunisé.

12. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur, utilisation, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 10 ou 11, ledit anticorps total ou fragment d'anticorps inhibant le clivage du complément C5.

13. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur, utilisation, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 10 à 12, ledit fragment d'anticorps étant choisi dans le groupe constitué de Fab, $F(ab')_2$, Fv, un anticorps à domaine, et un anticorps monocaténaire.

14. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur, utilisation, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 10, ledit fragment d'anticorps étant le pexélizumab.

15. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur, utilisation, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 10, ledit anticorps total étant l'éculizumab.

16. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur, utilisation, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 15, ledit éculizumab devant être administré une fois toutes les 2 semaines.

17. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 1 ou 4 à 16, utilisation de l'une quelconque des revendications 2 ou 4 à 16, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 3 à 16, ledit médicament immunosuppresseur inhibant l'activité des lymphocytes T ou l'activité des lymphocytes B.

18. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 1 ou 4 à 16, utilisation de l'une quelconque des revendications 2 ou 4 à 16, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 3 à 16, ledit médicament immunosuppresseur inhibant l'activité des lymphocytes T et l'activité des lymphocytes B.

19. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 1 ou 4 à 16, utilisation de l'une quelconque des revendications 2 ou 4 à 16, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 3 à 16, ledit médicament immunosuppresseur étant choisi dans le groupe constitué de la cyclosporine A, le tacrolimus, le sirolimus, OKT3, un corticostéroïde, le daclizumab, le basiliximab, l'azathioprène, le mycophénolate mofétil, le métho-

Samsung Bioepis Exhibit No. 1002
Page 1557

trexate, la 6-mercaptopurine, des anticorps anti-lymphocyte T, le cyclophosphamide, le léflunamide, le bréquinar, ATG, ALG, la 15-désoxyspergualine, et la brédinine.

20. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 1 ou 4 à 19, utilisation de l'une quelconque des revendications 2 ou 4 à 19, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 3 à 19, plus d'un médicament immunosuppresseur devant être administré.

21. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur de la revendication 1 ou utilisation de la revendication 2, ledit médicament devant être administré conjointement avec la cyclosporine A.

22. Anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 3, ledit médicament immunosuppresseur étant la cyclosporine A.

23. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur, utilisation, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 20, ledit médicament devant être administré conjointement avec la cyclosporine A et le cyclophosphamide.

24. Anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 3, ledit médicament immunosuppresseur comprenant à la fois de la cyclosporine A et du cyclophosphamide.

25. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur ou utilisation selon la revendication 21, ledit anticorps contre C5 étant un anticorps qui inhibe le clivage du complément C5.

26. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 1 ou 4 à 25, utilisation de l'une quelconque des revendications 2 ou 4 à 25, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 3 à 25, ladite allogreffe étant choisie dans le groupe constitué de i) le coeur, ii) les reins, iii) les poumons, iv) le pancréas, v) le foie, vi) le tissu vasculaire, vii) l'oeil, viii) la cornée, ix) le cristallin, x) la peau, xi) la moelle osseuse, xii) le muscle, xiii) le tissu conjonctif, xiv) le tissu gastro-intestinal, xv) le tissu nerveux, xvi) les os, xvii) les cellules souches, xviii) les îlots, xix) le cartilage, xx) les hépatocytes, et xxi) les cellules hématopoïétiques.

27. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 1 ou 4 à 25, utilisation de l'une quelconque des revendications 2 ou 4 à 25, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 3 à 25, ladite allogreffe survivant pendant un temps au moins 20 % plus long que celui qui serait obtenu si ladite utilisation était effectuée sans ledit anticorps contre C5.

28. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 1 ou 4 à 26, utilisation de l'une quelconque des revendications 2 ou 4 à 26, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 3 à 26, ladite allogreffe survivant pendant un temps au moins 40 % plus long que celui qui serait obtenu si ladite utilisation était effectuée sans ledit anticorps contre C5.

29. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 1 ou 4 à 26, utilisation de l'une quelconque des revendications 2 ou 4 à 26, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 3 à 26, ladite allogreffe survivant pendant le reste de la durée de vie dudit mammifère.

Samsung Bioepis Exhibit No. 1002
Page 1558

30. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 1 ou 4 à 26, utilisation de l'une quelconque des revendications 2 ou 4 à 26, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 3 à 26, ladite allogreffe survivant pendant au moins six mois.

31. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 1 ou 4 à 26, utilisation de l'une quelconque des revendications 2 ou 4 à 26, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 3 à 26, ladite allogreffe survivant pendant au moins un an.

32. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 1 ou 4 à 26, utilisation de l'une quelconque des revendications 2 ou 4 à 26, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 3 à 26, ladite allogreffe survivant pendant au moins cinq ans.

33. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur, utilisation, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 4, ladite allogreffe survivant pendant le reste de la durée de vie dudit humain.

34. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 1 ou 4 à 33, utilisation de l'une quelconque des revendications 2 ou 4 à 33, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 3 à 33, ledit anticorps contre C5 devant être administré chroniquement pendant au moins 14 jours.

35. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 1 ou 4 à 33, utilisation de l'une quelconque des revendications 2 ou 4 à 33, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 3 à 33, ledit anticorps contre C5 devant être administré chroniquement pendant au moins 28 jours.

36. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 1 ou 4 à 33, utilisation de l'une quelconque des revendications 2 ou 4 à 33, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 3 à 33, ledit anticorps contre C5 devant être administré chroniquement pendant au moins 3 mois.

37. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 1 ou 4 à 33, utilisation de l'une quelconque des revendications 2 ou 4 à 33, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 3 à 33, ledit anticorps contre C5 devant être administré chroniquement pendant au moins 6 mois.

38. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 1 ou 4 à 33, utilisation de l'une quelconque des revendications 2 ou 4 à 33, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 3 à 33, ledit anticorps contre C5 devant être administré chroniquement pendant au moins 1 an.

39. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 1 ou 4 à 33, utilisation de l'une quelconque des revendications 2 ou 4 à 33, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 3 à 33, ledit anticorps contre C5 devant être administré chroniquement pendant au moins 5 ans.

Samsung Bioepis Exhibit No. 1002
Page 1559

40. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 1 ou 4 à 33, utilisation de l'une quelconque des revendications 2 ou 4 à 33, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 3 à 33, ledit anticorps contre C5 devant être administré chroniquement pendant la durée de vie restante dudit mammifère.

41. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 1 ou 4 à 33, utilisation de l'une quelconque des revendications 2 ou 4 à 33, ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon l'une quelconque des revendications 3 à 33, au moins un médicament immunosuppresseur devant être administré chroniquement pendant la durée de vie restante dudit mammifère.

42. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur, utilisation ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 41, au moins la cyclosporine A devant être administrée chroniquement pendant la durée de vie restante dudit mammifère.

43. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 1 ou utilisation de la revendication 2, ledit anticorps contre C5 et ledit médicament immunosuppresseur étant dans une formulation adaptée pour administration concomitante audit mammifère.

44. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 1 ou utilisation de la revendication 2, ledit anticorps contre C5 et ledit médicament immunosuppresseur étant dans une formulation ou des formulations adaptées pour administration séquentielle audit mammifère.

45. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 1 ou utilisation de la revendication 2, ledit anticorps contre C5 étant dans une formulation adaptée pour administration chronique audit mammifère.

46. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 1 ou utilisation de la revendication 2, ledit médicament immunosuppresseur étant dans une formulation adaptée pour administration chronique audit mammifère.

47. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 1, utilisation de la revendication 2 ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 3, ledit médicament comprenant une formulation lyophilisée comprenant l'anticorps et un lyoprotecteur.

48. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 1, utilisation de la revendication 2 ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 3, ledit médicament immunosuppresseur étant dans une formulation lyophilisée comprenant le médicament immunosuppresseur et un lyoprotecteur.

49. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 1, utilisation de la revendication 2 ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 3, ledit anticorps et ledit médicament immunosuppresseur étant dans la même formulation lyophilisée comprenant ledit anticorps, ledit médicament immunosuppresseur, et un lyoprotecteur.

50. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 1, utilisation de la revendication 2 ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 3, ledit anticorps étant dans un système d'injection comprenant une seringue qui comprend une cartouche, ladite cartouche contenant ledit anticorps dans une formulation adaptée pour injection.

Samsung Bioepis Exhibit No. 1002
Page 1560

51. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 1, utilisation de la revendication 2 ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 3, ledit médicament immunosuppresseur étant dans un système d'injection comprenant une seringue qui comprend une cartouche, ladite cartouche contenant ledit médicament immunosuppresseur dans une formulation adaptée pour injection.

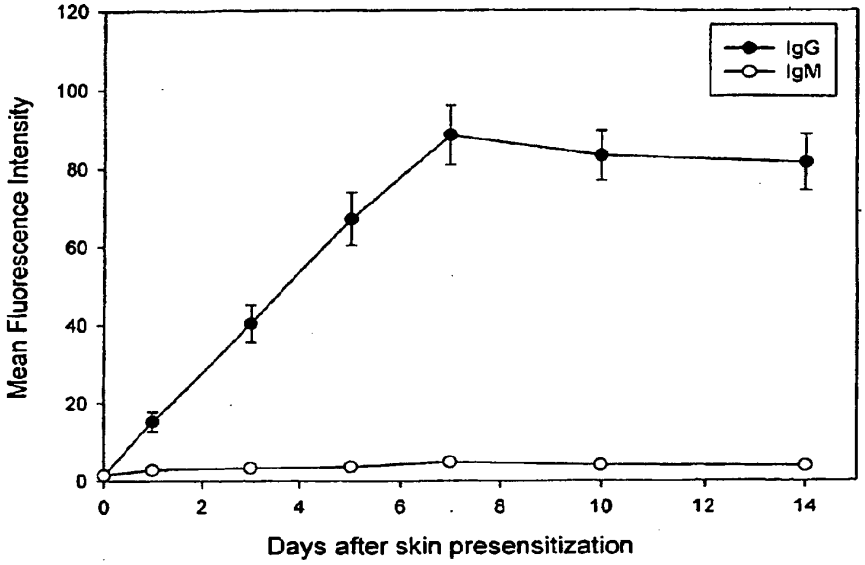
52. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 1, utilisation de la revendication 2 ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 3, ledit anticorps et ledit médicament immunosuppresseur étant dans un système d'injection comprenant une seringue qui comprend une cartouche, ladite cartouche contenant ledit anticorps et ledit médicament immunosuppresseur dans une formulation adaptée pour injection.

53. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 1, utilisation de la revendication 2 ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 3, ledit anticorps étant présent sous forme de dose unitaire.

54. Anticorps contre C5 pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 1, utilisation de la revendication 2 ou anticorps contre C5 et au moins un médicament immunosuppresseur pour utilisation dans la prolongation de la survie d'une allogreffe chez un mammifère receveur selon la revendication 3, ledit médicament immunosuppresseur étant présent sous forme de dose unitaire.

Samsung Bioepis Exhibit No. 1002
Page 1561

A



B

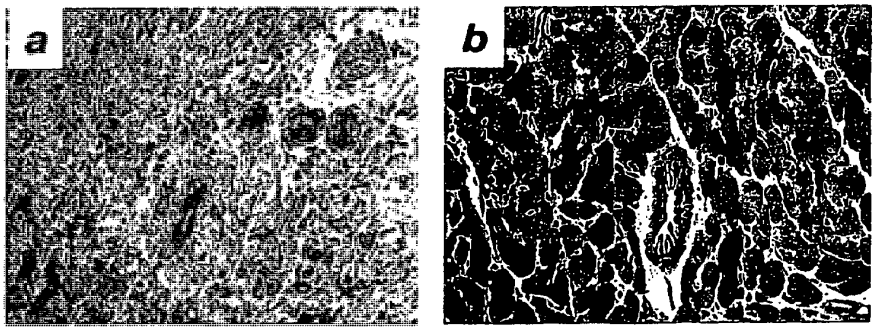


Figure 1A, B

Samsung Bioepis Exhibit No. 1002
Page 1562

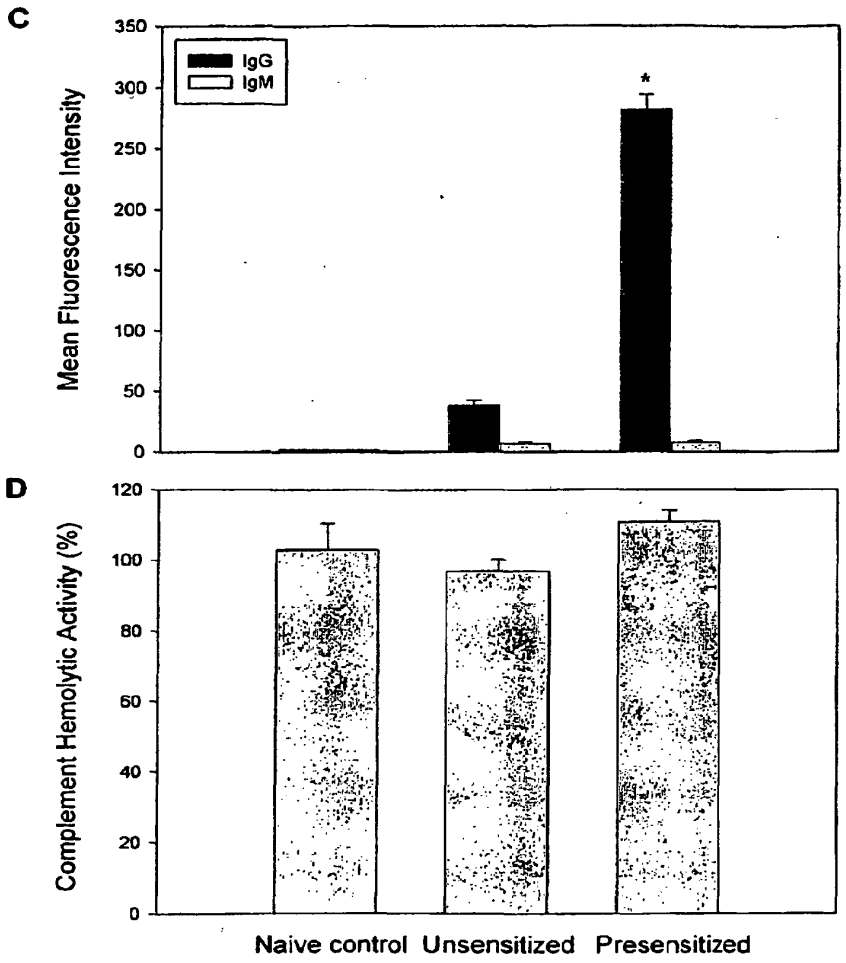


Figure 1C, D

Samsung Bioepis Exhibit No. 1002
Page 1563

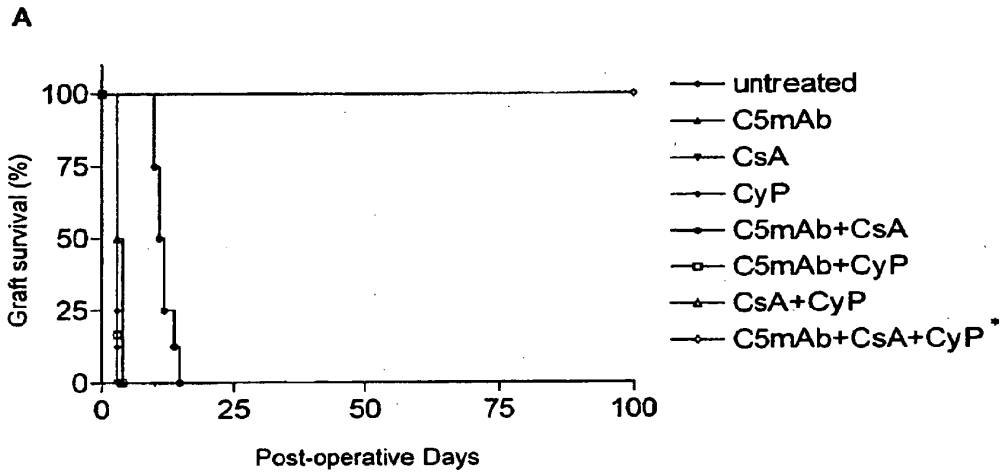


Figure 2A

Samsung Bioepis Exhibit No. 1002
Page 1564

B

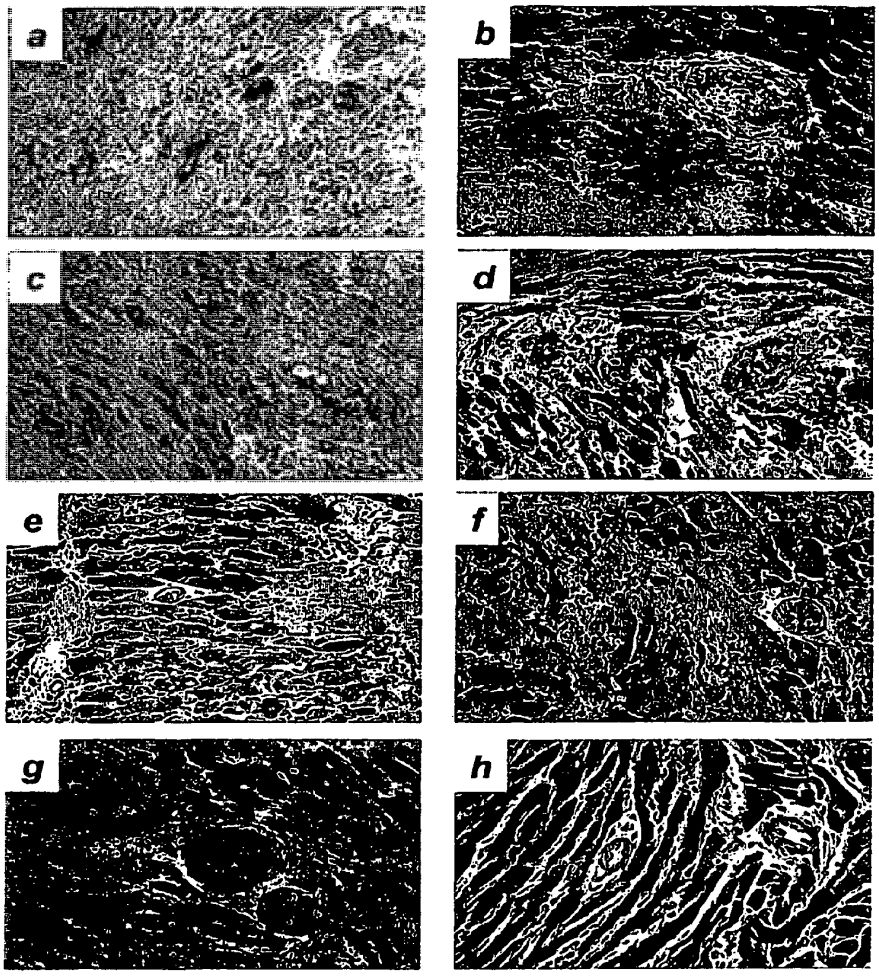


Figure 2B

Samsung Bioepis Exhibit No. 1002
Page 1565

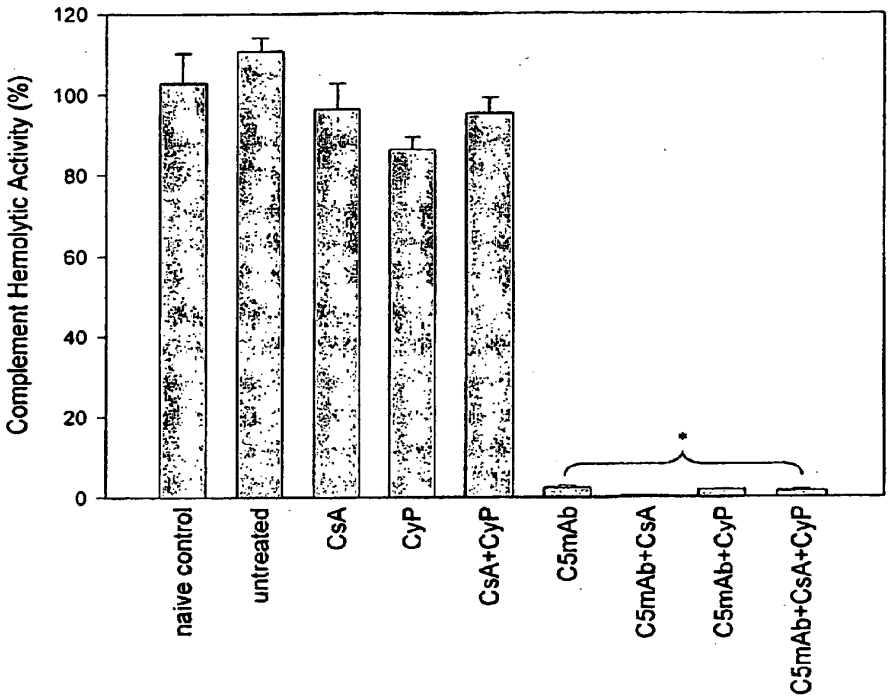


Figure 3

Samsung Bioepis Exhibit No. 1002
Page 1566

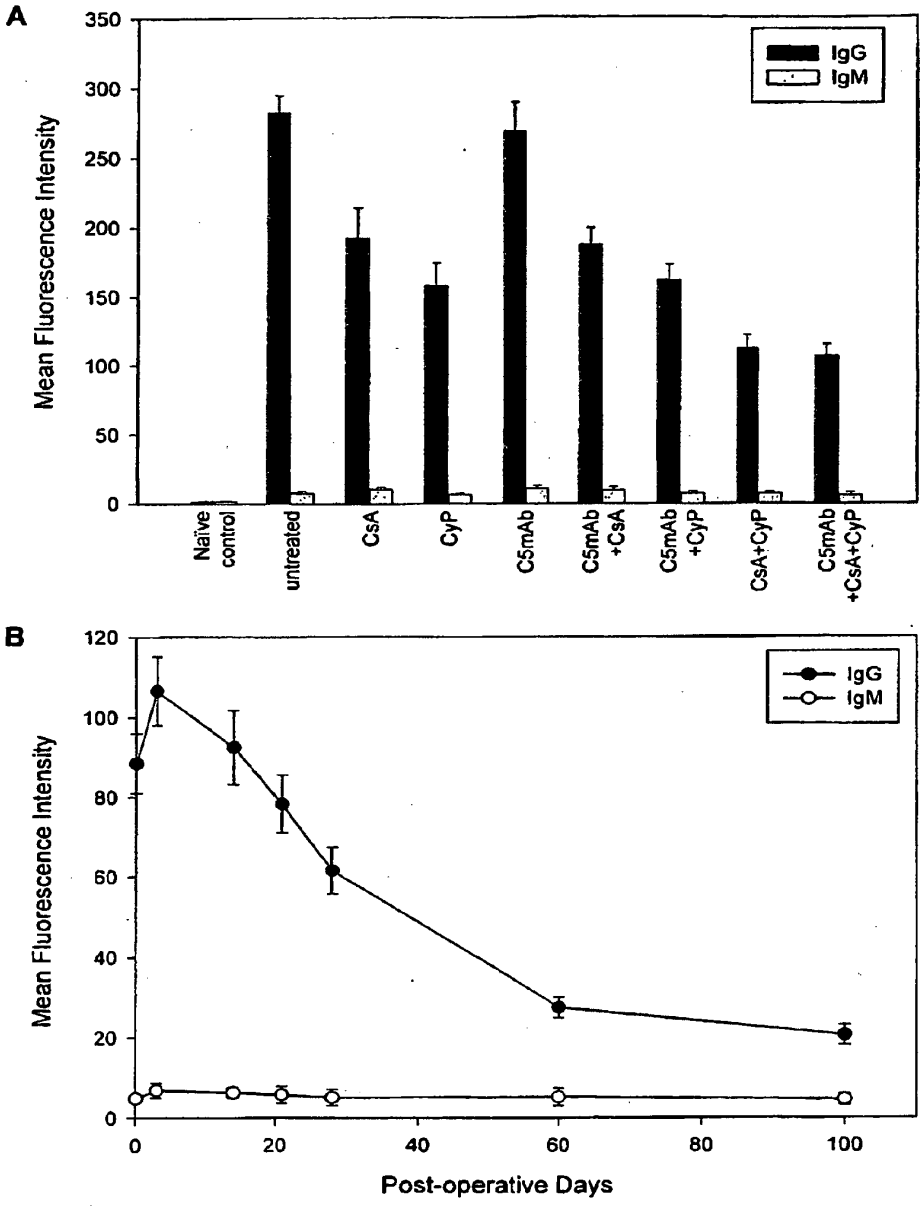
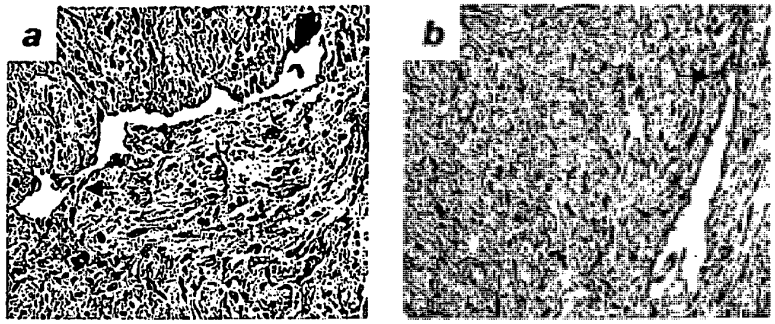


Figure 4A, B

Samsung Bioepis Exhibit No. 1002
Page 1567

C



D

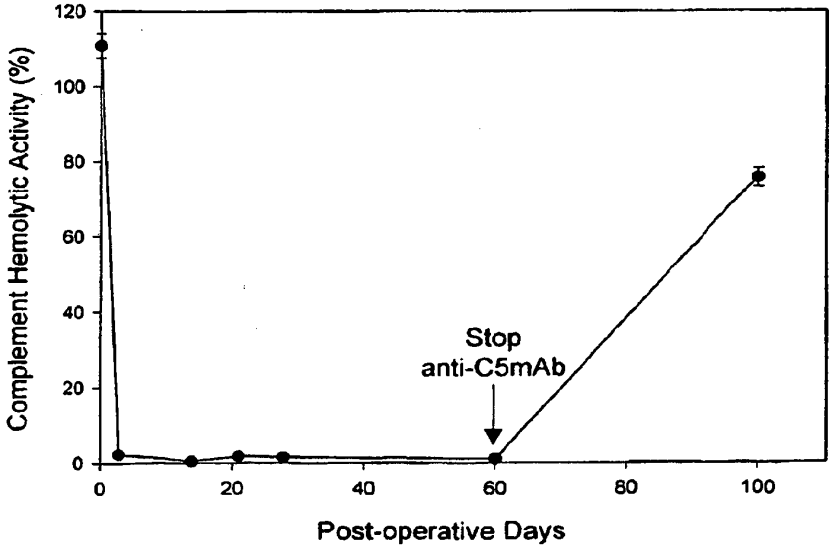


Figure 4C, D

Samsung Bioepis Exhibit No. 1002
Page 1568

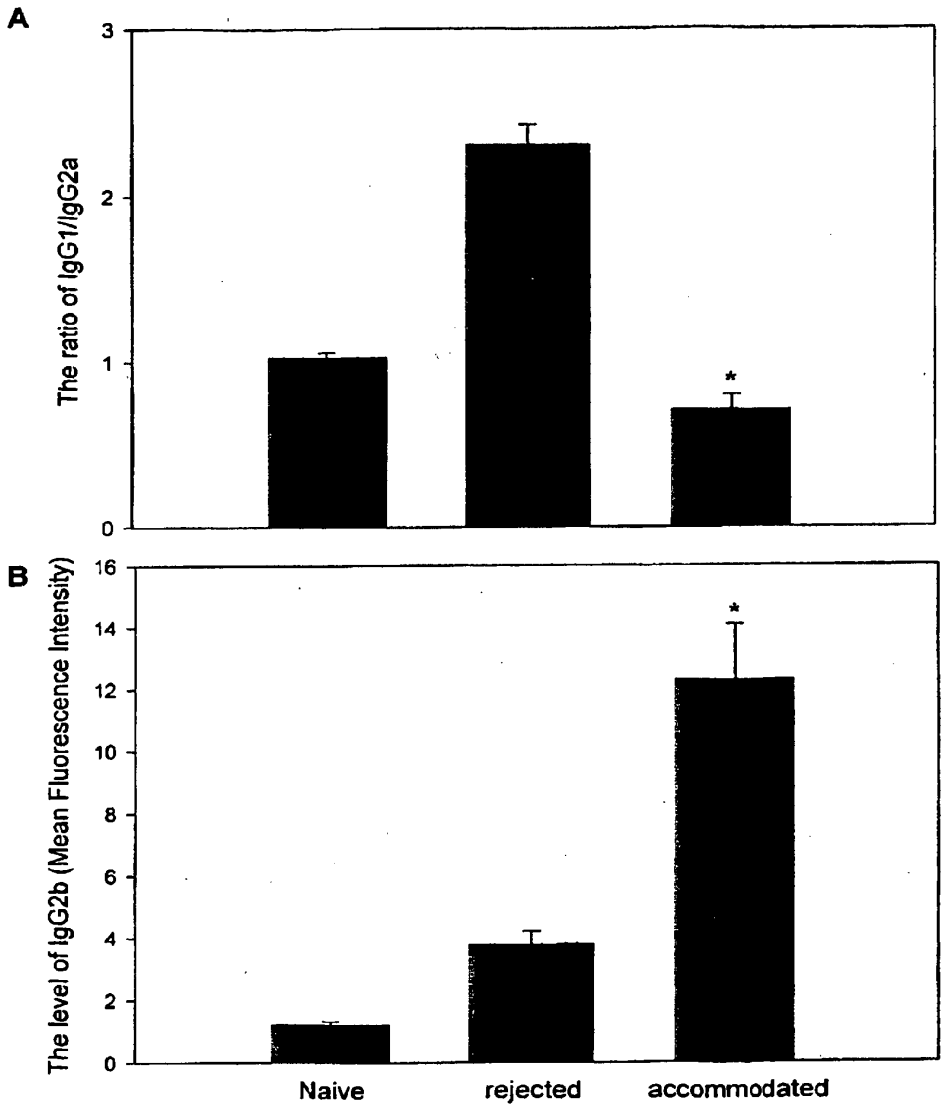


Figure 5A, B

Samsung Bioepis Exhibit No. 1002
Page 1569

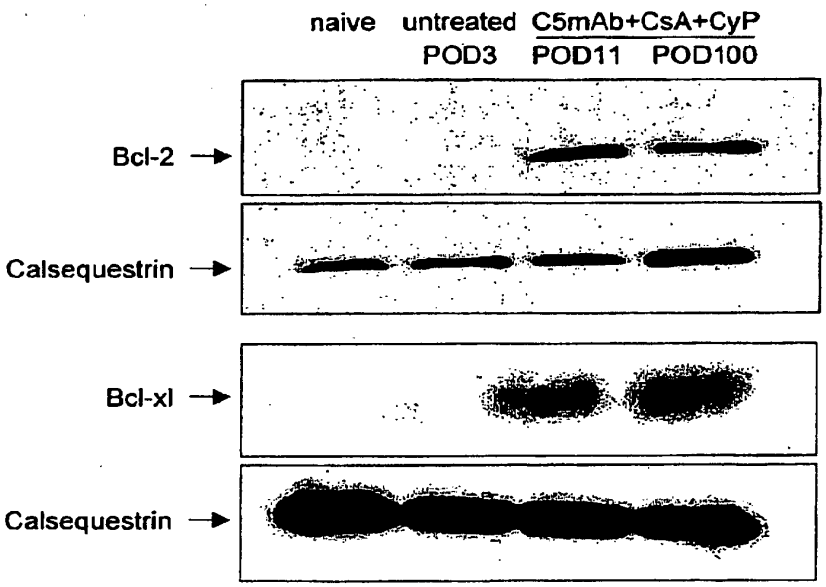


Figure 6

Samsung Bioepis Exhibit No. 1002
Page 1570

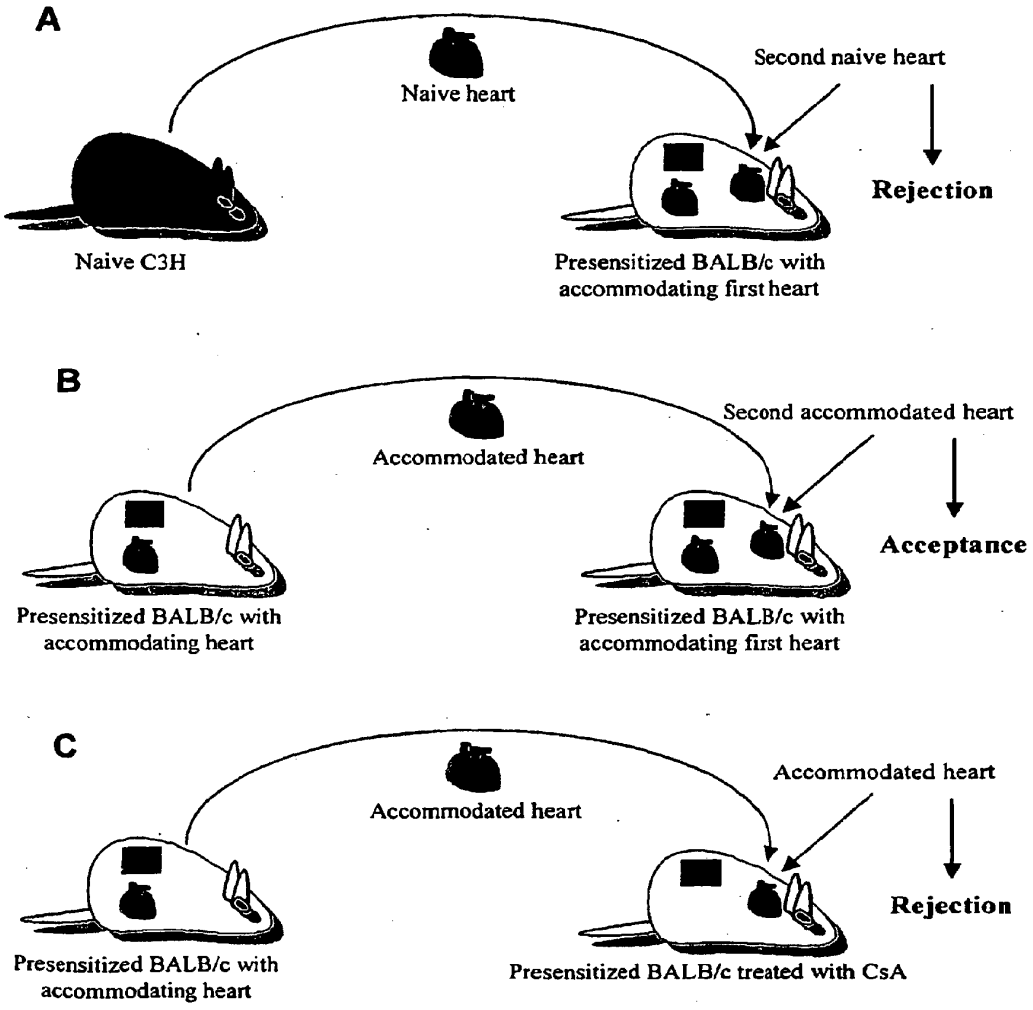
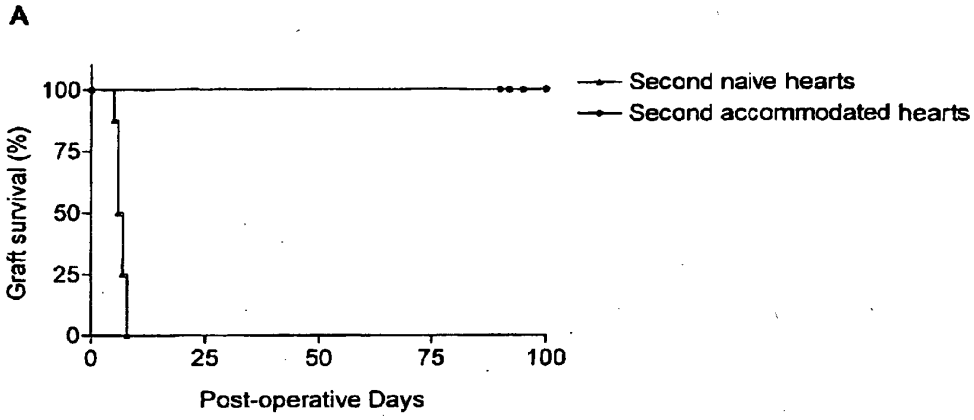


Figure 7

Samsung Bioepis Exhibit No. 1002
Page 1571



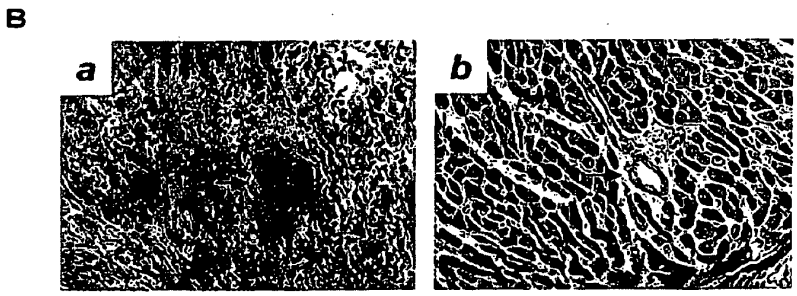


Figure 8

Samsung Bioepis Exhibit No. 1002
Page 1572

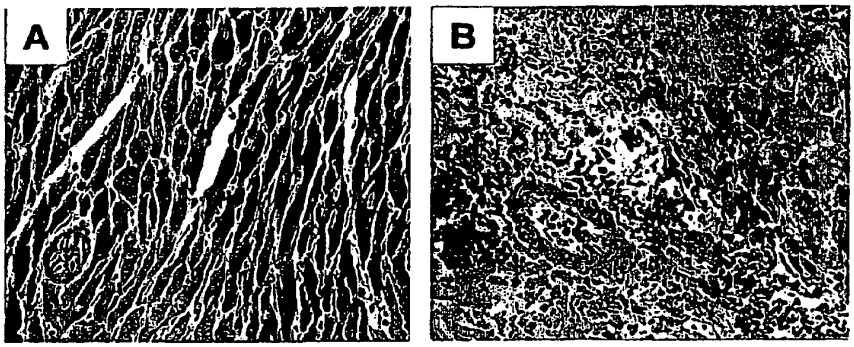


Figure 9

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Page 1573

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Samsung Bioepis Exhibit No. 1002
Page 1575

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(71) Applicant *(for all designated States except US)*: **ALEXION PHARMACEUTICALS, INC.** [US/US]; 352 Knotter Drive, Cheshire, CT 06410 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **YI, Wang** [US/US]; 270 Sylvan Valley Road, Orange, CT 06477 (US).

(74) Agent: **VARMA, Anita**; ROPES & GRAY LLP, One International Place, Boston, MA 02110-2624 (US).

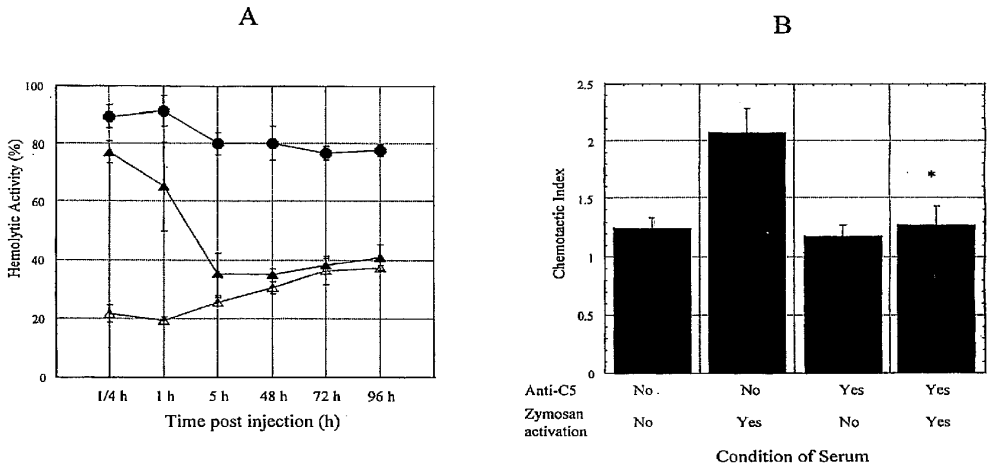
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(54) Title: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES



(57) Abstract: The present application relates to methods and compositions employing an antibody that inhibits activation of the complement system and can be used to prevent or treat a pulmonary disease or condition.

WO 2006/122257 A2

Nebulization of Monoclonal Antibodies for Treating Pulmonary Diseases

Related Applications

This application claims priority to and benefit of the filing date of U.S. Patent Application Serial No. 11/127,438, filed May 11, 2005, which is a continuation-in-part application of U.S. Patent Application Serial No. 10/655,861, filed September 5, 2003, which claims priority to and benefit of the filing date of U.S. Provisional Application Serial No. 60/469,189, filed May 9, 2003 and U.S. Provisional Application Serial No. 60/408,571, filed September 6, 2002, all of which are hereby incorporated by reference in their entirety.

Background

Asthma, bronchitis and emphysema are known collectively as Chronic Obstructive Pulmonary Diseases. These diseases are characterized as generalized airways obstruction, especially of small airways, associated with varying degrees of symptoms of chronic bronchitis, asthma, and emphysema. These diseases may often coexist in an individual, and it may be difficult to determine the primary cause of an airway obstruction. Airway obstruction is defined as an increased resistance to airflow during forced expiration. Obstruction of large airways may also occur in these diseases, particularly in asthma. Currently available therapy for asthma remains problematic. Similarly, improved therapy is also desirable for treating or preventing other pulmonary diseases, for example lung cancers.

Effective delivery to a patient is a critical aspect of any successful drug therapy. Various routes of delivery exist, and each has its own advantages and disadvantages. Oral drug delivery of pills, capsules, elixirs, and the like is perhaps the most convenient method, but many drugs are degraded in the digestive tract before they can be absorbed. Subcutaneous injection is frequently an effective route for systemic drug delivery, including the delivery of proteins, but enjoys a low patient acceptance. Since injection of drugs one or more times a day can frequently be a source of poor patient compliance, a variety of alternative routes of administration have also been developed, including transdermal, intranasal, intrarectal, intravaginal, and pulmonary delivery. Thus, it is desirable to improve

Samsung Bioepis Exhibit No. 1002
Page 1577

drug delivery methods and compositions, particularly for antibody-based therapeutics in treating or preventing pulmonary diseases.

Summary

Accordingly, this application provides compositions and methods suitable for delivering antibody-based therapeutics. These antibody-based therapeutics can be particularly useful in preventing or treating pulmonary diseases or conditions such as for example asthma. Examples of antibodies useful in this application include anti-C5 antibody or antibodies that inhibit activation of the complement cascade, for example, the antibodies as described in U.S. Patent No. 6,355,245. In addition to antibodies, other therapeutics are also contemplated to be employed with the compositions and methods of the present application, for example, the chimeric complement inhibitor proteins as described in U.S. Patent No. 5,627,264. Certain preferred embodiments employ pexelizumab or eculizumab as the antibody therapeutic. In certain embodiments, the methods suitable for delivering antibody-based therapeutics for treating or preventing pulmonary diseases are not designed for systemic delivery of the therapeutics, and therefore, systemic effect of the therapeutics may not be observed using these methods.

A first aspect of the application provides a method for preventing or treating a pulmonary disease or condition in a subject comprising administering to the subject a therapeutically effective amount of an antibody that inhibits activation of the complement cascade or blocks the function of one or more subsequently activated components of the complement system. In certain embodiments, an additional active agent is also administered to the same subject. Administration of the antibody and the additional active agent may occur simultaneously or sequentially in either order. In certain embodiments, the antibody and the additional active agent can be administered to the subject via the same delivery method or route, for example, by inhalation. In alternative embodiments, the antibody and the additional active agent can be administered to the subject via different delivery methods or routes, for example, the antibody delivered by inhalation and the additional active agent by injection or by oral intake.

Samsung Bioepis Exhibit No. 1002
Page 1578

A second aspect of the application provides an aerosol composition comprising an antibody that inhibits activation of the complement cascade, wherein the composition is suitable for preventing or treating a pulmonary disease or condition in a subject. The antibody is formulated in a composition suitable for aerosolization. The antibody may be formulated in combination with an additional active agent, and the combination formulation is suitable for aerosolization. Alternatively, the antibody and an additional active agent may be formulated separately, such that they will be combined after aerosolization occurs or after being administered to a subject.

A third aspect of the application provides a nebulization composition comprising an antibody that inhibits activation of the complement cascade, wherein the composition is suitable for preventing or treating a pulmonary disease or condition in a subject. The antibody is formulated in a composition suitable for nebulization. Similarly, the antibody may be formulated in combination with an additional active agent, and the combination formulation is suitable for nebulization. Alternatively, the antibody and an additional active agent may be formulated separately, such that they will be combined after nebulization occurs or after being administered to a subject.

A further aspect of the application provides a biopharmaceutical package comprising an antibody that inhibits activation of the complement cascade and a nebulizer, wherein the package is suitable for preventing or treating a pulmonary disease or condition in a subject. The biopharmaceutical package may further comprise an active agent in addition to the antibody. The biopharmaceutical package may also comprise instructions for use.

Pulmonary diseases or conditions contemplated by the application include, but are not limited to, asthma, bronchial constriction, bronchitis, a chronic obstructive pulmonary disease (COPD), interstitial lung diseases, lung malignancies, α-1 anti-trypsin deficiency, emphysema, bronchiectasis, bronchiolitis obliterans, sarcoidosis, pulmonary fibrosis, and collagen vascular disorders.

The timing of administering a therapeutic to a subject can vary, for example, depending on the identity of the subject or the pulmonary disease or condition to be

Samsung Bioepis Exhibit No. 1002
Page 1579

treated or prevented, or both. For example, the administration may occur before the manifestation of the pulmonary condition (e.g., pre-asthmatic attack), during the manifestation of the pulmonary condition (e.g., during the asthmatic attack), or after the manifestation of the pulmonary condition (e.g., post-asthmatic attack).

An antibody of the present application can be specific to C5 such that it prevents the cleavage of C5 into C5a and C5b. The antibody can be specific to the C5 convertase. Alternatively, the antibody may be specific to a component of the complement system, for example, C5a, C5b, or C5b-9, and the antibody specific to the component preferably inhibits the component's function, for example, by blocking the component's binding to its respective receptor, or by blocking its function in activating the subsequent signaling or events in the complement cascade. Certain embodiments employ eculizumab or pexelizumab, or both. An antibody or antibody therapeutic of the present application can be a full length immunoglobulin, a monoclonal antibody, a chimeric antibody (e.g., a humanized antibody), a single chain antibody, a domain antibody, an Fab fragment, or an antibody having an Fab fragment and a mutated Fc portion. In certain embodiments, the mutated Fc portion does not activate complement, or the mutation(s) in the Fc portion decreases the Fc portion's ability to activate complement. An antibody of the present application may be produced or processed in bulk and packaged in an ampule made of a suitable material (e.g., glass or plastic) at different doses.

An additional active agent (or an active agent in addition to the antibody therapeutic) of the present application can be another antibody therapeutic (e.g., an anti-IgE antibody such as Xolair® or omalizumab, an anti-IL-4 antibody or an anti-IL-5 antibody), an anti-IgE inhibitor (e.g., Singulair® or montelukast sodium), a sympathomimetic (e.g., albuterol), an antibiotic (e.g., tobramycin), a deoxyribonuclease (e.g., pulmozyme), an anticholinergic drug (e.g., ipratropium bromide), a corticosteroid (e.g., dexamethasone), a β-adrenoreceptor agonist, a leukotriene inhibitor (e.g., zileuton), a 5 Lipoxygenase inhibitor, a PDE inhibitor, a CD23 antagonist, an IL-13 antagonist, a cytokine release inhibitor, a histamine H1 receptor antagonist, an anti-histamine, an anti-inflammatory agent (e.g. cromolyn sodium) or a histamine release inhibitor.

Samsung Bioepis Exhibit No. 1002
Page 1580

An example of formulation suitable for aerosolization or nebulization of an antibody is in physiologic osmolarity (e.g., between 280 and 320 mM) at a suitable pH (e.g., pH 6 to 8). A formulation of the present application may further comprise an excipient, for example polysorbate 80 which can be used at 0.0015 to 0.02%.

A nebulizer of the present application can be a jet air nebulizer (e.g., Pari LC Jet Plus or Hudson T Up-draft II), an ultrasonic nebulizer (e.g., MABISMist II), a vibrating mesh nebulizer (e.g., Micro air by Omron) and a shockwave nebulizer (EvitLabs Sonik LDI20).

Brief Description of the Drawings

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

Fig. 1 illustrates blocking the generation of C5a and C5b-9 by anti-C5 mAb, BB5.1. Pharmacodynamic profile (A) of inhibition of C5b-9 mediated hemolysis from animals given a single i.v. or i.p. injection of anti-C5 mAb (40 mg/kg) or control mAb (40 mg/kg). ● represents data from 4 control animals that were given control mAb by either i.v. (n=2) or i.p. (n=2). ▲ represents i.p. anti-C5 mAb-treated animals (n=3). Δ represents i.v. anti-C5 mAb-treated animals (n=3). C5a-mediated neutrophil migration (B) was analyzed in an in vitro assay. Zymosan activated sera (20%) was used as the source of C5a. Anti-C5 mAb (100 μg/ml) was added to serum sample prior to zymosan activation as indicated (n=8). * indicates $p < 0.05$ when compared to zymosan activated serum samples. BALB/c mice were used in all figures except Figs. 4 C and D.

Fig. 2 shows airway response to OVA provocation and definition of three critical checkpoints. A: Representative DCP results indicating the longitudinal changes of sRaw. After aerosol challenge with 5% OVA for 10 min, the appearance of EAR was observed at 15 min, followed by the LAR at 5 h. B: Three critical points during the course of disease. Checkpoint 1: Anti-C5 mAb was given by i.p. injection on days 25, 29 and 31. Analysis of lower airway functions and quantification of airway inflammation were evaluated 5 h after aerosol challenge of 5% OVA on day 32. Checkpoint 2: Anti-C5 mAb was given by i.p. injection on day

Samsung Bioepis Exhibit No. 1002
Page 1581

33, after recovery from the first airway response on day 32. AHR was assessed by evaluating the changes of RL and Cdyn from baseline in response to aerosol Mch challenge on day 35. Checkpoint 3: C5 inhibition was given by either i.v. or aerosol administration of BB5.1 during EAR on day 35. Lower airway function was determined during the peak of LAR, 5 h after 5% OVA provocation.

Fig. 3 shows contribution of C5 at checkpoint 1, the initiation of airway inflammation. Mean serum OVA-specific antibody titers of 5-6 mice on day 32 were measured by ELISA (A). Cross hatched bars: OVA-specific IgG. Open bars: OVA-specific IgE. Also on day 32, animals were trachea cannulated for the measurement of RL (B) and Cdyn (C) 5 h after 5% aerosol OVA provocation, followed by histological analyses of lung (D). The mean histology score for control mAb-treated mice (F) is 2±0.28 compared to 0.64±0.21 for corticosteroid-treated animals (G) and 1.25±0.25 for anti-C5 mAb-treated animals (H) compared to total lack of inflammation, score =0, seen in sham-mice (E). * indicates $p<0.05$ when comparing treated animals (i.e. corticosteroid or anti-C5 mAb treatment) with control mAb treated animals, while ■ indicates $p<0.05$ when comparing corticosteroid treated mice with animals treated with antibody targeting C5 or C5aR in this and all following figures unless otherwise indicated. For clarity, all control mAb treated animals are labeled as control in all following figures. Arrow indicates eosinophil infiltration. Giemsa stain, magnification = 100X.

Fig. 4 shows contribution of C5 at checkpoint 2, the development of AHR. C5s BALB/cByJ mice (A&B) and C5d B10D2oSn mice (C&D) were immunized with OVA in identical manner (Fig. 2 B). Animals were randomized and given (i.p.) the indicated treatment on day 33. On day 35, aerosol Mch challenges were administered through a trachea cannula during measurement of RL (A&C) and Cdyn (B&D). Changes in RL and Cdyn are expressed as a percentage of baseline after each aerosol challenge. Subgroups of C5d mice with or without OVA immunization were reconstituted (i.v.) with 200μg of rmC5a 3 hours prior to Mch challenges. For C5s BALB/c mice, * indicates $p<0.05$ when comparing either steroid or anti-C5 mAb treated animals with control mAb treated animals. For C5d B10D2oSn mice, * indicates $p<0.05$ when comparing the mAb treated animals (n=6) given either control mAb (n=3) or anti-C5 mAb (n=3) with OVA immunized C5d B10D2oSn

Samsung Bioepis Exhibit No. 1002
Page 1582

mice reconstituted with rmC5a. There are no statistical differences between C5d animals treated with control mAb and anti-C5 mAb.

Fig. 5 shows contribution of C5 at checkpoint 3, during an on-going airway response. Animals that experienced previous airway response to allergen provocation on day 32 were given a second aerosol allergen challenge on day 35. During the peak of EAR, animals were randomized and given (i.v.) the indicated treatment. Animals were trachea cannulated for the measurement of RL (A) and Cdyn (B) 5 h after the OVA provocation. (n= 7-8 mice per group.)

Fig. 6 shows BALF WBC counts and differential analysis. BALF was obtained 5 h after aerosol allergen provocation at checkpoint 1 (A) or at checkpoint 3 (B&C). Total BALF WBC counts (A&B) and their differential analyses (C) were performed by a pathologist in a double blind fashion. For 6 (C), open bars: alveolar macrophages. Cross hatched bars: Eosinophils. Solid bars: Neutrophils. Hatched bars: Lymphocytes. (n= 6-8 mice per group.)

Fig. 7 shows BALF levels of inflammatory mediators. BALF was obtained 5 h after aerosol allergen provocation at checkpoint 1 (cross hatched bars) or checkpoint 3 (solid bars). BALF levels of IL-5 (A), IL-13 (B), histamine (C), eotaxin (D), RANTES (E), activated TGF-β (F), TNF-α (G) and proMMP9 (H) were measured by ELISA. (n= 6-8 samples per group.)

Fig. 8 shows contribution of intrapulmonary C5 activation at checkpoint 3. Results represent data pooled from 3 separate experiments with identical experimental procedures. Animals were aerosol challenged with 5% OVA and randomized. During EAR, animals

were then given aerosol administration of one of the indicated treatments through a jet nebulizer. Sham-immunized mice were treated with aerosol PBS. All animals were trachea cannulated for the measurement of RL (A) and Cdyn (B) 5 h after the OVA provocation. BALF WBC counts (C) and proMMP9 (D) were analyzed in the majority of animals. The data from control mAb (n=15) and control sIgG treated mice (n=4) were pooled together. Except anti-C5 Fab (n=3), n=6-19 animals in all other cohorts.

Samsung Bioepis Exhibit No. 1002
Page 1583

Fig. 9 shows the result of combined therapy on the development of AHR in subjects with established airway inflammation. The results were obtained based on checkpoint 2 study as described herein. Treatment was delivered on day 32 by nebulization instead of i.p. Mch challenge occurred on day 35. For anti-C5, BB5.1 at 3 mg/ml was subjected to nebulization for 10 minutes. The steroid at 2 mg/ml was nebulized for 10 minutes. For the combination, the final concentration was 3 mg/ml for BB5.1 and 2 mg/ml for the steroid, which was subjected to nebulization for 10 minutes.

Fig. 10 shows exemplary formulations suitable for nebulization of an antibody.

Fig. 11 shows the result of Mch challenge two days after nebulization treatment.

Fig. 12 shows the particle size distribution.

Fig. 13 shows nebulization and delivery efficiencies.

Fig. 14 shows SDS-PAGE analysis of aerosolized eculizumab.

Fig. 15 shows SEC HPLC analysis of aerosolized eculizumab using two different nebulizers.

Fig. 16 shows that repeated long term aerosol administrations of anti-C5 monoclonal antibody do not induce airway inflammation. The mice here were subject to 10-week treatments: sham mice were treated with PBS for 10 min twice per week for 10 weeks; control mice were treated with HFN7.1 (an isotype-matched mouse antibody) for 10 min by nebulization, also twice a week for 10 weeks; the anti-C5 antibody (BB5.1) treated mice were treated with BB5.1 for 10 min by nebulization, also twice a week for 10 weeks.

Detailed Description of the Application

Overview

The present application abbreviates certain terms as follows:

AHR: airway hyper-responsiveness; BALF: bronchoalveolar lavage fluid; Cdyn - dynamic lung compliance; C5aR: C5a receptor; C5d: C5 deficient; C5s: C5

Samsung Bioepis Exhibit No. 1002
Page 1584

sufficient; DCP: double chamber plethysmograph; EAR: Early phase airway response; IC: immune complexes; LAR: Late phase airway response; Mch: methacholine; rmC5a: recombinant mouse C5a; RL: lung resistance; sRaw: Specific airway resistance; sIgG: serum IgG.

The Complement System

The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of cellular and viral pathogens. There are at least 25 complement proteins, which are found as a complex collection of plasma proteins and membrane cofactors. The plasma proteins make up about 10% of the globulins in vertebrate serum. Complement components achieve their immune defensive functions by interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions.

The complement cascade progresses via the classical pathway or the alternative pathway. These pathways share many components, and while they differ in their initial steps, they converge and share the same "terminal complement" components (C5 through C9) responsible for the activation and destruction of target cells.

The classical complement pathway is typically initiated by antibody recognition of and binding to an antigenic site on a target cell. The alternative pathway is usually antibody independent, and can be initiated by certain molecules on pathogen surfaces. Both pathways converge at the point where complement component C3 is cleaved by an active protease (which is different in each pathway) to yield C3a and C3b. Other pathways activating complement attack can act later in the sequence of events leading to various aspects of complement function.

C3a is an anaphylatoxin (see discussion below). C3b binds to bacterial and other cells, as well as to certain viruses and immune complexes, and tags them for removal from the circulation. (C3b in this role is known as opsonin.) The opsonic function of C3b is considered to be the most important anti-infective action of the complement system. Patients with genetic lesions that block C3b function are prone

Samsung Bioepis Exhibit No. 1002
Page 1585

to infection by a broad variety of pathogenic organisms, while patients with lesions later in the complement cascade sequence, i.e., patients with lesions that block C5 functions, are found to be more prone only to Neisseria infection, and then only somewhat more prone (Fearon, in Intensive Review of Internal Medicine, 2d ed. Fanta and Minaker, eds. Brigham and Women's and Beth Israel Hospitals, 1983).

C3b also forms a complex with other components unique to each pathway to form classical or alternative C5 convertase, which cleaves C5 into C5a and C5b. C3 is thus regarded as the central protein in the complement reaction sequence since it is essential to both the alternative and classical pathways (Wurzner, et al., Complement Inflamm. 8:328-340, 1991). This property of C3b is regulated by the serum protease Factor I, which acts on C3b to produce iC3b. While still functional as opsonin, iC3b cannot form an active C5 convertase.

C5 is a 190 kDa beta globulin found in normal serum at approximately 75 μg/ml (0.4 μM). C5 is glycosylated, with about 1.5-3 percent of its mass attributed to carbohydrate. Mature C5 is a heterodimer of a 999 amino acid 115 kDa alpha chain that is disulfide linked to a 656 amino acid 75 kDa beta chain. C5 is synthesized as a single chain precursor protein product of a single copy gene (Haviland et al. J. Immunol. 1991, 146:362-368). The cDNA sequence of the transcript of this gene predicts a secreted pro-C5 precursor of 1659 amino acids along with an 18 amino acid leader sequence.

The pro-C5 precursor is cleaved after amino acid 655 and 659, to yield the beta chain as an amino terminal fragment and the alpha chain as a carboxyl terminal fragment, with four amino acids deleted between the two. C5a is cleaved from the alpha chain of C5 by either alternative or classical C5 convertase as an amino terminal fragment comprising the first 74 amino acids of the alpha chain. Approximately 20 percent of the 11 kDa mass of C5a is attributed to carbohydrate. The cleavage site for convertase action is at or immediately adjacent to amino acid residue 733 of the 1659-amino acid pro-C5 precursor sequence with an 18-amino acid leader sequence (SEQ ID NO:2 as described in U.S. Patent No. 6,355,245). A compound that would bind at or adjacent to this cleavage site would have the

Samsung Bioepis Exhibit No. 1002
Page 1586

potential to block access of the C5 convertase enzymes to the cleavage site and thereby act as a complement inhibitor.

C5 can also be activated by means other than C5 convertase activity. Limited trypsin digestion (Minta and Man, J. Immunol. 1977, 119:1597-1602; Wetsel and Kolb, J. Immunol. 1982, 128:2209-2216) and acid treatment (Yamamoto and Gewurz, J. Immunol. 1978, 120:2008; Damerau et al., Molec. Immunol. 1989, 26:1133-1142) can also cleave C5 and produce active C5b.

C5a is another anaphylatoxin. C5b combines with C6, C7, and C8 to form the C5b-8 complex at the surface of the target cell. Upon binding of several C9 molecules, the membrane attack complex (MAC, C5b-9, terminal complement complex--TCC) is formed. When sufficient numbers of MACs insert into target cell membranes the openings they create (MAC pores) mediate rapid osmotic lysis of the target cells. Lower, non-lytic concentrations of MACs can produce other effects. In particular, membrane insertion of small numbers of the C5b-9 complexes into endothelial cells and platelets can cause deleterious cell activation. In some cases activation may precede cell lysis.

As mentioned above, C3a and C5a are anaphylatoxins. These activated complement components can trigger mast cell degranulation, which releases histamine and other mediators of inflammation, resulting in smooth muscle contraction, increased vascular permeability, leukocyte activation, and other inflammatory phenomena including cellular proliferation resulting in hypercellularity. C5a also functions as a chemotactic peptide that serves to attract pro-inflammatory granulocytes to the site of complement activation.

Pulmonary Diseases or Conditions, and the Complement System

Certain pulmonary diseases or conditions such as asthma can be characterized by a combination of chronic airway inflammation, airway obstruction and airway hyper-responsiveness (AHR) to various stimuli. It is thought to be mediated primarily by adaptive immune responses including allergen-specific $CD4^+$ T cells, Th2 cytokines, and allergen specific IgE leading to pulmonary inflammation and AHR. Complement and its activated components, which form a central core of innate immune defense against bacterial, viral and fungal invasions (Nagy et al., J.

Samsung Bioepis Exhibit No. 1002
Page 1587

Allergy. Clin. Immunol. (2003) 112:729-734; Kasamatsu et al., Arerugi (1993) 42:1616-1622; Bjornson et al., Am. Rev. Respir. Dis. (1991) 143:1062-1066), can be activated through the classical pathway, alternative pathway and the lectin pathway (Lachmann, Res. Immunol. (1996) 147:69-70). All three activation pathways converge at complement component C5 prior to the generation of C5a and C5b-9, both of which induce potent biological responses including tissue injury, inflammation, anaphylatoxic responses, and cell lysis at very low concentrations (Takafuji et al., Int. Arch. Allergy Immunol. (1994) 104 Suppl. 1:27-29). In addition, C5 can be activated after allergen exposure (Nagata et al., J. Allergy Clin. Immunol. (1987) 80:24-32). Recent data from animal models of allergic asthma suggest that activated complement components, such as C5a, provide a critical link between innate and adaptive immunity (Karp et al., Nat. Immunol. (2000) 1:221-226). However, controversies remain in the art regarding the involvement of C5 and its activated components in the pathogenesis of asthma (Karp et al., *supra*; Gerard et al., Curr. Opin. Immunol. (2002) 14:705-708; Abe et al., J. Immunol. (2001) 167:4651-4660; Lukacs et al., Am. J. Physiol. Lung Cell. Mol. Physiol. (2001) 280(3):L512-L518).

Several experimental models for bronchial asthma have indicated that C5 and its activated components are involved in the development of airway inflammation and bronchoconstriction (or bronchial constriction). Inhibition studies with various complement inhibitors markedly reduced AHR or airway inflammation in rodents (Abe et al., *supra*; Lukacs et al., *supra*). The potential involvement of C5 activation was also extended to clinical observations that the severity of clinical symptoms was correlated with the extent of C5 activation (Gonczi et al., Allergy (1997) 52:1110-1114). On the other hand, studies have shown that C5 deficiency (C5d) leads to increased susceptibility to allergen-induced AHR in mice, and this finding is supported by evidence of decreased production of IL-12, a key Th1 cytokine reported to modulate the pathogenesis of asthma (Karp et al., *supra*). The key question at the center of the debate is whether C5 and its activated components are pro-inflammatory or anti-inflammatory during the sensitization phase and the effector phase of the pathogenesis as suggested respectively from studies of C5d animals (Karp et al., *supra*) and experiments with intervention during the course of

Samsung Bioepis Exhibit No. 1002
Page 1588

disease (Abe et al., *supra*; Lukacs et al., *supra*). In addition, another key issue is whether intrapulmonary-activated complement components play a significant role in the pathogenesis and overcome the potential anti-inflammatory effect of activated C5 components on the adaptive immune system (Karp et al., *supra*).

The present application relates to the study of the contribution of C5 and its activated components at three critical points during the course of disease, which outlines the key mechanisms of this essential component of the innate immune system in the pathogenesis of asthma. Comprehensive analyses of lower airway function and quantification of multiple parameters of airway inflammation have been conducted. As described herein, the study shows that C5 contributes to the initiation of airway inflammation, demonstrates the critical contribution of C5 in the development of AHR in animals with established airway inflammation and the significant role of activated C5 components in sustaining an on-going airway response to allergen challenge.

The pathophysiological hallmark necessary for the development of asthma-like symptoms is airway inflammation. Individuals with a genetic predisposition may have modulated adaptive immune responses to environmental exposures, including activation of allergen-specific $CD4^+$ T cells, polarization of Th2 cytokines, such as IL-4 and IL-5, which are critically involved in the production of allergen-specific IgE and the recruitment of eosinophils (Cieslewicz et al., J. Clin. Invest. (1999) 104:301-308). More recently, IL-13 was identified as a key player in the pathogenesis, and reported to be both necessary and sufficient to induce the airway response in animals (Wills-Karp et al., Science (1998) 282:2258-2261). As shown by the study described herein, the broad spectrum of anti-inflammatory activities of corticosteroid, including modulating the Th2 cytokine profile (Figs. 7 A and B) and blocking the recruitment of inflammatory cells into lower airways (Fig. 3 D), correlates with its potent antiasthmatic activity. Consistent with its anti-inflammatory activities reported earlier (Wang et al., Proc. Natl. Acad. Sci.U.S.A (1996) 93:8563-8568), C5 inhibition at checkpoint 1 resulted in significantly less airway inflammation without significant impact on the adaptive immune system's responses to allergen exposures. Further, C5d animals developed a similar degree of airway inflammation as that developed by C5s animals. One possible explanation is

Samsung Bioepis Exhibit No. 1002
Page 1589

that airways are directly exposed to allergens and infections. These exposures may activate other complement components, such as C3a, which has very similar proinflammatory properties (Takafuji et al., Int. Arch. Allergy Immunol. (1994) 104 Suppl. 1:27-29; Nagata et al., J. Allergy Clin. Immunol. (1987) 80:24-32), and was known to contribute to the pathogenesis (Gerard et al., Curr. Opin. Immunol. (2002) 14:705-708). The data provided in the present application indicate that activated C5 components are critically involved but are not necessary for the initiation of airway inflammation, which requires the activation of adaptive immune responses to aerosol exposures of allergen (Cieslewicz et al., *supra*). However, at checkpoints 2 and 3 when airway inflammation was already well established, the data described herein indicate that C5 inhibition has similar or better efficacy than corticosteroid in ameliorating airway inflammation and improving lower airway functions (see, e.g., Figs. 4-8).

The second hallmark of asthma, the development of AHR in response to non-specific stimuli, was selected as another critical checkpoint. By pre-screening several strains of C5s and C5d mice, the genetic influences on the function of muscarinic acetylcholine receptor responsible for the intrinsic AHR (De Sanctis et al., Am. J. Respir. Crit. Care Med. (1997) 156:S82-S88; Levitt et al., FASEB J. (1988) 2:2605-2608.) are eliminated from the study described herein. Using animals that do not have intrinsic AHR, C5 inhibition is shown to be sufficient to prevent the development of AHR to aerosol Mch challenges in C5s BALB/c mice with established airway inflammation (Figs. 4 A and B) while reconstitution of C5d B10D2oSn mice with rmC5a restored AHR, also in the presence of established airway inflammation
(Figs. 4 C and D). These data suggest that C5, most likely C5a, serves as a direct link between the innate immune responses and the key components of the airways responsible for AHR. C5aR was found on airway smooth muscle cells and airway epithelia (Drouin et al., J. Immunol. (2001) 166:2025-2032). Although the direct interaction of activated C5 components, in particular C5a, with receptors on airway smooth muscles and epithelium was demonstrated previously (Irvin et al., Am. Rev. Respir. Dis. (1986) 134:777-783; Larsen, Annu. Rev. Immunol. (1985) 3:59-85.), the data provided herein indicate that the maintenance of AHR depends upon

Samsung Bioepis Exhibit No. 1002
Page 1590

complex interactions among airway epithelia, airway smooth muscle cells, inflammatory cells and their inflammatory mediators as suggested by minimal impact on the functions of lower airways during Mch challenges after systemic reconstitution (i.v.) of sham B10D2.oSn mice with rmC5a (Figs. 4 C and D).

The on-going airway responses after aerosol allergen provocation, serve as the third critical checkpoint in the study described herein. The development of EAR and LAR together with massive production and release of multiple inflammatory mediators provide a good opportunity to examine the complex interaction of activated C5 components with other components of innate and adaptive immune responses and their role in pathogenesis. The data described herein demonstrate that C5 inhibition by either anti-C5 monoclonal antibody (mAb) or its Fab fragment achieved similar in vivo efficacy in ameliorating intrapulmonary inflammatory activities as well as in improving functions of lower airways.

The data here also suggest that in the presence of airway inflammation, activated C5 components function as the key regulators of the downstream inflammatory cascade. This regulatory effect is demonstrated by its ability to influence the migration of inflammatory cells into airway lumen as well as the activation and the release of multiple harmful mediators corresponding to the significant changes of lower airway functions at checkpoint 3. C5a is probably the most potent activator of inflammatory cells (Takafuji et al., *supra*) and C5aR has been identified on circulating leukocytes, mast cells, macrophages, and endothelial cells (Chenoweth et al., Proc. Natl. Acad. Sci. U.S.A. (1978) 75:3943-3947). The disassociation of significant improvement of lower airway functions with significant presence of intrapulmonary inflammatory activities after anti-C5aR sIgG treatment is consistent with the hypothesis that the direct engagement of C5a with its receptors expressed on airway smooth muscle cells and epithelia is, in part, responsible for airway constriction (Irvin et al., *supra*). The data also indicate that the chemotactic activity and cell activation properties of C5b-9 (Czermak et al., Am. J. Pathol. (1999) 154:1513-1524) are responsible for the significant intrapulmonary inflammatory activities in the absence of C5aR engagement. In the presence of established airway inflammation and IC, C5b-9 can sufficiently regulate the downstream inflammatory cascade by triggering releases of multiple mediators such

Samsung Bioepis Exhibit No. 1002
Page 1591

as proMMP9 independently of the engagement of C5a with its receptors expressed on inflammatory cells (Fig. 8 D). The presence of an elevated level of C5b-9 together with the presence of multiple inflammatory mediators contributed to migration of inflammatory cells into the bronchial lumen in animals treated with anti-C5aR sIgG (Fig. 8 C).

Both the adaptive and innate immune systems contributed in the complex process of the production and release of multiple inflammatory mediators, including leukotrienes, prostaglandins, histamine, alkaline proteins, cytokines, chemokines, and enzymes, which are responsible for bronchospasm, altered vascular permeability, adherence and migration of inflammatory cells, mucosal edema and excessive mucous secretion and ultimately lead to three unique but interdependent phenomena: airway inflammation, airway obstruction and AHR. Since there are many redundant biological mechanisms, under the right circumstances, such as in genetically deficient animals, these redundant mechanisms may compensate and contribute in a more significant fashion. The complex relationship and interactions of these components are responsible for airway inflammation, obstruction and AHR, as well as other pulmonary diseases or conditions.

Lung parenchymal cells possess the ability to produce C5 and cleave C5 into activated fragments (Desai et al, J. Exp. Pathol. (1984) 1:201-216). The activation of C5 as part of innate immune responses may well be an innocuous by-product of a self-defensive response, due in part to direct enzymatic cleavage of C5 (Maruo et al, J. Allergy Clin. Immunol. (1997) 100:253-260) after exposure to allergens (Nagata et al., *supra*), house dust (Maruo et al., *supra*), smoke (Robbins et al., Am. J. Physiol. (1991) 260:L254-L259.) or airway infections (Nagy et al., J. Allergy. Clin. Immunol. (2003) 112:729-734; Kasamatsu et al., Arerugi (1993) 42:1616-1622; Bjornson et al., Am. Rev. Respir. Dis. (1991) 143:1062-1066). The formation of intrapulmonary IC after allergen exposure may also activate complement cascade (Larsen, *supra*). This hypothesis proposes that activated C5 components are primarily located at the epithelial side of the airway and, in non-asthmatic individuals, the intrapulmonary activation of complement is well regulated (Varsano et al., Thorax (2000) 55:364-369). Consistent with this hypothesis, our data demonstrated that intrapulmonary C5 inhibition achieved similar efficacy as

Samsung Bioepis Exhibit No. 1002
Page 1592

systemic C5 inhibition at checkpoint 3 (Fig. 8) and at checkpoint 2. These data further suggest that intrapulmonary activated C5 components are major pro-inflammatory rather than anti-inflammatory forces.

The study also shows the effect of C5 inhibition on the inflammatory mediators produced or released by neutrophils or eosinophils (Fig. 7). These inflammatory mediators, in particular TGF-β, RANTES and proMMP9, may cause irreversible damage to airways by inducing bronchial smooth muscle hypertrophy and deposition of collagen under the basal membranes of respiratory epithelium, which leads to the remodel and repair of lower airways, the focal point for those individuals with a poor long term prognosis (Boulet et al., Am. J. Respir. Crit. Care Med. (2000) 162:1308-1313). Accordingly, C5 inhibition, which utilizes a unique and different anti-inflammatory mechanism, may complement the potent anti-inflammatory effect of corticosteroid and other active agents, and have an added benefit for long term prognosis.

This study clearly demonstrates that the complex functional changes of airways seen in individuals with asthma coexist with the complex inflammatory processes. On one hand, activated C5 components, such as C5a through its binding with airway C5aR, serve as the direct link between the innate immune system and AHR. On the other hand, both C5a and C5b-9 regulate the downstream inflammatory cascade through their chemotactic and cell activation activities in the presence of IC and established airway inflammation. Blocking the generation of C5a and C5b-9 not only improves the functioning of lower airways but also ameliorates intrapulmonary inflammatory activities. In contrast, C5aR antagonist significantly improves functions of lower airways without a dramatic impact on intrapulmonary inflammatory activities.

Aerosol or Nebulization Compositions

In one respect, this application is directed to pulmonary drug delivery compositions and/or devices for delivering an antibody that inhibits the activation of the complement cascade. The pulmonary drug delivery compositions are useful for treating a pulmonary disease or condition. For example, aerosol compositions are provided for the delivery of an antibody or an antibody combined with an additional

Samsung Bioepis Exhibit No. 1002
Page 1593

active agent to the respiratory tract. The respiratory tract includes the upper airways, including the oropharynx and larynx, followed by the lower airways, which include the trachea followed by bifurcations into the bronchi and bronchioli. The upper and lower airways are called the conductive airways. The terminal bronchioli then divide into respiratory bronchioli which then lead to the ultimate respiratory zone, the alveoli, or deep lung.

Pulmonary drug delivery may be achieved by inhalation, and administration by inhalation herein may be oral and/or nasal. Examples of pharmaceutical devices for pulmonary delivery include metered dose inhalers (MDIs), dry powder inhalers (DPIs), and nebulizers. Exemplary delivery systems by inhalation which can be adapted for delivery of the subject antibody and/or active agent are described in, for example, U.S. Patent Nos. 5,756,353; 5,858,784; and PCT applications WO98/31346; WO98/10796; WO00/27359; WO01/54664; WO02/060412. Other aerosol formulations that may be used for delivering the antibody and/or active agent are described in U.S. Patent Nos. 6,294,153; 6,344,194; 6,071,497, and PCT applications WO02/066078; WO02/053190; WO01/60420; WO00/66206.

Pressurized metered dose inhalers (pMDIs) are the most commonly used inhaler worldwide. The aerosol is created when a valve is opened (usually by pressing down on the propellant canister), allowing liquid propellant to spray out of a canister. Typically, a drug or therapeutic is contained in small particles (usually a few microns in diameter) suspended in the liquid propellant, but in some formulations the drug or therapeutic may be dissolved in the propellant. The propellant evaporates rapidly as the aerosol leaves the device, resulting in small drug or therapeutic particles that are inhaled. Propellants typically used in such pMDIs include but are not limited to hydrofluoroalkanes (HFAs). A surfactant may also be used, for example, to formulate the drug or therapeutic, with pMDIs. Other solvents or excipients may also be employed with pMDIs, such as ethanol, ascorbic acid, sodium metabisulfate, glycerin, chlorobutanol, and cetylpyridium chloride. Such pMDIs may further include add-on devices such as, for example, spacers, holding chambers and other modifications.

Samsung Bioepis Exhibit No. 1002
Page 1594

Nebulizers produce a mist of drug-containing liquid droplets for inhalation. They are usually classified into two types: ultrasonic nebulizers and jet nebulizers. A new type of nebulizer is also available, which does not require ultrasound or air pressure to function. Single breath atomizers have also been developed (e.g., Respimat®), which is used to deliver a drug in a single inhalation and may be preferred because of less contamination. Jet nebulizers are more common and use a source of pressurized air to blast a stream of air through a drug-containing water reservoir, producing droplets in a complex process involving a viscosity-induced surface instability that leads to nonlinear phenomena in which surface tension and droplet breakup on baffles play a role. Ultrasonic nebulizers produce droplets by mechanical vibration of a plate or mesh. In either type of nebulizer, the drug is usually contained in solution in the liquid in the nebulizer and so the droplets being produced contain drug in solution. However, for some formulations (e.g., Pulmicort) the drug is contained in small particles suspended in the water, which are then contained as particles suspended inside the droplets being produced. Certain excipients are usually included in formulations suitable for nebulization, such as sodium chloride (e.g., to maintain isotonicity), mineral acids and bases (e.g., to maintain or adjust pH), nitrogen headspace sparging, benzalkonium chloride, calcium chloride, sodium citrate, disodium edtate, and polysorbate 80.

The third type of inhaler is the dry powder inhaler (DPI). In DPIs, the aerosol is usually a powder, contained within the device until it is inhaled. The therapeutic or drug is manufactured in powder form as small powder particles (usually a few millionths of a meter, or micrometers, in diameter). In many DPIs, the drug or therapeutic is mixed with much larger sugar particles (e.g., lactose monohydrate), that are typically 50-100 micrometers in diameter. The increased aerodynamic forces on the lactose/drug agglomerates improve entrainment of the drug particles upon inhalation, in addition to allowing easier filling of small individual powder doses. Upon inhalation, the powder is broken up into its constituent particles with the aid of turbulence and/or mechanical devices such as screens or spinning surfaces on which particle agglomerates impact, releasing the small, individual drug powder particles into the air to be inhaled into the lung. The

Samsung Bioepis Exhibit No. 1002
Page 1595

sugar particles are usually intended to be left behind in the device and/or in the mouth-throat.

One aspect of the application provides an aerosol composition comprising an antibody that inhibits activation of the complement cascade, wherein the composition is suitable for preventing or treating a pulmonary disease or condition in a subject. An aerosol antibody composition can be a composition comprising aerosolized antibody or a composition comprising an antibody in a formulation suitable for aerosolization. The antibody may be formulated in combination with an additional active agent, and the combination formulation is suitable for aerosolization. Alternatively, the antibody and an additional active agent may be formulated separately, such that they will be combined after aerosolization occurs or after being administered to a subject.

Another aspect of the application provides a nebulization composition comprising an antibody that inhibits activation of the complement cascade, wherein the composition is suitable for preventing or treating a pulmonary disease or condition in a subject. A nebulization antibody composition can be a composition comprising a nebulized antibody or a composition comprising an antibody in a formulation suitable for nebulization. Similarly, the antibody may be formulated in combination with an additional active agent, and the combination formulation is suitable for nebulization. Alternatively, the antibody and an additional active agent may be formulated separately, such that they will be combined after nebulization occurs or after being administered to a subject.

A further aspect of the application provides a biopharmaceutical package comprising an antibody that inhibits activation of the complement cascade and a nebulizer, wherein the package is suitable for preventing or treating a pulmonary disease or condition in a subject. The biopharmaceutical package may further comprise an active agent in addition to the antibody. The biopharmaceutical package may also comprise instructions for use.

An antibody of the present application can be specific to C5 such that it prevents the cleavage of C5 into C5a and C5b. The antibody can be specific to the C5 convertase. Alternatively, the antibody may be specific to a component of the

Samsung Bioepis Exhibit No. 1002
Page 1596

complement system, for example, C5a, C5b, or C5b-9, and the antibody specific to the component preferably inhibits the component's function, for example, by blocking the component's binding to its respective receptor, or by blocking its function in activating subsequent signaling or events in the complement cascade. Certain embodiments employ eculizumab or pexelizumab, or both. An antibody or antibody therapeutic of the present application can be a full length immunoglobulin, a chimeric antibody (e.g., a humanized antibody), a single chain antibody, a domain antibody (e.g., a domain antibody as developed by Domantis, defined as the smallest functional binding units of antibodies, corresponding to the variable regions of either the heavy (VH) or light (VL) chains of human antibodies), an Fab fragment, or an antibody having an Fab fragment and a mutated Fc portion. In certain embodiments, the mutated Fc portion does not activate complement, or the mutation(s) in the Fc portion decreases the Fc portion's ability to activate complement. An antibody of the present application may be produced or processed in bulk and packaged in an ampule made of a suitable material (e.g., glass or plastic) at different doses. The antibody may be stable in a formulation at a concentration ranging from 1 mg/ml to 200 mg/ml.

An additional active agent (or an active agent in addition to the antibody therapeutic) of the present application can be another antibody therapeutic (e.g., an anti-complement or anti-C5 antibody, an anti-IgE antibody such as Xolair® or omalizumab, an anti-IL-4 antibody or an anti-IL-5 antibody), an anti-IgE inhibitor (e.g., Singulair® or montelukast sodium), a sympathomimetic (e.g., albuterol), an antibiotic (e.g., tobramycin), a deoxyribonuclease (e.g., pulmozyme), an anticholinergic drug (e.g., ipratropium bromide), a corticosteroid (e.g., dexamethasone), a β-adrenoreceptor agonist, a leukotriene inhibitor (e.g., zileuton), a 5 Lipoxygenase inhibitor, a PDE inhibitor, a CD23 antagonist, an IL-13 antagonist, a cytokine release inhibitor, a histamine H1 receptor antagonist, an anti-histamine, an anti-inflammatory agent (e.g. cromolyn sodium) or a histamine release inhibitor. As used herein, an active agent may also be referred to as a therapeutic or a drug.

An example of formulation suitable for aerosolization or nebulization of an antibody is in physiologic osmolarity (e.g., between 280 and 320 mM) at a suitable

Samsung Bioepis Exhibit No. 1002
Page 1597

pH (e.g., pH 6 to 8). A formulation of the present application may further comprise an excipient, for example polysorbate 80 which can be used at 0.0015 to 0.02%.

U.S. Patent No. 5,474,759 discloses aerosol formulations that are substantially free of chlorofluorocarbons, and having particular utility in medicinal applications. The formulations contain a propellant (such as 1,1,1,2,3,3,3,-heptafluoropropane), a medium-chain fatty acid propylene glycol diester, a medium-chain triglyceride, optionally a surfactant, and optionally auxiliary agents such as antioxidants, preservatives, buffers, sweeteners and taste masking agents.

Other pharmaceutically acceptable carriers may also be used in a formulation of the present application. The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. The phrase "pharmaceutically acceptable carrier" as used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject antagonists from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18)

Samsung Bioepis Exhibit No. 1002
Page 1598

Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

Methods of preparing these formulations or compositions include the step of bringing into association a compound of the present application with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association a compound of the present application with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

A nebulizer of the present application can be a jet air nebulizer (e.g., Pari LC Jet Plus or Hudson T Up-draft II), an ultrasonic nebulizer (e.g., MABISMist II), a vibrating mesh nebulizer (e.g., Micro air by Omron) and a shockwave nebulizer (EvitLabs Sonik LDI20).

"Aerosol composition" means an antibody and/or an active agent described herein in a form or formulation that is suitable for pulmonary delivery. The aerosol composition may be in the dry powder form, it may be a solution, suspension or slurry to be nebulized, or it may be in admixture with a suitable low boiling point, highly volatile propellant. It is to be understood that more than one antibody and optionally other active agents or ingredients may be incorporated into the aerosolized formulation or aerosol composition and that the use of the term "antibody" or "active agent" in no way excludes the use of two or more such antibodies or other agents or ingredients.

In certain preferred embodiments, an antibody or active agent retains more than 50% of its activity after nebulization, preferably more than 70%. In certain preferred embodiments, an antibody or active agent retains more than 50% of its purity after nebulization, preferably more than 70%.

Active agent formulations suitable for use in the present application include dry powders, solutions, suspensions or slurries for nebulization and particles suspended or dissolved within a propellant. Dry powders suitable for use in the present application include amorphous active agents, crystalline active agents and mixtures of both amorphous and crystalline active agents. The dry powder active agents have a particle size selected to permit penetration into the alveoli of the lungs,

Samsung Bioepis Exhibit No. 1002
Page 1599

that is, preferably 10 μm mass median diameter (MMD), preferably less than 7.5 μm, and most preferably less than 5 μm, and usually being in the range of 0.1 μm to 5 μm in diameter. The delivered dose efficiency (DDE) of these powders is >30%, usually >40%, preferably >50 and often >60% and the aerosol particle size distribution is about 1.0-5.0 μm mass median aerodynamic diameter (MMAD), usually 1.5-4.5 μm MMAD and preferably 1.5-4.0 μm MMAD. These dry powder active agents have a moisture content below about 10% by weight, usually below about 5% by weight, and preferably below about 3% by weight. Such active agent powders are described in WO 95/24183 and WO 96/32149, which are incorporated by reference herein.

Dry powder active agent formulations are preferably prepared by spray drying under conditions which result in a substantially amorphous powder. Bulk active agent, usually in crystalline form, is dissolved in a physiologically acceptable aqueous buffer, typically a citrate buffer having a pH range from about 2 to 9. The active agent is dissolved at a concentration from 0.01% by weight to 1% by weight, usually from 0.1% to 0.2%. The solutions may then be spray dried in a conventional spray drier available from commercial suppliers such as Niro A/S (Denmark), Buchi (Switzerland) and the like, resulting in a substantially amorphous powder. These amorphous powders may also be prepared by lyophilization, vacuum drying, or evaporative drying of a suitable active agent solution under conditions to produce the amorphous structure. The amorphous active agent formulation so produced can be ground or milled to produce particles within the desired size range. Dry powder active agents may also be in a crystalline form. The crystalline dry powders may be prepared by grinding or jet milling the bulk crystalline active agent.

The active agent powders of the present application may optionally be combined with pharmaceutical carriers or excipients which are suitable for respiratory and pulmonary administration. Such carriers may serve simply as bulking agents when it is desired to reduce the active agent concentration in the powder which is being delivered to a patient, but may also serve to improve the dispersability of the powder within a powder dispersion device in order to provide more efficient and reproducible delivery of the active agent and to improve handling

Samsung Bioepis Exhibit No. 1002
Page 1600

characteristics of the active agent such as flowability and consistency to facilitate manufacturing and powder filling. Such excipients include but are not limited to (a) carbohydrates, e.g., monosaccharides such as fructose, galactose, glucose, D-mannose, sorbose, and the like; disaccharides, such as lactose, trehalose, cellobiose, and the like; cyclodextrins, such as 2-hydroxypropyl-.beta.-cyclodextrin; and polysaccharides, such as raffinose, maltodextrins, dextrans, and the like; (b) amino acids, such as glycine, arginine, aspartic acid, glutamic acid, cysteine, lysine, and the like; (c) organic salts prepared from organic acids and bases, such as sodium citrate, sodium ascorbate, magnesium gluconate. sodium gluconate, tromethamin hydrochloride, and the like; (d) peptides and proteins such as aspartame, human serum albumin, gelatin, and the like; and (e) alditols, such as mannitol, xylitol, and the like. A preferred group of carriers includes lactose, trehalose, raffinose, maltodextrins, glycine, sodium citrate, human serum albumin and mannitol.

The dry powder active agent formulations may be delivered using Inhale Therapeutic Systems' dry powder inhaler as described in WO 96/09085 which is incorporated herein by reference, but adapted to control the flow rate at a desirable level or within a suitable range. The dry powders may also be delivered using a metered dose inhaler as described by Laube et al. in U.S. Patent No. 5,320,094, which is incorporated by reference herein.

Nebulized solutions may be prepared by aerosolizing commercially available active agent formulation solutions. These solutions may be delivered by a jet nebulizer such as the Raindrop, produced by Puritan Bennett, the use of which is described by Laube et al., *supra*. Other methods for delivery of solutions, suspensions of slurries are described by Rubsamen et al, U.S. Patent No. 5,672,581. A device that uses a vibrating, piezoelectric member is described in Ivri et al., U.S. Patent No. 5,586,550, which is incorporated by reference herein.

Propellant systems may include an active agent dissolved in a propellant or particles suspended in a propellant. Both of these types of formulations are described in Rubsamen et al., U.S. Patent No. 5,672,581, which is incorporated herein by reference.

Samsung Bioepis Exhibit No. 1002
Page 1601

In certain embodiments, an aerosol or nebulization antibody composition can be combined with one or more other aerosol or nebulization treatments, such as sympathomimetics (e.g., albuterol), antibiotics (e.g., tobramycin), deoxyribonucleases (e.g., pulmozyme), anticholinergic drugs (e.g., ipratropium bromide), or corticosteroids.

In certain embodiments, an aerosol or nebulization antibody composition can be combined with one or more other therapies (concurrently or sequentially) administered via nebulization, inhalation, intravenous or oral routes, such as sympathomimetics (e.g., albuterol), anticholinergic drugs (e.g., ipratropium bromide), inflammation inhibitors (e.g., cromolyn sodium), leukotriene inhibitors (e.g., zileuton), anti-IgE inhibitors (e.g., Singulair®) or corticosteroids.

As described herein, an antibody or therapeutic may be formulated as microparticles. Microparticles having a diameter of between 0.5 and 10 microns can penetrate the lungs, passing through most of the natural barriers. A diameter of less than ten microns is generally required to bypass the throat; a diameter of 0.5 microns or greater is usually required to avoid being exhaled.

In certain embodiments, the subject antibody or therapeutic is formulated in a supramolecular complex, which may have a diameter of between 0.5 and 10 microns, which can be aggregated into particles having a diameter of between 0.5 and 10 microns.

In other embodiments, the subject antibodies or therapeutics are provided in liposomes or supramolecular complexes appropriately formulated for pulmonary delivery.

(i). Polymers for forming Microparticles

In addition to the supramolecular complexes described above, a number of other polymers can be used to form the microparticles. As used herein, the term "microparticles" includes microspheres (uniform spheres), microcapsules (having a core and an outer layer of polymer), and particles of irregular shape.

Polymers are preferably biodegradable within the time period over which release of the antibody or therapeutic is desired or relatively soon thereafter,

Samsung Bioepis Exhibit No. 1002
Page 1602

generally in the range of one year, more typically a few months, even more typically a few days to a few weeks. Biodegradation can refer to either a breakup of the microparticle, that is, dissociation of the polymers forming the microparticles and/or of the polymers themselves. This can occur as a result of change in pH from the carrier in which the particles are administered to the pH at the site of release, as in the case of the diketopiperazines, hydrolysis, as in the case of poly(hydroxy acids), by diffusion of an ion such as calcium out of the microparticle, as in the case of microparticles formed by ionic bonding of a polymer such as alginate, and by enzymatic action, as in the case of many of the polysaccharides and proteins. In some cases linear release may be most useful, although in others a pulse release or "bulk release" may provided more effective results.

Representative synthetic materials are: diketopiperazines, poly(hydroxy acids) such as poly(lactic acid), poly(glycolic acid) and copolymers thereof, polyanhydrides, polyesters such as polyorthoesters, polyamides, polycarbonates, polyalkylenes such as polyethylene, polypropylene, poly(ethylene glycol), poly(ethylene oxide), poly(ethylene terephthalate), poly vinyl compounds such as polyvinyl alcohols, polyvinyl ethers, polyvinyl esters, polyvinyl halides, polyvinylpyrrolidone, polyvinylacetate, and poly vinyl chloride, polystyrene, polysiloxanes, polymers of acrylic and methacrylic acids including poly(methyl methacrylate), poly(ethyl methacrylate), poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate), polyurethanes and co-polymers thereof, celluloses including alkyl cellulose, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, nitro celluloses, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxy-propyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxylethyl cellulose, cellullose triacetate, and cellulose sulphate sodium salt, poly(butic acid), poly(valeric acid), and poly(lactide-co-caprolactone).

Natural polymers include alginate and other polysaccharides including dextran and cellulose, collagen, albumin and other hydrophilic proteins, zein and

Samsung Bioepis Exhibit No. 1002
Page 1603

other prolamines and hydrophobic proteins, copolymers and mixtures thereof. As used herein, chemical derivatives thereof refer to substitutions, additions of chemical groups, for example, alkyl, alkylene, hydroxylations, oxidations, and other modifications in the art.

Bioadhesive polymers include bioerodible hydrogels described by H. S. Sawhney, C. P. Pathak and J .A. Hubell in Macromolecules, 1993, 26, 581-587, polyhyaluronic acids, casein, gelatin, glutin, polyanhydrides, polyacrylic acid, alginate, chitosan, and polyacrylates.

To further illustrate, the matrices can be formed of the polymers by solvent evaporation, spray drying, solvent extraction and other methods known to those skilled in the art. Methods developed for making microspheres for drug delivery are described in the literature, for example, as described by Mathiowitz and Langer, J. Controlled Release 5,13-22 (1987); Mathiowitz, et al., Reactive Polymers 6, 275-283 (1987); and Mathiowitz, et al., J. Appl. Polymer Sci. 35, 755-774 (1988). The selection of the method depends on the polymer selection, the size, external morphology, and crystallinity that is desired, as described, for example, by Mathiowitz, et al., Scanning Microscopy 4,329-340 (1990); Mathiowitz, et al., J. Appl. Polymer Sci. 45, 125-134 (1992); and Benita, et al., J. Pharm. Sci. 73, 1721-1724 (1984).

In solvent evaporation, described for example, in Mathiowitz, et al., (1990), Benita, and U.S. Pat. No. 4,272,398 to Jaffe, the polymer is dissolved in a volatile organic solvent. The antibody and/or therapeutic, either in soluble form or dispersed as fine particles, is added to the polymer solution, and the mixture is suspended in an aqueous phase that contains a surface active agent such as poly(vinyl alcohol). The resulting emulsion is stirred until most of the organic solvent evaporates, leaving solid microspheres.

In general, the polymer can be dissolved in methylene chloride. Several different polymer concentrations can be used, for example, between 0.05 and 0.20 g/ml. After loading the solution with drug, the solution is suspended in 200 ml of vigorously stirring distilled water containing 1% (w/v) poly(vinyl alcohol) (Sigma Chemical Co., St. Louis, Mo.). After four hours of stirring, the organic solvent will

Samsung Bioepis Exhibit No. 1002
Page 1604

have evaporated from the polymer, and the resulting microspheres will be washed with water and dried overnight in a lyophilizer.

Microspheres with different sizes (1-1000 microns, though less than 10 microns for aerosol applications) and morphologies can be obtained by this method which is useful for relatively stable polymers such as polyesters and polystyrene. However, labile polymers such as polyanhydrides may degrade due to exposure to water. For these polymers, hot melt encapsulation and solvent removal may be preferred.

In hot melt encapsulation, the polymer is first melted and then mixed with the solid particles of antibodies or therapeutics, preferably sieved to appropriate size. The mixture is suspended in a non-miscible solvent such as silicon oil and, with continuous stirring, heated to
5 °C above the melting point of the polymer. Once the emulsion is stabilized, it is cooled until the polymer particles solidify. The resulting microspheres are washed by decantation with petroleum ether to give a free-flowing powder. Microspheres with diameters between one and 1000 microns can be obtained with this method. The external surface of spheres prepared with this technique are usually smooth and dense. This procedure is useful with water labile polymers, but is limited to use with polymers with molecular weights between 1000 and 50000.

In spray drying, the polymer is dissolved in an organic solvent such as methylene chloride (0.04 g/ml). A known amount of antibody and/or therapeutic is suspended (if insoluble) or co-dissolved (if soluble) in the polymer solution. The solution or the dispersion is then spray-dried. Microspheres ranging in diameter between one and ten microns can be obtained with a morphology which depends on the selection of polymer.]

Hydrogel microspheres made of gel-type polymers such as alginate or polyphosphazines or other dicarboxylic polymers can be prepared by dissolving the polymer in an aqueous solution, suspending the material to be incorporated into the mixture, and extruding the polymer mixture through a microdroplet forming device, equipped with a nitrogen gas jet. The resulting microspheres fall into a slowly stirring, ionic hardening bath, as described, for example, by Salib, et al.,

Samsung Bioepis Exhibit No. 1002
Page 1605

Pharmazeutische Industrie 40-111A, 1230 (1978). The advantage of this system is the ability to further modify the surface of the microspheres by coating them with polycationic polymers such as polylysine, after fabrication, for example, as described by Lim, et al., J. Pharm. Sci. 70, 351-354 (1981). For example, in the case of alginate, a hydrogel can be formed by ionically crosslinking the alginate with calcium ions, then crosslinking the outer surface of the microparticle with a polycation such as polylysine, after fabrication. The microsphere particle size will be controlled using various size extruders, polymer flow rates and gas flow rates.

Chitosan microspheres can be prepared by dissolving the polymer in acidic solution and crosslinking with tripolyphosphate. For example, carboxymethylcellulose (CMC) microsphere are prepared by dissolving the polymer in an acid solution and precipitating the microspheres with lead ions. Alginate/polyethyleneimine (PEI) can be prepared to reduce the amount of carboxyl groups on the alginate microcapsules.

(ii). Pharmaceutical Compositions

The microparticles can be suspended in any appropriate pharmaceutical carrier, such as saline, for administration to a patient. In the most preferred embodiment, the microparticles will be stored in dry or lyophilized form until immediately before administration. They can then be suspended in sufficient solution, for example an aqueous solution for administration as an aerosol, or administered as a dry powder.

(iii). Targeted Administration

The microparticles can be delivered to specific cells or organ, especially phagocytic cells and organs. Where systemic delivery of the drug or therapeutic is desirable, endocytosis of the microparticles by macrophages in the lungs can be used to target the microparticles to the spleen, bone marrow, liver, lymph nodes, and other parts of the body.

The microparticles can also be targeted by attachment of ligands, such as those described above, which specifically or non-specifically bind to particular targets, for example, particular targets within the pulmonary system or associated with a pulmonary disease or condition (e.g., a target specific for cancerous cells in

Samsung Bioepis Exhibit No. 1002
Page 1606

the lung). Examples of such ligands also include antibodies and fragments including the variable regions, lectins, and hormones or other organic molecules having receptors on the surfaces of the target cells.

(iv). Storage of the Microparticles

In certain embodiments, the microparticles are stored lyophilized. The dosage is determined by the amount of encapsulated antibodies and/or therapeutics, the rate of release within the pulmonary system, and the pharmacokinetics of the antibodies and/or therapeutics.

(v). Delivery of Microparticles

The microparticles can be delivered using a variety of methods, ranging from administration directly into the nasal passages so that some of the particles reach the pulmonary system, to the use of a powder instillation device, to the use of a catheter or tube reaching into the pulmonary tract. As described above, dry powder inhalers are commercially available, although those using hydrocarbon propellants are no longer used and those relying on the intake of a breath by a patient can result in a variable dose. Examples of suitable propellants include hydrofluoroalkane propellants, such as 1,1,1,2-tetrafluoroethane (CF3CH2F) (HFA-134a) and 1,1,1,2,3,3,3-heptafluoro-n-propane (CF3CHFCF3) (HFA-227), perfluoroethane, monochloroifluoromethane, 1,1 difluoroethane, and combinations thereof.

Therapeutic Methods

A first aspect of the application provides a method for preventing or treating a pulmonary disease or condition in a subject comprising administering to the subject a therapeutically effective amount of an antibody that inhibits activation of the complement cascade. In certain embodiments, an additional active agent is also administered to the same subject. Administration of the antibody and the additional active agent may occur simultaneously or sequentially in either order.

The term "preventing" is art-recognized, and when used in relation to a condition, such as a local recurrence (e.g., pain), a disease such as cancer, a syndrome complex such as heart failure or any other medical condition, is well understood in the art, and includes administration of a composition which reduces the frequency of, or delays the onset of, symptoms of a medical condition (e.g.,

Samsung Bioepis Exhibit No. 1002
Page 1607

asthma) in a subject relative to a subject which does not receive the composition. Thus, prevention of cancer includes, for example, reducing the number of detectable cancerous growths in a population of patients receiving a prophylactic treatment relative to an untreated control population, and/or delaying the appearance of detectable cancerous growths in a treated population versus an untreated control population, e.g., by a statistically and/or clinically significant amount. Prevention of an infection includes, for example, reducing the number of diagnoses of the infection in a treated population versus an untreated control population, and/or delaying the onset of symptoms of the infection in a treated population versus an untreated control population. Prevention of pain includes, for example, reducing the magnitude of, or alternatively delaying, pain sensations experienced by subjects in a treated population versus an untreated control population.

The term "treating" includes prophylactic and/or therapeutic treatments. The term "prophylactic or therapeutic" treatment is art-recognized and includes administration to the host of one or more of the subject compositions. If it is administered prior to clinical manifestation of the unwanted condition (e.g., disease or other unwanted state of the host animal) then the treatment is prophylactic, (i.e., it protects the host against developing the unwanted condition), whereas if it is administered after manifestation of the unwanted condition, the treatment is therapeutic, (i.e., it is intended to diminish, ameliorate, or stabilize the existing unwanted condition or side effects thereof).

Pulmonary diseases or conditions contemplated by the application include, but are not limited to, asthma, bronchial constriction, bronchitis, a chronic obstructive pulmonary disease (COPD), interstitial lung diseases, lung malignancies, α-1 anti-trypsin deficiency, emphysema, bronchiectasis, bronchiolitis obliterans, sarcoidosis, pulmonary fibrosis, and collagen vascular disorders.

The subject or patient receiving this treatment is any animal in need, including primates, in particular humans, and other mammals such as equines, cattle, swine and sheep, and poultry and pets in general.

The phrase "therapeutically effective amount" as used herein means that amount of a compound, material, or composition comprising a compound of the

Samsung Bioepis Exhibit No. 1002
Page 1608

present invention which is effective for producing some desired therapeutic effect by inhibiting C5 or activation of the complement cascade in at least a sub-population of cells or an organ in an animal and thereby blocking the biological consequences of that function in the treated cells or an organ, at a reasonable benefit/risk ratio applicable to any medical treatment.

The timing of administering a therapeutic to a subject can vary, for example, depending on the identity of the subject or the pulmonary disease or condition to be treated or prevented, or both. For example, the administration may occur before the manifestation of the pulmonary condition (e.g., pre-asthmatic attack or pre-bronchial constriction), during the manifestation of the pulmonary condition (e.g., during the asthmatic attack or bronchial constriction), or after the manifestation of the pulmonary condition (e.g., post-asthmatic attack or post-bronchial constriction).

The phrase "inhibits the activation of complement cascade" refers to any inhibitory effect or action on any component of the complement system as described above conferred by an antibody or active agent of the present application. The inhibitory effect may be manifested by decrease in or lack of a response typical to activation of complement. For example, typical cellular and other biological responses to activation of complement include, but are not limited to, release of histamine, smooth muscle contraction, increased vascular permeability, leukocyte activation, chemotaxis (e.g., movement of granulocytes to the site of complement activation), and other inflammatory phenomena including cellular proliferation. In certain embodiments, the inhibitory effect is specific to the C5 component. In certain embodiments, the inhibitory effect is specific to C5 convertase, for example, blocking C5 convertase's function to convert C5 into C5a and C5b-9. In certain embodiments, the inhibitory effect is specific to C5a. In certain embodiments, the inhibitory effect is specific to C5b or C5b-9.

Examples

The following examples are offered by way of illustration and not by way of limitation.

Samsung Bioepis Exhibit No. 1002
Page 1609

Materials and Methods

Animals: Male C5s BALB/cByJ, C57BL/6J mice and C5d B10D2oSn/J, AKR/J and SWR/J mice were purchased from the Jackson Laboratory and housed in a pathogen free facility. In addition to serum C5b-9 mediated hemolysis, the C5 genotype was further confirmed by PCR performed on tail DNA using a pair of primers (5'-CAC GAT AAT GGG AGT CAT CTG GG-3' (SEQ ID NO:1) and 5'-AAG TTG GAG TGT GGT CTT TGG GCC-3' (SEQ ID NO:2) that amplify a 280-bp DNA fragment from both C5s and C5d DNA. This fragment encodes a HindIII site that is selectively destroyed by a mutation in the C5 gene, such that HindIII (New England BioLabs,MA) digestion selectively cleaves the C5s but not the C5d PCR products into 150 and 130-bp fragments. All animal protocols were reviewed by Institutional Committee and were in accordance with NIH guidelines.

Reagents: Anti-mouse C5 mAb (BB5.1) (Wang et al., Proc. Natl. Acad. Sci. U.S.A (1996) 93:8563-8568) and an isotype matched irrelevant control mAb (HFN7.1), which is specific to human fibronectin, were purified from ascites. BB5.1 Fab, which was purified with a kit (Pierce Biotechnology), had retained similar activity of BB5.1 and was more than 97% in purity. The anti-C5aR sIgG, which was shown to prevent mortality in experimental sepsis (Riedemann et al., J. Clin. Invest. (2002) 110:101-108), was able to prevent zymosan induced neutrophil migration. Both anti-C5aR sIgG and the control sIgG were purified from sera harvested from C5aR deficient mice (Gerard et al., Curr. Opin. Immunol. (2002) 14:705-708) that were repeatedly immunized with either PBS or peptide spanning the N-terminus of the mouse C5aR and one extra cysteine (Riedemann et al., *supra*) emulsified with CFA. Antibodies from either ascites or serum samples were purified by protein A affinity chromatography (Amersham). Corticosteroid (dexamethasone) was purchased from Sigma. Recombinant mouse C5a, which was free of endotoxin contamination and had potent chemotactic activity in neutrophil migration assay, was cloned and purified as previously reported (Riedemann et al., *supra*).

Samsung Bioepis Exhibit No. 1002
Page 1610

Induction of Airway Inflammation and Severe Airway Response

BALB/c and B10D2oSn Mice 10-12 wks of age were sensitized on day 1 and day 14 with i.p. injection of 20 μg OVA (Grade V; Sigma) emulsified in 2 mg aluminum hydroxide (Alum Inject; Pierce Biotechnology). Mice were immunized with 1% OVA for 10 min via airway on days 28, 29 and 30 with a jet nebulizer (Harvard Apparatus) connected to a single housing chamber (Buxco Electronics, Inc.). On day 32, all sensitized animals were aerosol challenged with 5% OVA for 10 min. In checkpoint 3 experiments, BALB/c mice were aerosol challenged with 5% OVA again on day 35. Sham C5s or C5d mice were immunized with Alum only and aerosol challenged with PBS instead of OVA.

Non-Invasive Measurement of sRaw

sRaw, which measures the resistance of both upper and lower airways, was measured by a DCP in conscious animals with spontaneous breathing (Buxco). The DCP was used to monitor the longitudinal changes of sRaw prior to and after each 5% OVA aerosol challenge. In checkpoint 3 experiments, a DCP was used to ensure the appearance of EAR prior to randomizing animals into different treatment groups.

Invasive Measurement of Lower Airway Functions

Changes in RL and Cdyn were measured by using Buxco Biosystem software and a Buxco whole body plethysmograph. Mice were anesthetized (Avertin, 160 mg/kg) and tracheas cannulated. Spontaneous breathing was blocked by pancuronium bromide (0.3 mg/kg i.p.). Respirations were maintained by a Harvard Apparatus Inspira ventilator, which calculated a tidal volume and respiratory rate according to body weight. Measurements of RL and Cdyn were performed during the peak of LAR, 5 h after aerosol challenge with 5% OVA. Real-time changes of RL and Cdyn were recorded by Buxco software and reported as the mean value of five min of recording. AHR was evaluated based on changes of RL and Cdyn during aerosol Mch challenges. Changes in RL and Cdyn were expressed as a percentage of baseline after each aerosol challenge. A Buxco aerosol control and sonicating nebulizer unit was attached to the ventilator for aerosol delivery of Mch through tracheal cannulation. PBS or Mch (1.6 mg/ml) was delivered at the

Samsung Bioepis Exhibit No. 1002
Page 1611

rate of 10 puffs or 20 puffs per 10 seconds, with each puff of aerosol delivery lasting 15 ms.

C5 Inhibition

(1) For C5 inhibition at checkpoint 1, anti-C5 mAb (BB5.1) or a control mAb (HFN7.1) was administered i.p. at 40 mg/kg on days 25, 29, and 31. Dexamethasone (2 mg/kg) was used as a positive control. (2) Animals that experienced airway response to 5% OVA provocation on day 32 were randomized for the following studies. For C5 inhibition at checkpoint 2, animals were given one i.p. injection of either dexamethasone (2 mg/kg), anti-C5 mAb (40 mg/ml) or control mAb (40 mg/kg) on day 33. On day 35, animals were anesthetized and tracheas cannulated for measurement of RL and Cdyn during aerosol Mch challenges. (3) At checkpoint 3, anti-C5 mAb (40 mg/kg), control mAb (40 mg/kg), or dexamethasone (2 mg/kg) was administered by i.v. injection 20 min after aerosol challenging animals with 5% OVA on day 35. For intrapulmonary C5 inhibition at this critical point, animals were given aerosol administration of either anti-C5 mAb (3 mg/ml), anti-C5 Fab (3 mg/ml), control mAb (3 mg/ml), anti-C5aR sIgG (3 mg/ml), control sIgG (2 mg/ml), or corticosteroid (2 mg/ml) for 10-30 min by using a jet nebulizer connected to a single chamber housing the animal. Sham-immunized mice were given sham treatment with the same volume of PBS solution administered through the same route. The results from three intrapulmonary C5 inhibition experiments, which were conducted with identical protocols, were pooled together.

Analysis of Bronchoalveolar Lavage Fluid (BALF)

Approximately 5.5 h after allergen provocation, after the measurement of RL and Cdyn, BALF was harvested by instilling 1 ml PBS through the tracheal cannula, followed by gentle aspiration. The BALF was resuspended in 400 μl PBS. The total numbers of WBC in BALF were counted by using a hemocytometer or an automatic cell counter as in the aerosol experiments (Cell-Dyn 3700 Abbott). Cytospin slides were prepared, fixed and stained using Diff-Quik (VWR International, Inc.). The WBC differential was determined by a certified pathologist after counting a total of 300 WBC per slide on a 100x microscopic lens. The BALF levels of proMMP9, activated TGF-β, RANTES, eotaxin and IL-13 (R&D Systems,

Samsung Bioepis Exhibit No. 1002
Page 1612

Inc.), IL-5 (Amersham Pharmacia Biotech, Inc.), and histamine (Immunotech, Inc.) were measured by ELISA according to the manufacturer's instructions.

Analysis of Serum OVA Specific Antibodies

Serum samples were harvested 5 h after 5% allergen provocation on day 32 for OVA specific IgG and IgE by ELISA.

Lung Histology

Lung was inflated with 10% buffered formalin (1 ml) through tracheal cannulation and fixed in 10% formalin at least 24 h. Lung samples were stained with Giemsa. Double blind histological analysis was performed to quantify airway inflammation according to the following criteria. 0: No detectable airway inflammation; 1: Less than 25% bronchials and surrounding vasculature were found with either perivascular or peribronchial inflammatory cell infiltration; 2: Approximately 25-50% of bronchials and surrounding vasculature were affected; 3: Approximately 50-75% bronchials and surrounding vasculature were affected; 4: >75% of bronchials and surrounding vasculature were affected.

C5b-9 Mediated Hemolytic Assay and in Vitro C5a Mediated Neutrophil Migration Assay

Serum harvested at the indicated time was used as the source of C5b-9, which mediated the hemolysis of chicken RBC as previously reported (Wang et al., *supra*). Mouse sera (Sigma) were incubated with either BB5.1 (100 μg/ml) or the same volume of PBS prior to activation with zymosan (1 mg/ml; Sigma). Diluted sera (20%) were used as the source of C5a for neutrophil chemotaxis experiments.

Statistical Analysis

The data were expressed as the means ± S.E.M. Student's one-tailed t-test assuming

equal variance was used (MS Windows). A P value less than 0.05 was considered significant.

Samsung Bioepis Exhibit No. 1002
Page 1613

Blocking the generation of C5a and C5b-9 by anti-C5 mAb, BB5.1

Functional inhibition of complement component C5 by anti-C5 mAb was determined by the pharmacodynamic profile of blocking serum C5b-9 mediated hemolytic activity (Fig. 1 A). In addition, C5a mediated neutrophil migration was determined in an in vitro assay (Fig. 1 B). The pharmacodynamics of C5 inhibition by a single i.v. injection of anti-C5 mAb, BB5.1, is very different from the pharmacodynamics of a single i.p. injection during the first 5 h after administration. More than 80% of C5b-9 mediated hemolysis was inhibited within 1 h of i.v. administration of BB5.1, compared to less than 30% of inhibition by i.p. injection. Administration of anti-C5 mAb continued blocking more than 60% of hemolysis between 5 h and 48 h after i.v. injection while approximately 56% of hemolysis was blocked during this period of time after i.p. injection. Serum samples harvested from control mAb treated mice had more than 80% of normal hemolytic activity during the entire period of the study (Fig. 1 A). Hemolytic activity gradually returned to the normal range one wk after a single injection of anti-C5 mAb.

An in vitro neutrophil migration assay was employed to test the ability of anti-C5 mAb on blocking the generation of C5a (Fig. 1 B). Inhibition of serum samples with anti-C5 mAb prior to zymosan activation significantly blocked human neutrophil chemotaxis which was mediated by the presence of C5a after zymosan activation, as seen in zymosan activated control sera (Fig.1, B). Since anti-C5 mAb, BB5.1, blocked the generation of both C5a and C5b-9 and did not bind to C5a directly, this anti-C5 mAb is probably specific to blocking cleavage of C5 by C5 convertase.

Development of Severe Airway Response in OVA Sensitized Animals and Definition of Three Critical Points

OVA-sensitized animals immediately developed a severe airway response after aerosol challenge with 5% OVA (Fig. 2 A). A typical airway response to aerosol provocation with allergen consisted of an early-phase airway response (EAR), which is known to be mediated by the release of histamine by mast cells, and a late-phase airway response (LAR), which is mediated by the combination of effects of infiltration of inflammatory cells, edema and bronchial constriction

Samsung Bioepis Exhibit No. 1002
Page 1614

(Larsen, Annu. Rev. Immunol. (1985) 3:59-85). The airway response after 5% OVA provocation was monitored by a non-invasive double chamber plethysmograph (DCP) in each mouse to measure the longitudinal changes of specific airway response (sRaw), which indicates the appearance of EAR and LAR (Fig. 2 A). EAR was typically a brief elevation of sRaw 15 min after 5% OVA provocation and LAR was typically observed 5 h later, consistent with an earlier report (Cieslewicz et al., J. Clin. Invest. (1999)104:301-308). Both EAR and LAR have magnitudes between 5 to 30 fold over the baseline. The severe airway response during the peak of LAR was generally evident as animals showed obvious labored breathing. Since inhibition of C5 can be achieved by the administration of anti-C5 mAb, we sought to dissect the critical involvement of C5 and its activated components at three critical points

(Fig. 2 B) during critical stages of the disease. The three critical checkpoints are: 1) initiation of airway inflammation, one of the two hallmarks of asthma's pathogenesis; 2) development of AHR to nonspecific stimuli, another hallmark of asthma; and 3) sustainment of an on-going airway response after allergen provocation. Both checkpoint 2 and checkpoint 3 are designed to evaluate the contribution of C5 in subjects that had experienced severe airway response previously (Fig. 2 B) and had established airway inflammation. Checkpoint 3 studies were conducted during an on-going airway response after allergen provocation by administering treatment via either an i.v. or aerosol route during the EAR. Functions of lower airways and quantification of airway inflammation were evaluated during the peak of LAR, 5 h after 5% OVA provocation.

Checkpoint 1: Involvement of C5 in the initiation of airway inflammation

C5 inhibition at checkpoint 1, at the time of repeat aerosol sensitization of 1% OVA, had no significant impact on serum levels of OVA-specific IgE or IgG on day 32 (Fig. 3 A). Corticosteroid treated mice also had elevated levels of OVA-specific IgE and IgG, that may have been generated long before the corticosteroid or anti-C5 mAb was administered on day 25. Sham treated mice had a negligible level of OVA specific IgG or IgE. As a result of repeated administration (i.p.) of anti-C5 mAb, approximately 80% of C5b-9 mediated hemolysis was inhibited on day 32 (21.1%±4) as compared to the normal hemolytic activity seen in control mAb and

Samsung Bioepis Exhibit No. 1002
Page 1615

corticosteroid treated mice (92.7%±6.8). Two key functions of lower airways were examined by tracheal cannulation at 5 h after 5% OVA provocation. The increase in lung resistance (RL) is commonly associated with significant obstruction of lower airways (Fig. 3 B) and a decrease in the dynamic lung compliance (Cdyn) is associated with the loss of lung elasticity, an important characteristic of the return to normal volume after pressure changes seen in a normal lung (Fig. 3 C). The significantly increased RL and reduced Cdyn seen in control mAb-treated mice indicated an increased airway obstruction (Figs. 3 B and C). Sham-mice provided normal ranges of RL and Cdyn at 5 h after exposure to aerosol PBS solution.

Correlating to its potent anti-inflammatory and anti-asthmatic activities, corticosteroid treatment at checkpoint 1 significantly blocked the increase of RL and prevented the loss of Cdyn. Similarly, C5 inhibition at checkpoint 1 markedly reduced the increase of RL 5 h after allergen provocation with an RL of 1.66 cm H2O/ml/second ±0.2, compared to an RL of 2.17 cm H_2O/ml/second ±0.33 seen in control mAb treated animals (Fig. 3 B). C5 inhibition also slightly reduced the loss of Cdyn (Fig. 3 C). Double blind histological analysis (Fig. 3 D) of lung tissue confirmed data from the function analysis and demonstrated that control mAb-treated BALB/c mice had severe perivascular and peribronchial infiltration of inflammatory cells (Fig. 3 F). A significant reduction of histology score was observed in corticosteroid treated mice (Fig. 3 G) while less impressive reduction was observed in anti-C5 mAb-treated mice (Fig. 3 H). Similar to severe allergic asthma in humans, eosinophils were the predominant inflammatory cells (more than 50%) in the perivascular and peribronchial lesions along the airways (Figs. 3 F-H).

Checkpoint 2: Contribution of C5 in the development of AHR in animals with established airway inflammation

In addition to airway inflammation, AHR to non-specific stimuli is another hallmark of asthma. AHR was evaluated as changes of RL and Cdyn expressed as a percentage of baseline in response to increasing doses of aerosol methacholine (Mch) challenges (Figs. 4 A and B). Control mAb-treated BALB/c mice had significant increases of RL and significant losses of Cdyn during the course of aerosol Mch challenges, while sham-mice responded only modestly to aerosol Mch

Samsung Bioepis Exhibit No. 1002
Page 1616

challenges. Two of eight among control mAb-treated mice died during high doses of aerosol Mch challenge due to severe respiratory stress. In contrast, C5 inhibition prevented dramatic increases of RL and the reduction of Cdyn during the course of aerosol Mch challenges, similar to those animals treated with corticosteroid (Figs. 4 A and B). The loss of Cdyn and increase of RL during the course of aerosol Mch challenges compared to sham-mice were probably due to the presence of established airway inflammation in these animals. There were no noticeable differences in the degree of airway inflammation among the three treated cohorts. Anti-C5 mAb treated mice had approximately 45% of normal hemolytic activity on day 35.

In order to compare our results of C5 inhibition at checkpoint 2 with data from a previous report (Karp et al., Nat. Immunol. (2000) 1:221-226), the presence or absence of native intrinsic AHR was examined in some strains of normal C5d mice as previously reported (De Sanctis et al., Am. J. Respir. Crit. Care Med. (1997) 156:S82-S88; Levitt et al., FASEB J. (1998) 2:2605-2608). Non-immunized C5d AKR/J mice have dramatic airway responses to increasing doses of aerosol Mch challenges with significant increases of RL over baseline (84.2%±16.7 with 20 puffs Mch) and a significant reduction of Cdyn under baseline (40% ±5.4 with 20 puffs of Mch) whereas C5d SWR/J and B10D2oSn mice have minimal changes of RL and Cdyn, indistinguishable from C5 sufficient (C5s) BALB/c mice and C57BL/6 mice, with no more than a maximal change of 15% of either RL or Cdyn away from baseline (n=3 for each strain, data not shown). Next, C5d B10D2oSn mice, which do not have intrinsic AHR, were immunized and challenged with aerosol Mch in the identical manner as C5s BALB/c mice in Figs. 4 A and B. Subgroups of sensitized C5d animals were also given either control or anti-C5 mAb treatment on day 33. C5d B10D2oSn mice developed a similar degree of airway inflammation as C5s mice, with an average histology score of 1.91 ± 0.37 (n=6) compared to a histology score of 2.25 ± 0.25 in C5s BALB/c mice (n=4). No noticeable histological difference was observed between anti-C5 mAb and control mAb treated C5d mice. In contrast to a previous report (Karp et al., *supra*), increasing doses of aerosol Mch challenges did not induce significant changes of lower airway functions in OVA immunized B10D2.oSn mice (Figs. 4 C and D). Treatment with either anti-C5 mAb or control mAb on day 33 had no impact on the development of AHR. The data

Samsung Bioepis Exhibit No. 1002
Page 1617

from both mAb treated cohorts were pooled together as an mAb treated cohort (Figs. 4 C and D). Furthermore, reconstitution of OVA immunized B10D2.oSn mice with recombinant mouse C5a (rmC5a) completely restored AHR in response to aerosol Mch challenge as indicated by the significant increase of RL and reduction of Cdyn (Figs. 4 C and D).

Reconstitution of rmC5a was not sufficient for the development of AHR since sham-B10D2.oSn mice reconstituted with rmC5a did not have significant changes of lower airway functions during Mch challenge (Figs. 4 C and D). Analysis of serum samples harvested from each mouse after Mch challenges confirmed the C5 deficient status of B10D2.oSn mice with average hemolytic activity of 8.1% ± 0.9 compared to 108%±3.8 of C5s BALB/c mice. Furthermore, random tail samples (n=2) from each lot of C5s and C5d animals were analyzed to confirm the C5 genotype status.

Checkpoint 3: Contribution of C5 during an on-going airway response

The role of C5 in sustaining an on-going airway response was also examined. Intervention was given during the peak of EAR, which was monitored by DCP at 15 min after aerosol exposure to 5% OVA on day 35. All control mAb-treated BALB/c mice developed severe LAR with significant increases of RL and reductions of Cdyn 5 h after allergen provocation (Figs. 5 A and B).

Administration of treatment through i.v. injection during EAR was selected to achieve rapid systemic C5 inhibition, which completely blocked the development of LAR with minimal increase of RL 5 h after 5% OVA provocation (Fig. 5 A) and prevented much of the loss of lung elasticity indicated by the minimal reduction of Cdyn (Fig. 5 B). Interestingly, corticosteroid treatment did not eliminate the development of LAR with significant elevation of RL and reduction of Cdyn at 5 h.

Although no immediate impacts (within 5 h) on lower airway functions were observed after i.v. corticosteroid treatment (Fig. 5 A), this treatment significantly modulated the intrapulmonary IL-13 (Fig. 7 B) and significantly improved lower airway functions 24 h later. Double blind histologic analysis of lung tissue showed comparable levels of perivascular and peribronchial infiltration of inflammatory

Samsung Bioepis Exhibit No. 1002
Page 1618

cells from the animals 5 h after i.v. intervention with either corticosteroid, anti-C5 mAb or control mAb, similar to the histological sample shown in Fig. 3 F.

Effect of C5 Inhibition on the Migration of Inflammatory Cells

The migration of inflammatory cells from airway tissue inflammation into bronchial lumen as enumerated was also examined by bronchial alveolar lavage fluid (BALF) WBC analysis. There was a significant reduction of total BALF inflammatory cells in anti-C5 mAb-treated BALB/c mice compared to control mAb-treated mice at checkpoint 1 (Fig. 6 A). The blockade on the migration of inflammatory cells by C5 inhibition exceeded the degree of improved lower airway function (Figs. 3 B and C) and reduced tissue inflammation (Fig. 3 D). Similarly, when BALF was harvested at checkpoint 3 (Fig. 6 B), C5 inhibition again had significant blockade on the migration of inflammatory cells into bronchial lumen, with significantly lower BALF WBC counts in anti-C5 treated animals than in animals treated with either corticosteroid or control mAb. This result is in dramatic contrast to the prominent presence of perivascular and peribronchial infiltration of inflammatory cells in lung tissue harvested before allergen provocation on day 35. The significant blockade on the migration of inflammatory cells by C5 inhibition correlated with the significantly enhanced efficacy of C5 inhibition over corticosteroid therapy at checkpoint 3 (Figs. 5 A and B).

Sham mice provided the baseline level of BALF WBC counts, which are primarily normal alveolar macrophages (82.3%±10.3) in WBC differential analysis (Fig. 6 C). Eosinophils constituted more than 45% of BALF inflammatory cells from all OVA immunized animals, regardless of the therapeutic intervention. There was a significant reduction in the percentage of neutrophils recovered in BALF from the animals treated at checkpoint 3 with either corticosteroid (4.5%±2.8) or anti-C5 mAb (6.3%±3.3) versus control mAb treated mice (18.3%±4.4). No significant reductions occurred in the percentages of eosinophils or lymphocytes in BALF samples from the animals given intervention at checkpoint 3 (Fig. 6 C).

Samsung Bioepis Exhibit No. 1002
Page 1619

Effect of C5 Inhibition on Intrapulmonary Th1/Th2 Cytokine Profile and Inflammatory Mediators

BALF harvested from control mAb treated mice had markedly increased levels of IL-5 and IL-13 (Figs. 7 A and B) as compared to sham mice. Corticosteroid treatment markedly reduced the BALF levels of IL-5 and IL-13, however, this did not translate into an immediate improvement of lower airway function (Figs. 5 A and B) during the 5 h period of treatment. The dramatic improvement of lower airway functions (Figs. 5 A and B) seen after C5 inhibition at checkpoint 3 did not correlate with any reduction of BALF level of IL-5 and IL-13. The statistical difference of the BALF level of IL-13 between anti-C5- and corticosteroid-treated mice equals a p value of 0.051 C5 inhibition or corticosteroid therapy at checkpoint 3 did not influence the BALF level of histamine (Fig. 7 C). This was expected since histamine had probably already been released into the airway lumen upon the engagement of IgE receptors of mast cells by aerosol allergen provocation and was responsible for the appearance of EAR when therapeutic intervention was given. Eosinophils, the most prominent inflammatory cells along lower airways (Figs. 3 F-H), are probably responsible for the production of eotaxin, RANTES and TGF-β (Figs. 7 D-F) in asthmatic individuals. Control mAb-treated animals had significantly elevated BALF levels of eotaxin (Fig. 7 D), RANTES (Fig. 7 E), or activated TGF-β (Fig. 7 F), compared to negligible or non-detectable levels of these mediators in sham-mice. In contrast, C5 inhibition had a marked impact on BALF levels of eotaxin, RANTES, and activated TGF-β when given either at checkpoint 1 (Figs. , D and E) or checkpoint 3 (Fig. 7 F). Corticosteroid treatment only reduced the BALF level of TGF-β when given at checkpoint 3 but had no obvious impact on the production and release of eotaxin or RANTES when given at checkpoint 1.

Neutrophils are commonly believed to be responsible for the production of bronchial TNF-α and proMMP9. C5 inhibition significantly reduced BALF levels of TNF-α (Fig. 7 G) when given at checkpoint 1, significantly different from control mAb-treated mice. The bronchial level of proMMP9 was dramatically reduced and was significantly different from control mAb-treated and corticosteroid-treated mice when C5 inhibition was given at checkpoint 3 (Fig. 7 H).

Samsung Bioepis Exhibit No. 1002
Page 1620

Checkpoint 3: Contribution of Intrapulmonary Activation Of C5 During An On-Going Airway Response

The massive migration of inflammatory cells from airway tissue inflammation into bronchial lumen probably results from powerful chemotactic forces from the epithelial mucosa of the airway. Thus, C5 may be activated intrapulmonarily during an airway response to allergen provocation. The functions of lower airways and parameters of inflammation were evaluated after blocking the intrapulmonary activation of C5. The potential impact at checkpoint 3 by inhibitory receptors, such as FcγRIIB (Katz, Curr. Opin. Immunol. (2002) 14:698-704; Ravetch et al., Annu. Rev. Immunol. (2001) 19:275-90), were also evaluated, as the result of interaction between intrapulmonary C5 and anti-C5 mAb and the subsequent formation of immune complexes (IC).

Effect of intrapulmonary C5 inhibition with anti-C5 mAb was directly compared to anti-C5 Fab in order to achieve this goal. Furthermore, the efficacy of an anti-C5aR serum IgG (sIgG) was evaluated in order to dissect the role of intrapulmonary C5a versus C5b-9 at this checkpoint. As demonstrated in Figs. 8 A and B, control mAb-treated animals developed severe LAR with significant increases of RL and significant reductions of Cdyn, in significant contrast to the normal RL and Cdyn seen in sham-mice. Aerosol administration of corticosteroid blocked the development of LAR as evidenced by the reduction of RL with corresponding preservation of Cdyn. Blocking intrapulmonary C5 activation with aerosol administration of either anti-C5 mAb or anti-C5 Fab during the peak of EAR also prevented the development of LAR with minimal increase of RL and minimal reduction of Cdyn (Figs. 8 A and B). RL and Cdyn of both anti-C5 treated cohorts were significantly different from control mAb treated animals. Blocking the binding of intrapulmonary C5a to its receptors with an anti-C5aR sIgG also prevented the development of LAR with minimal increase of RL and minimal reduction of Cdyn similar to animals treated with anti-C5 mAb. There was no statistical difference between the three cohorts treated with either anti-C5 mAb, anti-C5 Fab, or anti-C5aR sIgG (Figs. 8 A and B).

Samsung Bioepis Exhibit No. 1002
Page 1621

When the parameters of intrapulmonary inflammatory activities were examined, such as the migration of inflammatory cells (Fig. 8 C) and the BALF level of proMMP9 (Fig. 8 D), significant differences were found between blocking intrapulmonary activation of C5 versus blocking the binding of C5a to its C5aR. Aerosol administration of either anti-C5 mAb or its Fab fragment during EAR significantly blocked both the migration of inflammatory cells into bronchial airway lumen and the elevation of BALF level of proMMP9, whereas blocking the engagement of C5aR had no significant impact on these two key inflammatory parameters similar to the animals treated with control mAb (Figs. 8 C and D). Consistent with its ability to block the development of LAR (Figs. 8 A and B), aerosol administration of corticosteroid also ameliorated intrapulmonary inflammatory activities with marked reduction of the migration of inflammatory cells and the production of proMMP9 (Figs. 8 C and D) indicating the importance of inflammatory activities at the epithelial side of the bronchial lumen. Aerosol administration of anti-C5 mAb, anti-C5 Fab or anti-C5aR sIgG did not have any impact on serum C5b-9 mediated hemolytic activity.

Combination Therapy:

As demonstrated in Fig. 9, animals treated with a non-specific control antibody had significant increases of lung resistance during the course of Mch challenges. In this study, two animals challenged with high doses of Mch died due to severe respiratory stress. In contrast, both the steroid and anti-C5 treated animals have only moderate increases of lung resistance, higher than normal sham immunized animals but significantly lower than control placebo treated mice. Combined treatment with both anti-C5 antibody and steroid significantly further reduced the increases of lung resistance during the course of Mch challenges. Based on this result, both steroid and C5 inhibitor can prevent the development of airway hyper-responsiveness to non-specific aerosol stimuli in asthmatic individuals with established airway inflammation.

Nebulization Formulations

Examples of formulations suitable for nebulization of an antibody are shown in Fig. 10. Fig. 11 further demonstrates the effectiveness of nebulization treatment.

Samsung Bioepis Exhibit No. 1002
Page 1622

The results shown in Fig. 11 were obtained following a study that employed the same protocol as the Checkpoint 2 study described herein, except that the treatment here was delivered by nebulization on day 32 instead of i.p. For anti-C5 antibody, 3 mg/ml of BB5.1 was subject to 10 min nebulization, and 2 mg/ml steroid was also subject to 10 min nebulization. For the combination therapy, the final concentrations subjected to 10 min nebulization were: 3 mg/ml for BB5.1 and 2 mg/ml for the steroid.

Fig. 12 further shows that eculizumab in Formulation 1 (Fig. 10) at 30 mg/ml can be effectively and efficiently nebulized by a conventional nebulizer such as the Pari LC Jet Plus or a specialty nebulizer such as Sonik LDI. Fig. 12 shows that a majority of the particles after nebulization were less than 5 μM, which is suitable for deep lung delivery.

As shown in Fig. 13, eculizumab in Formulation 1 (Fig. 10) can be effectively and efficiently delivered by a conventional nebulizer, comparable to the aerosol characteristics of an existing pulmonary drug delivered by inhalation, Pulmozyme®, also using a conventional nebulizer. Eculizumab in Formulation 1 (Fig. 10) can also be effectively and efficiently delivered by a specialty nebulizer, for example, Sonik LDI.

Using SDS-PAGE and HPLC analyses, Fig. 14 and Fig. 15 demonstrate that eculizumab can be effectively and efficiently delivered by nebulization. Eculizumab in a suitable formulation was nebulized using either the Pari-Jet Air nebulizer or the Sonik LDI nebulizer. The nebulized antibody, in a spray form, was collected from the mouth piece of the nebulizer (e.g., F1D2F), and different sizes of mouth pieces (e.g., F1D2C1-C5) were used. The SDS-PAGE and HPLC analyses demonstrated the integrity of the nebulized antibody through various sizes of the spray mouth piece, as shown by the purity of the samples analyzed.

References

All publications and patents mentioned herein, are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

Samsung Bioepis Exhibit No. 1002
Page 1623

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the application described herein. Such equivalents are intended to be encompassed by the following claims.

Samsung Bioepis Exhibit No. 1002
Page 1624

We claim:

1. A method for preventing or treating a pulmonary condition in a subject comprising administering to the subject a therapeutically effective amount of an antibody that inhibits activation of the complement cascade and an additional active agent.

2. The method of claim 1, wherein the pulmonary condition is selected from the group consisting of: asthma, bronchitis, a chronic obstructive pulmonary disease (COPD) interstitial lung diseases, lung malignancies, α-1 anti-trypsin deficiency, emphysema, bronchiectasis, bronchiolitis obliterans, sarcoidosis, pulmonary fibrosis, and collagen vascular disorders.

3. The method of claim 1, wherein the subject is a human.

4. The method of claim 1, wherein the antibody is specific to C5.

5. The method of claim 1, wherein the antibody is specific to C5 convertase.

6. The method of claim 1, wherein the antibody is specific to C5a or C5b-9.

7. The method of claim 1, wherein the antibody is eculizumab or pexelizumab.

8. The method of claim 1, wherein the antibody is selected from the group consisting of: a full length immunoglobulin, a single chain antibody, a domain antibody, a Fab fragment, and an antibody having a Fab fragment and a mutated Fc portion.

9. The method of claim 7, wherein the mutated Fc portion does not activate complement.

10. The method of claim 1, wherein the antibody is administered prior to manifestation of the pulmonary condition, during manifestation of the pulmonary condition, or after manifestation of the pulmonary condition.

11. The method of claim 1, wherein the additional active agent is a corticosteroid.

12. The method of claim 1, wherein the corticosteroid is dexamethasone.

13. The method of claim 1, wherein the antibody and the additional active agent may be administered to the subject by the same delivery method or route.

Samsung Bioepis Exhibit No. 1002
Page 1625

14. The method of claim 1, wherein the antibody and the additional active agent may be administered to the subject by different delivery methods or routes.

15. An aerosol composition comprising an antibody that inhibits activation of the complement cascade, wherein the composition is suitable for preventing or treating a pulmonary condition in a subject.

16. The aerosol composition of claim 15, wherein the antibody is formulated in a composition suitable for aerosolization.

17. The aerosol composition of claim 15 further comprising an additional active agent.

18. A biopharmaceutical package comprising: a) an antibody that inhibits activation of the complement cascade; and b) a nebulizer, wherein the package is suitable for preventing or treating a pulmonary condition in a subject.

19. The biopharmaceutical package of claim 18, wherein the nebulizer is selected from the group consisting of a jet air nebulizer, an ultrasonic nebulizer, a vibrating mesh nebulizer and a shockwave nebulizer.

20. The biopharmaceutical package of claim 19 further comprising instructions for use.

21. The biopharmaceutical package of claim 19 further comprising an additional active agent.

22. A pulmonary drug delivery kit comprising: a) an inhaler; and b) an antibody formulated for inhalation delivery.

23. The kit of claim 22, wherein said inhaler is a metered dose inhaler.

24. The kit of claim 23, whereon the metered dose inhaler is a pressurized metered dose inhaler.

25. The kit of any one of claims 22 or 23, wherein said inhaler further comprises a holding chamber.

26. The kit of claim 22, wherein said inhaler is a nebulizer.

27. The kit of claim 26, wherein said nebulizer is an ultrasonic nebulizer.

Samsung Bioepis Exhibit No. 1002
Page 1626

28. The kit of claim 26, wherein said nebulizer is a jet air nebulizer.

29. The kit of claim 22, wherein said inhaler is a dry powder inhaler.

30. A pulmonary drug delivery kit comprising a) an anti C5 antibody composition comprising 1 mg/ml to 200 mg/ml; and b) a label indicating administration by nebulization.

Samsung Bioepis Exhibit No. 1002
Page 1627

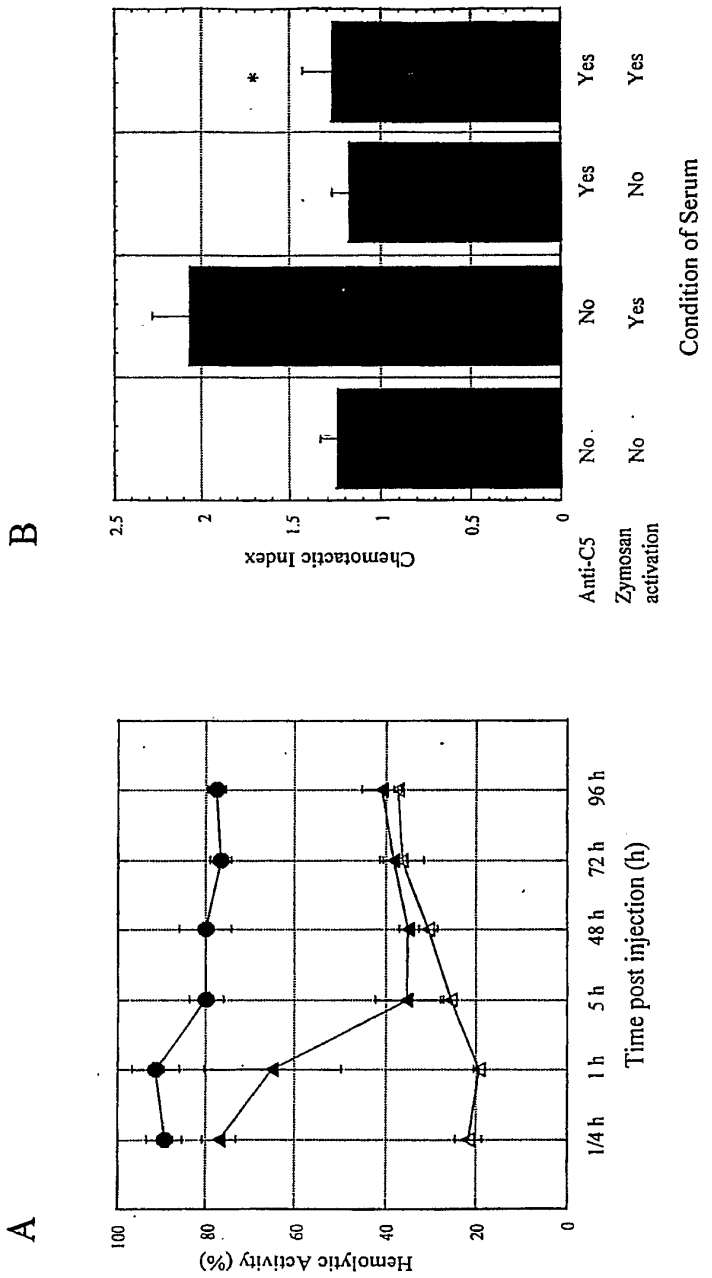

A
Hemolytic Activity (%)
0
20
40
60
80
100
1/4 h
1 h
5 h
48 h
72 h
96 h
Time post injection (h)
B
Chemotactic Index
0
0.5
1
1.5
2
2.5
*
Anti-C5
Zymosan activation
No
No
No
Yes
Yes
No
Yes
Yes
Condition of Serum

Fig. 1

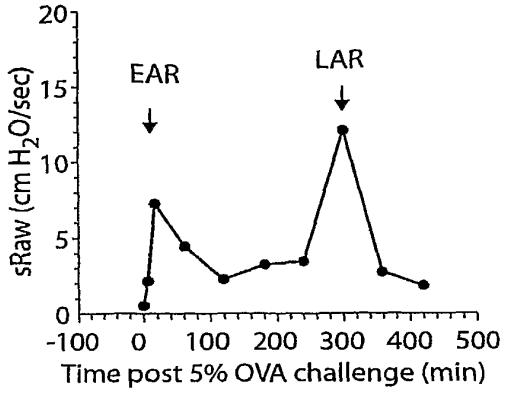


Fig. 2A

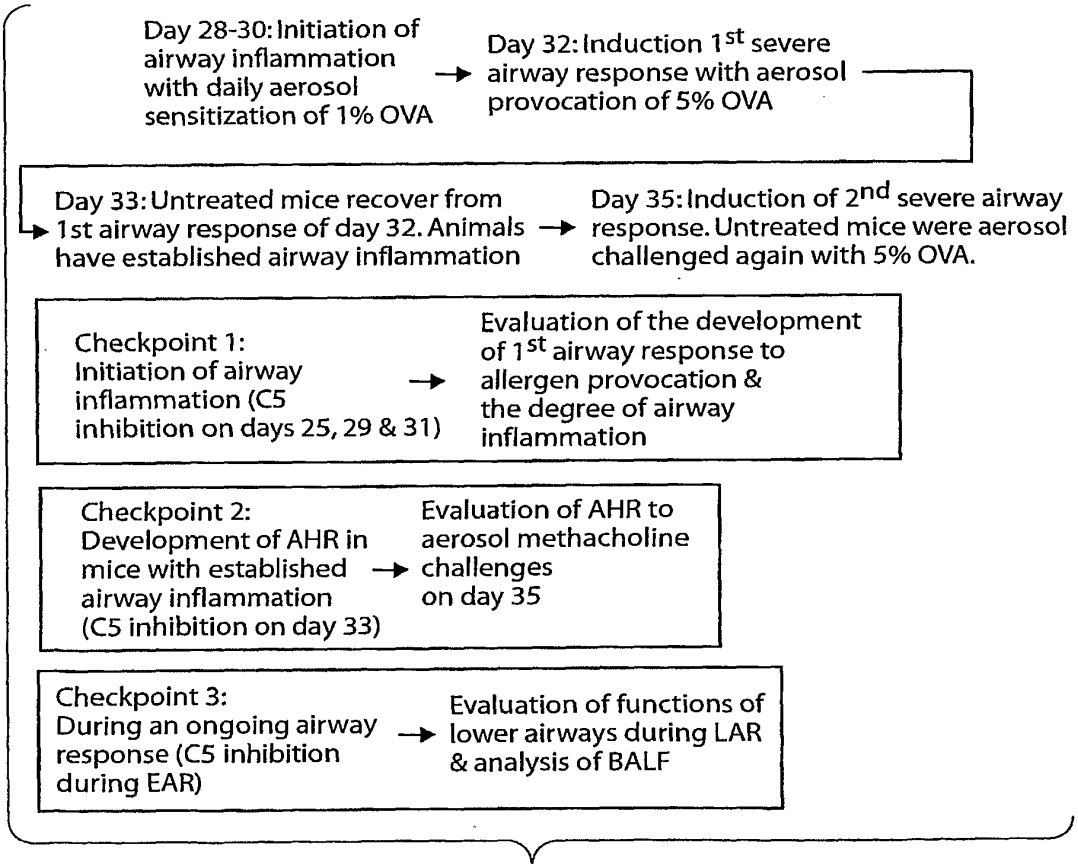


Fig. 2B

Samsung Bioepis Exhibit No. 1002
Page 1629

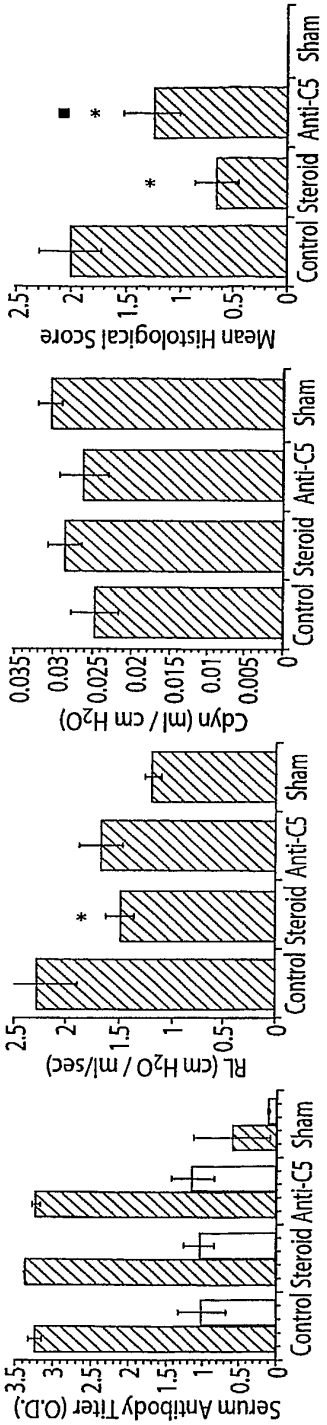


Fig. 3A

Fig. 3B

Fig. 3C

Fig. 3D



Fig. 3E

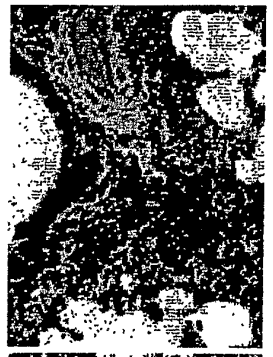


Fig. 3F



Fig. 3G



Fig. 3H

Samsung Bioepis Exhibit No. 1002
Page 1630

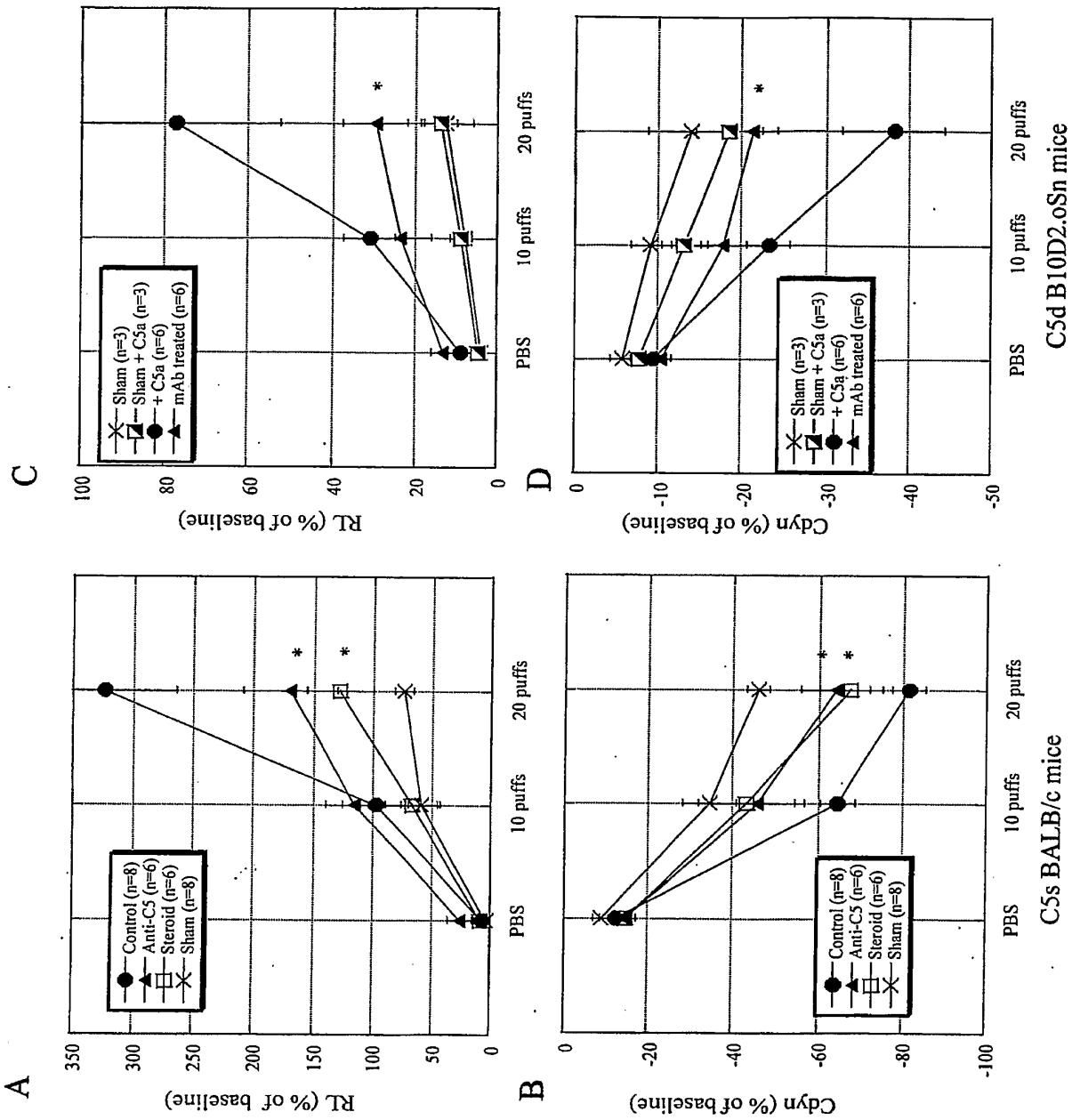

A
RL (% of baseline)
Control (n=8)
Anti-C5 (n=6)
Steroid (n=6)
Sham (n=8)
PBS
10 puffs
20 puffs
B
Cdyn (% of baseline)
C
RL (% of baseline)
Sham (n=3)
Sham + C5a (n=3)
+ C5a (n=6)
mAb treated (n=6)
D
Cdyn (% of baseline)
C5s BALB/c mice
C5d B10D2.oSn mice

Fig. 4

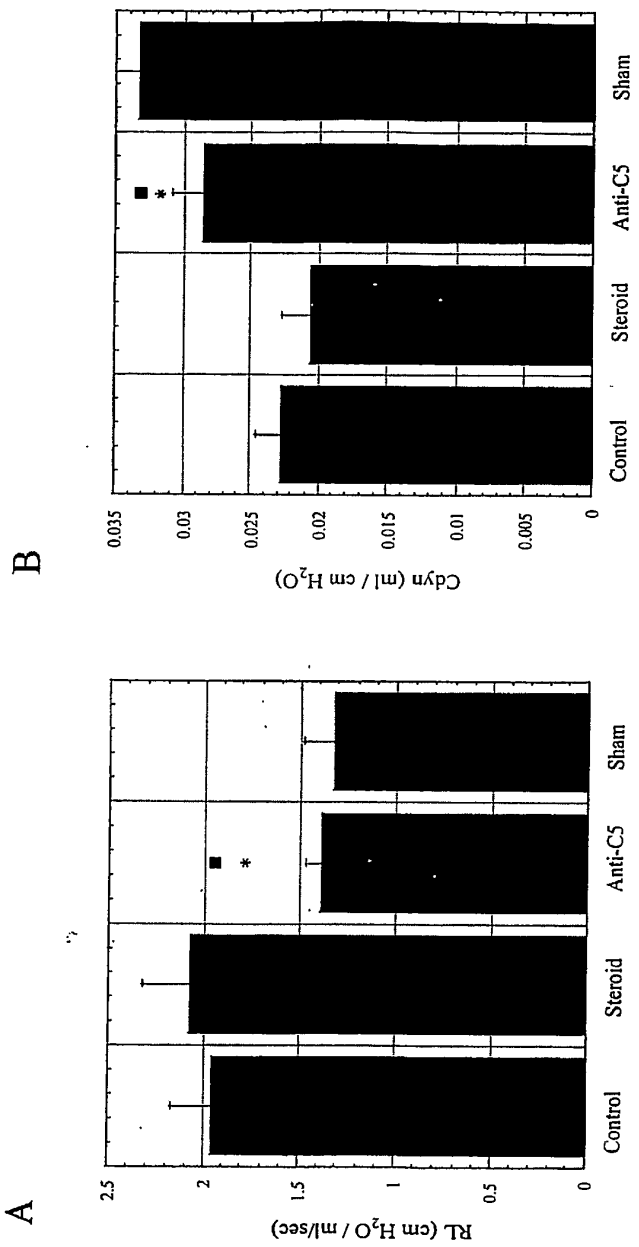

A
RL (cm H2O / ml/sec)
2.5
2
1.5
1
0.5
0
Control
Steroid
Anti-C5
Sham
*
B
Cdyn (ml / cm H2O)
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
Control
Steroid
Anti-C5
Sham
*

Fig. 5

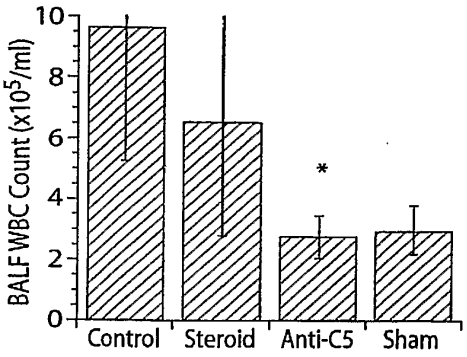


Fig. 6A

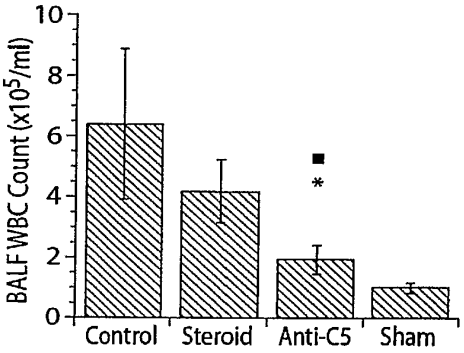


Fig. 6B

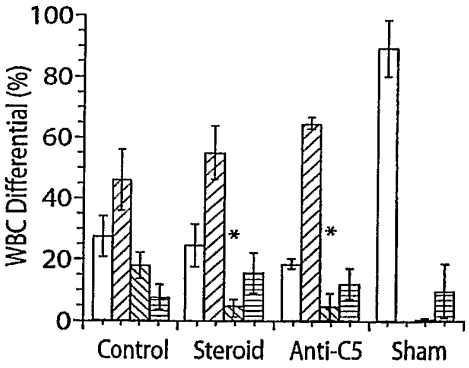


Fig. 6C

Samsung Bioepis Exhibit No. 1002
Page 1633

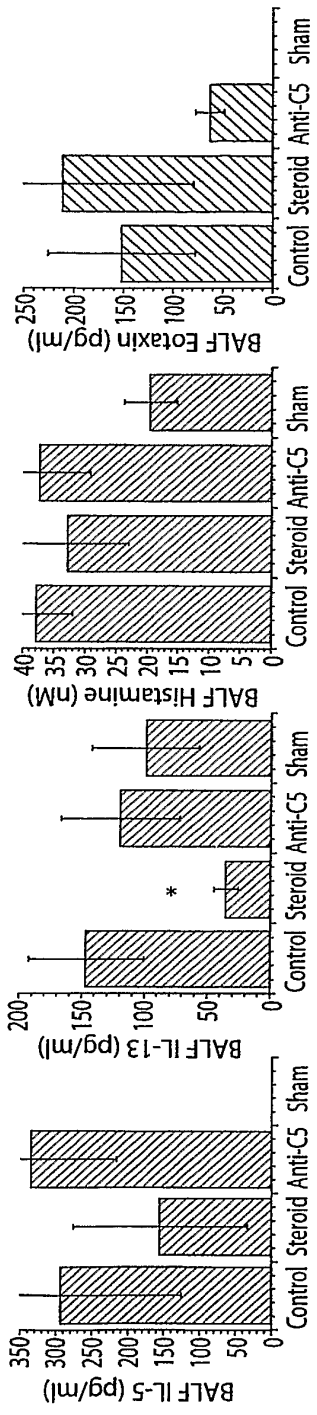


Fig. 7A

Fig. 7B

Fig. 7C

Fig. 7D

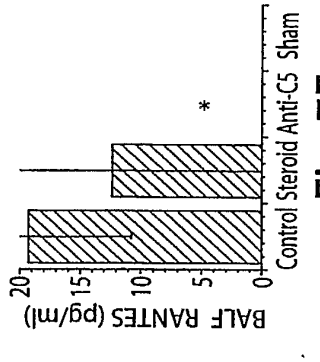


Fig. 7E

Fig. 7F

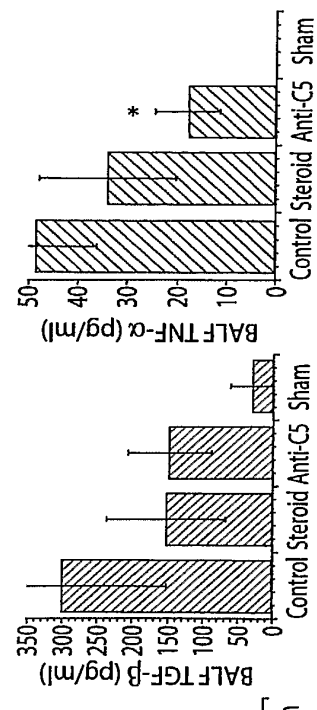


Fig. 7G

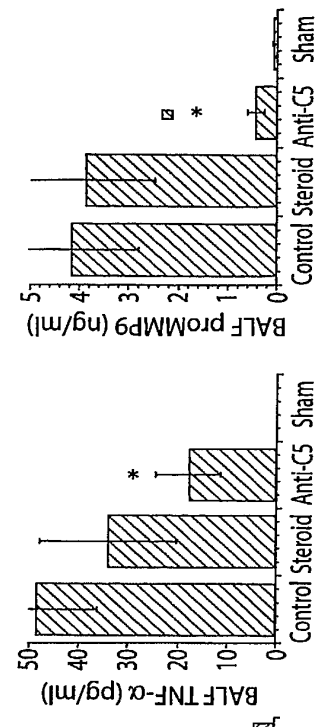


Fig. 7H

Samsung Bioepis Exhibit No. 1002
Page 1634

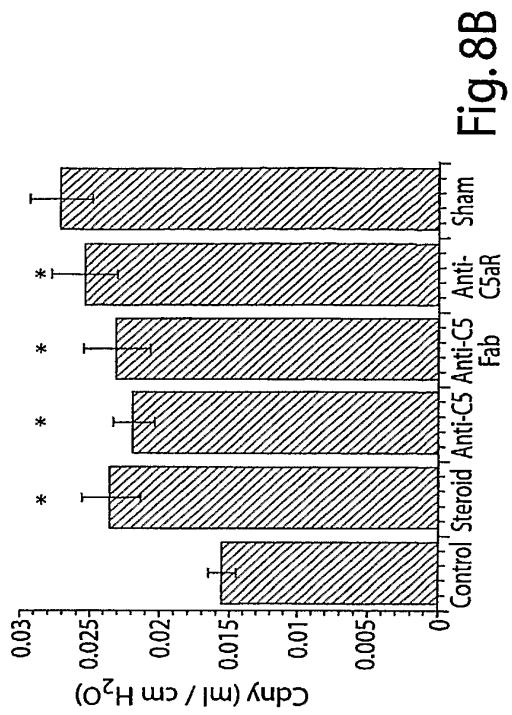


Fig. 8B

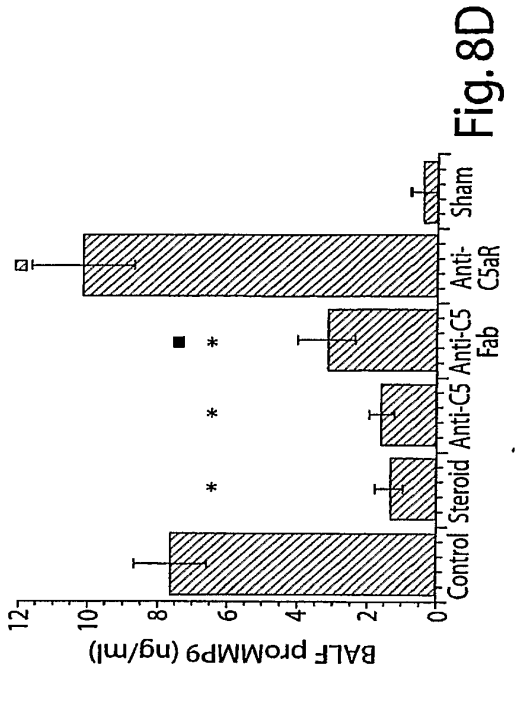


Fig. 8D

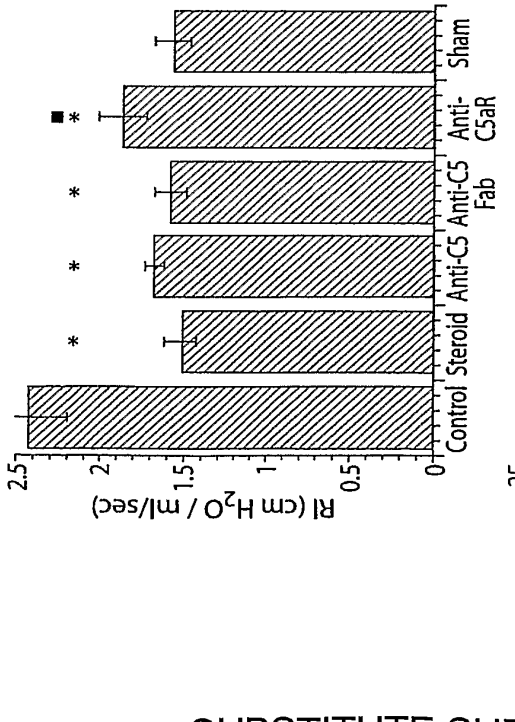


Fig. 8A

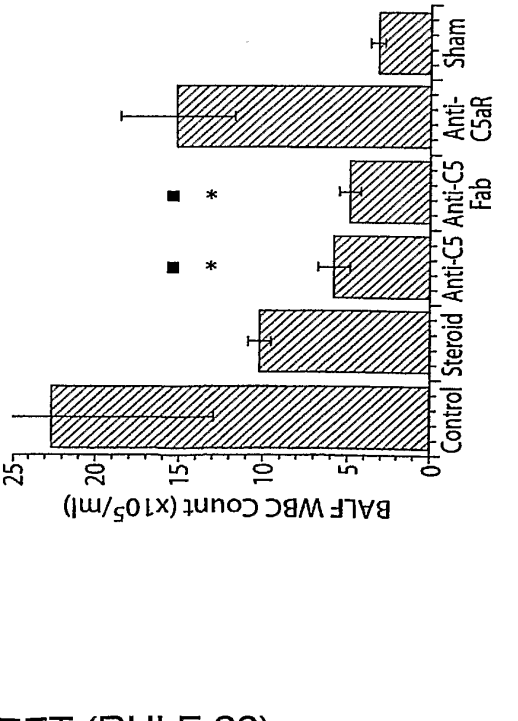


Fig. 8C

Samsung Bioepis Exhibit No. 1002
Page 1635

Fig. 9
2 days after aerosol Treatment

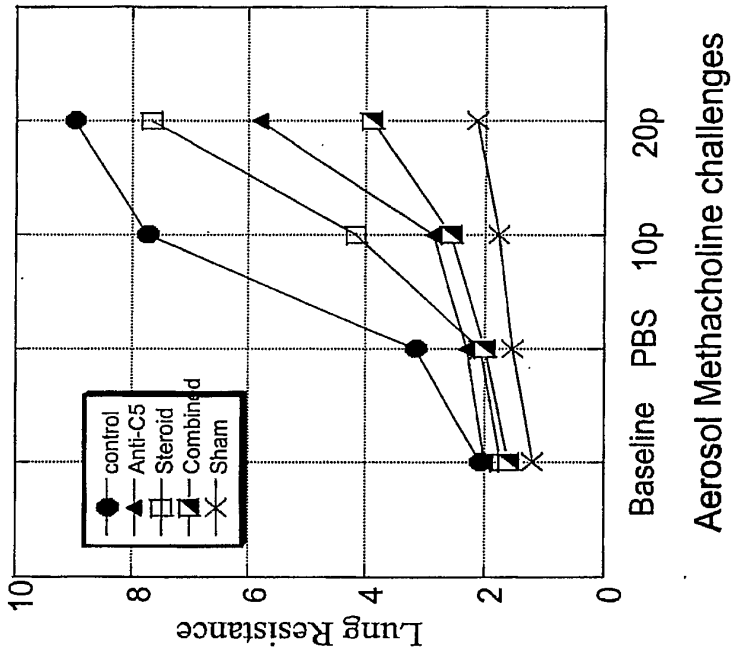


Fig. 10 Example of Suitable Formulations

Formulation 1

- Mab 1- 30 mg/ml
- 10 mM sodium phosphate
- 150 mM NaCl
- pH 6-8
- 0.001 to 0.02% Tween 80 v/v
- physiologic osmolality

Formulation 2

- 20mM Histidine
- 50mM Glycine
- 3% (w/v) Sorbital
- 1.5% (w/v) Mannitol
- pH 6-8
- 0.001 to 0.02% Tween 80
- 40mg/ml-200mg/ml
- physiologic osmolality

Samsung Bioepis Exhibit No. 1002
Page 1637

Fig. 11 Mch challenge two days after nebulization treatment

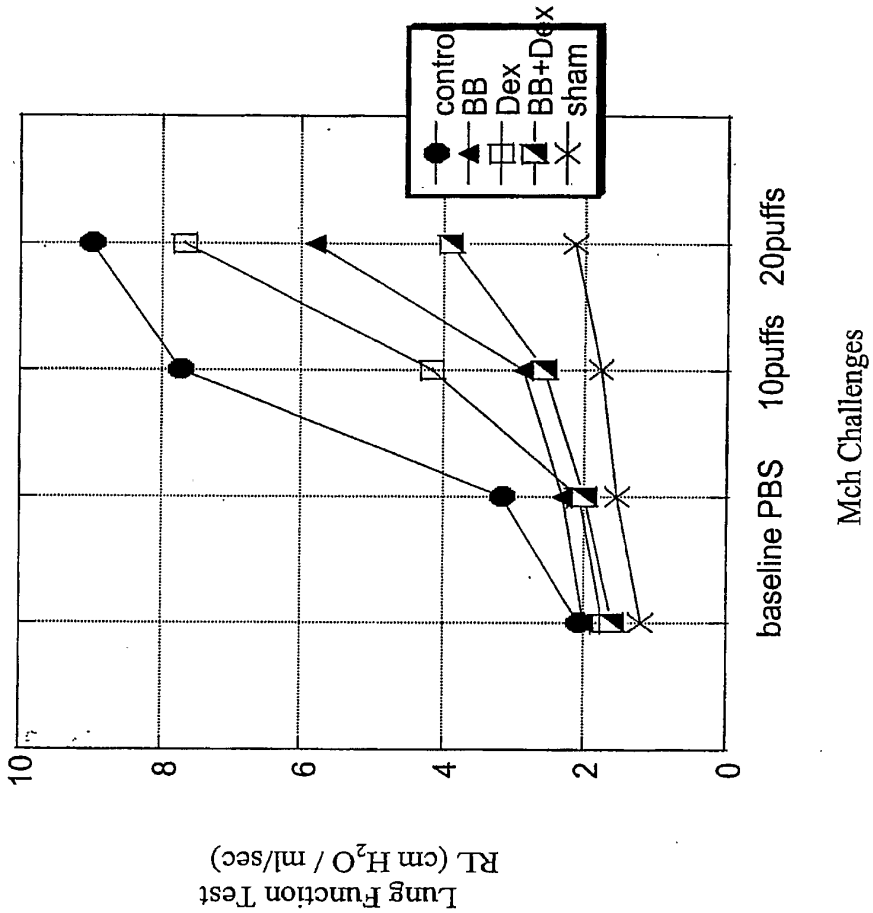


Fig. 12 Particle Size Distribution

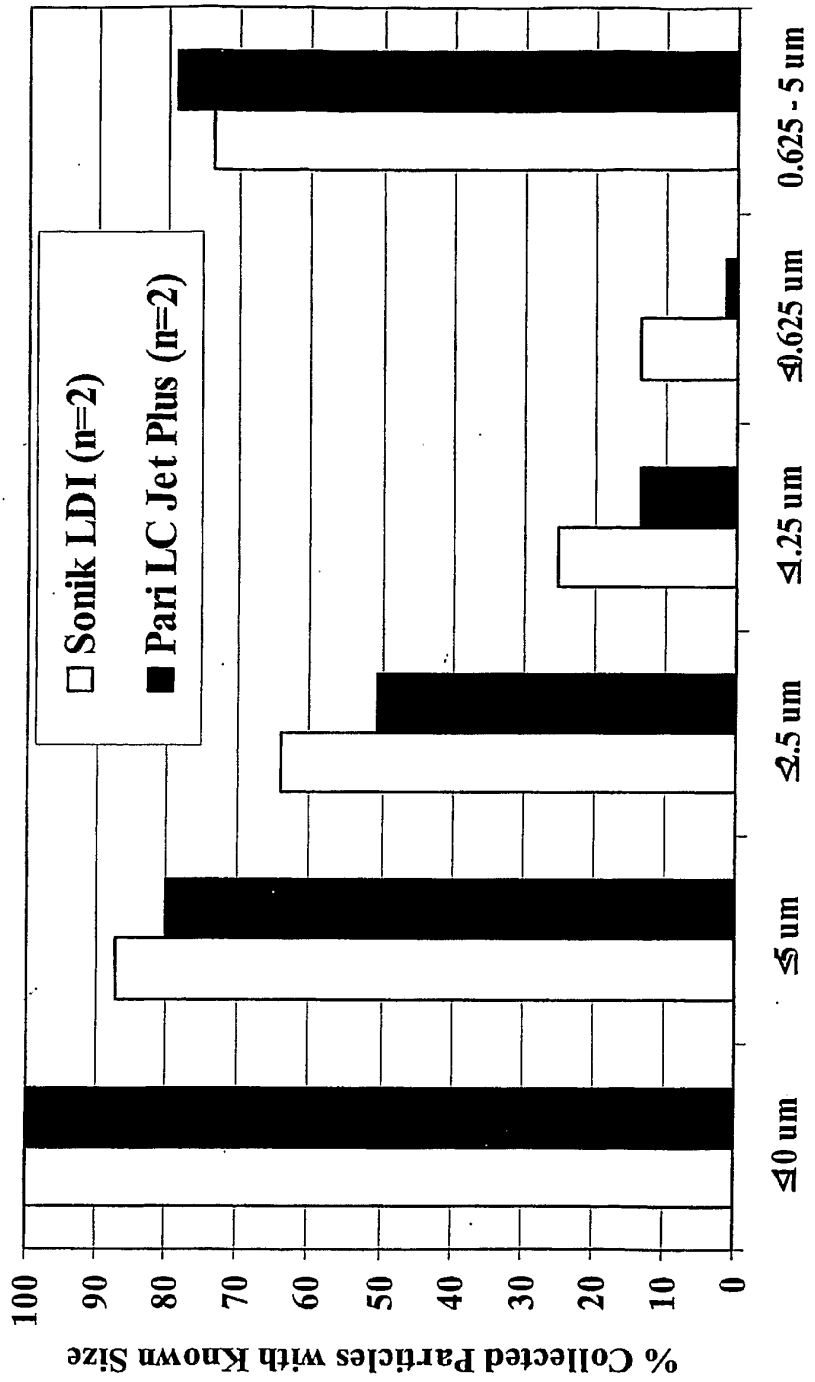


Fig. 13 Nebulization and Delivery Efficiency

	Sonik LDI Eculizumab	Pari LC Jet Plus Eculizumab	Pari LC Jet Plus Pulmozyme
Respirable fraction (%)	44.91 – 59.63	71.88 – 74.21	47.8 (1 – 5 µm)
Nebulizer efficiency (%)	11.24 – 19.91	42.51 – 52.46	51.4
Delivery (%)	6.70 – 8.94	31.55 – 37.71	24.6 (1 – 5 µm)

Respirable fraction: the fraction of the droplets delivered to the exit from the mouth piece in the range of 0.625 – 5 µm

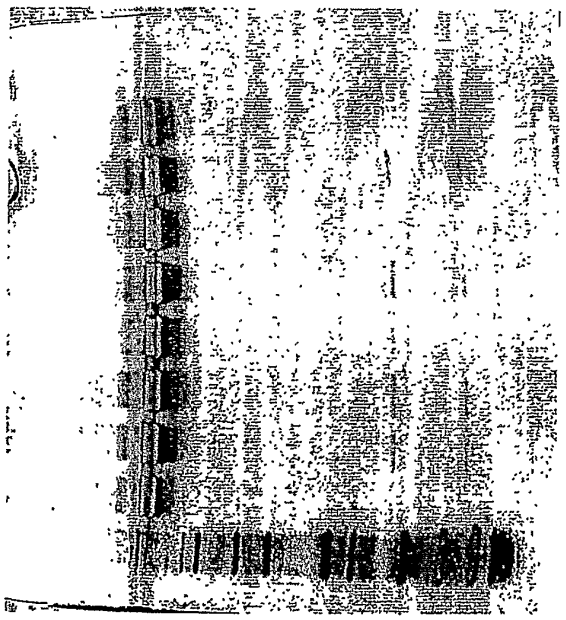
Nebulizer efficiency: the fraction of the dose initially placed in the nebulizer that reaches the exit from the mouthpiece

Delivery: the fraction of the initial dose that reaches the exit from the mouthpiece in the size range of 0.625 – 5 µm

Samsung Bioepis Exhibit No. 1002
Page 1640

Eculizumab Aerosolization

SDS-PAGE Analysis
4-20% TG, 1.5mm, 10 Well, Non Reduced
Reference NDG #1383 page 38-42
Pari-Jet Air



Lane	Sample	load (ug)	Purity(%)	% Peak Response
	MW marker	NA	NA	N/A
1	Ecu Ref Std	1.5	99.3	N/A
2	TR_CTRL-1 (30mg/mL)	1.5	99.1	102.7
3	16FEB05, F1D2C1	1.5	97.7	99.8
4	16FEB05, F1D2C2	1.5	98.5	98.6
5	16FEB05, F1D2C3	1.5	96.8	99.3
6	16FEB05, F1D2C4	1.5	94.3	94.1
7	16FEB05, F1D2C5	1.5	88.9	92.3
8	16FEB05, F1D2F	1.5	N/A	N/A
9	Blank	N/A	N/A	N/A

Fig. 14

Samsung Bioepis Exhibit No. 1002
Page 1641

Fig. 15 Eculizumab Aerosolization

SEC HPLC Purity Summaries

	15 Feb 05		16Feb 05	
	Sonik LDI	Pari Jet Air	Sonik LDI	Pari Jet Air
F1P1-W1	99.1	98.4	99.0	98.4
F1P2-W1	99.2	99.2	98.2	98.5
F1P3-W1	99.1	99.2	99.1	99.1
F1P4-W1	95.7	NA	93.8	n/a
F1C1-W1	[95.6]	97.5	94.2	97.7
F1C2-W1	[96.1]	98.2	95.8	98.5
F1C3-W1	[94.9]	96.9	[96.3]	96.8
F1C4-W1	[93.0]	94.0	95.2	94.3
F1C5-W1	[90.8]	89.3	[92.7]	88.9
[Peak responses < 90%]				

Samsung Bioepis Exhibit No. 1002
Page 1642

Fig. 16 Repeat Long Term Aerosol Administrations of Anti-C5
mAb Do Not Induce Airway Inflammation (2x per week for 10wks
Sham Treated (PBS)
Control mAb
Ant-C5 (BB5.1)

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(71) Applicant *(for all designated States except US)*: **ALEXION PHARMACEUTICALS, INC.** [US/US]; 352 Knotter Drive, Cheshire, CT 06410 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **SPULER, Simone** [DE/DE]; Akazienallee 32, 14050 Berlin (DE). **ROTHER, Russell** [US/US]; 25 Blue Trail Drive, Prospect, CT 06712 (US). **WENZEL, Katrin** [DE/DE]; Fröbelweg 23, 16321 Bernau (DE).

(74) Agents: **VARMA, Anita** et al.; ROPES & GRAY LLP, One International Place, Boston, MA 02110-2624 (US).

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WO 2007/130031 A1

(54) Title: METHODS FOR THE TREATMENT OF MUSCULAR DYSTROPHY ASSOCIATED WITH DYSFERLIN-DEFICIENCY

(57) Abstract: The use of therapeutics capable of inhibiting complement such as an anti-C5 antibody to treat muscular dystrophy associated with dysferlin-deficiency is disclosed.

Methods for the Treatment of Muscular Dystrophy Associated with Dysferlin-Deficiency

FIELD OF THE INVENTION

The present invention relates to methods for the treatment of muscular dystrophy associated with dysferlin-deficiency. In specific embodiments, the invention relates to the use of antibodies capable of inhibiting complement as therapeutic agents to treat muscular dystrophy associated with dysferlin-deficiency.

BACKGROUND OF THE INVENTION

Muscular dystrophy represents a family of inherited diseases of the muscles. To date, there is no known treatment, medicine, or surgery that will cure muscular dystrophy, or stop the muscles from weakening. There has thus been a long felt need for new approaches and better methods to treat muscular dystrophy, including muscular dystrophy associated with dysferlin-deficiency.

SUMMARY OF THE INVENTION

Accordingly, the disclosure provides methods and compositions useful for treating muscular dystrophy, particularly muscular dystrophy associated with dysferlin-deficiency.

In certain embodiments, the disclosure provides a method of treating muscular dystrophy associated with dysferlin-deficiency in a mammal comprising administering to said mammal a therapeutically effective amount of an agent (e.g., an antibody or fragment thereof) that inhibits complement, such as for example by inhibiting the formation of the membrane attack complex (MAC). In specific embodiments, the agent is an antibody that comprises anti-C5 antibody, such as for example an antibody that binds C5 and prevents the cleavage of C5 to form C5a and C5b. In certain embodiments, the mammal is a human.

In certain embodiments, the antibody is a whole antibody or an antibody fragment. In certain embodiments, the whole antibody or antibody fragment is the group consisting of a polyclonal antibody, a monoclonal antibody or antibody fragment, a diabody, a chimerized or chimeric antibody or antibody fragment, a

humanized antibody or antibody fragment, a deimmunized human antibody or antibody fragment, a fully human antibody or antibody fragment, a single chain antibody, an Fv, an Fab, an Fab', and an $F(ab')_2$. In certain embodiments, the antibody is pexelizumab. In certain embodiments, the antibody is eculizumab.

In certain embodiments, the agent is administered chronically to said mammal. In certain embodiments, said mammal receives a one-time administration or multiple administrations of the agent during a limited time period such as a week, a month, a year or longer.

In certain embodiments, the agent is administered systemically to said mammal. In certain embodiments, the agent is administered locally to said mammal.

In certain embodiments, the agent is administered in combination with another therapeutic agent to said mammal.

In certain embodiments, the disclosure provides a method of treating muscular dystrophy associated with dysferlin-deficiency in a mammal comprising administering to said mammal a therapeutically effective amount of a CD55 agonist. In certain embodiments, the mammal is a human. In certain embodiments, the CD55 agonist increases the expression level and/or activity of CD55.

In certain embodiments, the CD55 agonist is selected from: a polypeptide, a polypeptide analog, a nucleic acid, a nucleic acid analog, and a small molecule. In certain embodiments, the CD55 agonist comprises an agonistic ligand of CD97. In certain embodiments, the said CD55 agonist comprises an agent that is capable of preventing the association C4b and C3b to form the C3 or C5 convertase. In certain embodiments, the polypeptide comprises an amino acid sequence of greater than 90% sequence identity to the amino acid sequence of a soluble portion of a naturally occurring CD55 protein. In certain embodiments, the nucleic acid comprises a polynucleotide sequence of greater than 90% sequence identity to the nucleotide sequence of a naturally occurring CD55 mRNA.

In certain embodiments, the polypeptide CD55 agonist is an antibody. In certain embodiments, the antibody is a whole antibody or an antibody fragment. In certain embodiments, the whole antibody or antibody fragment is the group

Samsung Bioepis Exhibit No. 1002
Page 1646

consisting of a polyclonal antibody, a monoclonal antibody or antibody fragment, a diabody, a chimerized or chimeric antibody or antibody fragment, a humanized antibody or antibody fragment, a deimmunized human antibody or antibody fragment, a fully human antibody or antibody fragment, a single chain antibody, an Fv, an Fab, an Fab', and an $F(ab')_2$. In certain embodiments, the antibody is an agonistic antibody that specifically binds CD55 and increases CD55 activity.

In certain embodiments, the CD55 agonist is administered chronically to said mammal. In certain embodiments, said mammal receives a one-time administration or multiple administrations of the antibody during a limited time period such as a week, a month, a year or longer.

In certain embodiments, the CD55 agonist is administered systemically to said mammal. In certain embodiments, the antibody is administered locally to said mammal.

In certain embodiments, the CD55 agonist is administered in combination with another therapeutic agent to said mammal.

In certain embodiments, the disclosure provides the use of an anti-C5 antibody in the manufacture of a medicament or medicament package for the treatment of muscular dystrophy associated with dysferlin-deficiency in a mammal.

In certain embodiments, the disclosure provides the use of a CD55 agonist in the manufacture of a medicament or medicament package for the treatment of muscular dystrophy associated with dysferlin-deficiency in a mammal.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. TaqMan RT-PCR amplification plots of DAF/CD55. *A* and *C*, DAF1 (*A*) and DAF2 (*C*) expression in skeletal muscle (M. quadriceps) of a 30-wk-old SJL/J mouse compared with a C57BL/6 control mouse of the same age (Ctrl). *E*, DAF/CD55 expression in skeletal muscle of a patient with LGMD2B (patient 4, Table III) compared with a healthy control. Housekeeping genes porphobilinogen deaminase *(B* and *D)* and β_2-microglobulin (*F*) were used as internal standards.

Samsung Bioepis Exhibit No. 1002
Page 1647

FIGURE 2. DAF/CD55 protein expression in dysferlin deficiency. Immunofluorescence staining using anti-DAF/CD55 Abs. *A–D*, Murine skeletal muscle. *A–D* have the same scale as indicated in *B*. *E* and *F*, Murine cardiac muscle. *E* and *F* have the same scale as indicated in *F*. *G* and *H*, Human skeletal muscle. *G* and *H* have the same scale as indicated in *H*. *A*, SJL/J, wk 28; *B*, A/J wk 16; *C*, *Dysf*$^{-/-}$, wk 16; *D*, C57BL/6; *E*, SJL/J; *F*, C57BL/6; *G*, LGMD2B patient 2 (Table III); *H*, control skeletal muscle without detectable neuromuscular disorder.

FIGURE 3. Complement lysis assay and binding of C5b9-MAC to nonnecrotic muscle cells. *A*, Quantification of PI uptake of myotubes after exposure to complement (ratio of PI-positive cells after exposure to complement to Veronal buffer control). n, number of wells counted. Normal human (*B* and *C*) and dysferlin-deficient (*D* and *E*) human myoblasts after exposure to complement (*B* and *D*) and after preincubation with anti-CD55 Ab and subsequent exposure to complement (*C* and *E*). *F* and *G*, Serial sections of quadriceps muscle in LGMD2B (patient 1), demonstrating dystrophic changes with increase in connective tissue and pathological variation in fiber size (Gomori-TriChrome stain). There was sarcolemmal expression of C5b9-MAC on nonnecrotic muscle fibers. Staining was performed with anti-C5b9 mAb and Cy3-labeled donkey anti-mouse Ab.

FIGURE 4. Expression of regulatory factors in skeletal muscle of dysferlin-deficient patients and SJL/J mice (aged 20–30 wk). *A*, Unpooled TaqMan analysis of myostatin, SMAD3, SMAD4, CARP, and EGR1 (only human). The *y*-axis demonstrates the fold change compared with healthy individuals and C57BL/6 mice, respectively. *B* and *C*, Double-immunofluorescent staining of SMAD2 protein (FITC) and nuclear membrane with anti-lamin A/C mAb (Cy3) on dysferlin-deficient (patient 4, Table III; *B*) and normal (*C*) human skeletal muscle.

DETAILED DESCRIPTION OF THE INVENTION

Overview

The present invention relates, in part, to the discovery that CD55 is down-regulated in the skeletal muscle of dysferlin-deficient mice or human patients

Samsung Bioepis Exhibit No. 1002
Page 1648

suffering from LGMD2B. Accordingly, methods and compositions are provided for treating muscular dystrophy, particularly muscular dystrophy associated with dysferlin deficiency. The term "treating" includes prophylactic and/or therapeutic treatments. The term "prophylactic or therapeutic" treatment is art-recognized and includes administration to the host of one or more of the subject therapeutic agents or pharmaceutical compositions. If it is administered prior to clinical manifestation of the unwanted condition (e.g., disease or other unwanted state of the host animal) then the treatment is prophylactic, (i.e., it protects the host against developing the unwanted condition), whereas if it is administered after manifestation of the unwanted condition, the treatment is therapeutic, (i.e., it is intended to diminish, ameliorate, or stabilize the existing unwanted condition or side effects thereof).

In certain embodiments, the methods and compositions of the disclosure employ a therapeutic agent that can inhibit complement activity, such as for example, by preventing the formation of MAC; in specific embodiments, such a therapeutic agent may comprise an antibody that binds to C5 and inhibits C5 activity (for example, by preventing the cleavage of C5 into C5a and C5b).

In certain embodiments, the methods and compositions of the disclosure employ a therapeutic agent that is a CD55 agonist and capable of directly or indirectly increasing or potentiating an activity and/or expression of CD55.

In previous investigations, activation of the complement cascade has been identified on the surface of nonnecrotic muscle fibers in some patients with LGMD (1) and, in particular, in dysferlinopathies (2). Deposition of MAC on nonnecrotic muscle fibers in muscular dystrophies was surprising, in particular because this mechanism does not play a role in inflammatory muscle diseases (1). The complement system consists of >30 plasma and cell surface proteins. It is activated by three different pathways, named classical, alternative, and lectin pathway, respectively (3,4). All pathways require the proteolytic cleavage of C3, followed by the last phase of the complement cascade that leads to the formation of the C5b9-MAC. To provide an immediate defense against infection, there is a constant low level of C3 activation in the alternative pathway, a background "tick-over" (5). To prevent uncontrolled rapid amplification of the complement cascade and complement-mediated damage of self, numerous soluble and membrane-bound

Samsung Bioepis Exhibit No. 1002
Page 1649

complement inhibitory and regulatory proteins have evolved. Among the membrane-bound inhibitors are decay-accelerating factor (DAF/CD55), membrane cofactor protein (CD46), and CD59 (6).

The Complement System

The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of cellular and viral pathogens. There are at least 25 complement proteins, which are found as a complex collection of plasma proteins and membrane cofactors. The plasma proteins (which are also found in most other body fluids, such as lymph, bone marrow, synovial fluid, and cerebrospinal fluid) make up about 10% of the globulins in vertebrate serum. Complement components achieve their immune defensive functions by interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions.

The complement cascade progresses via the classical pathway or the alternative pathway. These pathways share many components, and, while they differ in their early steps, both converge and share the same terminal complement components responsible for the destruction of target cells and viruses.

The classical complement pathway is typically initiated by antibody recognition of and binding to an antigenic site on a target cell. This surface bound antibody subsequently reacts with the first component of complement, C1. The C1 thus bound undergoes a set of autocatalytic reactions that result in, inter alia, the induction of C1 proteolytic activity acting on complement components C2 and C4.

This activated C1 cleaves C2 and C4 into C2a, C2b, C4a, and C4b. The function of C2b is poorly understood. C2a and C4b combine to form the C4b,2a complex, which is an active protease known as classical C3 convertase. C4b,2a acts to cleave C3 into C3a and C3b. C3a and C4a are both relatively weak anaphylatoxins that may induce degranulation of mast cells, resulting in the release of histamine and other mediators of inflammation.

C3b has multiple functions. As opsonin, it binds to bacteria, viruses and other cells and particles and tags them for removal from the circulation. C3b can

Samsung Bioepis Exhibit No. 1002
Page 1650

also form a complex with C4b,C2a to produce C4b,2a,3b, or classical C5 convertase, which cleaves C5 into C5a (another anaphylatoxin) and C5b. Alternative C5 convertase is C3b, Bb, C3b and performs the same function. C5b combines with C6 yielding C5b,6, and this complex combines with C7 to form the ternary complex C5b,6,7. The C5b,6,7 complex binds C8 at the surface of a cell membrane. Upon binding of C9, the complete membrane attack complex (MAC) is formed (C5b-9) which mediates the lysis of foreign cells, microorganisms, and viruses.

Decay accelerating factor (DAF) (CD55) (NM_000574) can bind C4b and C3b dissociating the C3 and C5 convertases in both the classical and alternative pathways (Markrides, Pharmacol Rev. 1998 Mar;50(1):59-87). CD55 is also a known agonist of CD97 (Visser et al., Pharmacol Rev. 1998 Mar;50(1):59-87; and Hamann et al., J Exp Med. 1996 Sep 1;184(3):1185-9). Soluble versions of DAF (sDAF) have been shown to inhibit complement activation (Christiansen et al., Eur J Immunol. 1996 Mar;26(3):578-85; and Moran et al., J Immunol. 1992 Sep 1;149(5):1736-43).

Further discussions of the classical complement pathway, as well as a detailed description of the alternative pathway of complement activation, can be found in numerous publications including, for example, Muller-Eberhard, Annu Rev Biochem. 1988;57:321-47.

Muscular dystrophy

Muscular dystrophy represents a family of inherited diseases of the muscles. The following are the most common symptoms of muscular dystrophy. Symptoms may include: clumsy movement, difficulty climbing stairs, frequently trips and falls, unable to jump or hop normally, tip toe walking, leg pain, facial weakness, inability to close eyes or whistle, and shoulder and arm weakness.

Some forms affect children (e.g., Duchenne dystrophy) and are lethal within two to three decades. Other forms present in adult life and are more slowly progressive. The genes for several dystrophies have been identified, including Duchenne dystrophy (caused by mutations in the dystrophin gene) and the teenage and adult onset Miyoshi dystrophy or its variant, limb girdle dystrophy 2B or LGMD-2B (caused by mutations in the dysferlin gene). These are "loss of function"

Samsung Bioepis Exhibit No. 1002
Page 1651

mutations that prevent expression of the relevant protein in muscle and thereby cause muscle dysfunction.

Dysferlin is a 230-kDa membrane-spanning protein consisting of a single C-terminal transmembrane domain and six C2 domains (7). In normal muscle, sarcolemma injuries lead to accumulation of dysferlin-enriched membrane patches and resealing of the membrane in the presence of Ca^{2+}. Dysferlin deficiency results in defective membrane repair mechanisms (8, 9). An impaired interaction between dysferlin and annexins A1 and A2 has been discussed as a possible mechanism (9). Although dysferlin is expressed in human skeletal and cardiac muscles (7), mutations in the encoding gene (*DYSF*) lead only to skeletal muscle phenotypes without myocardial involvement, namely limb girdle muscular dystrophy 2B (LGMD2B) and Miyoshi myopathy (10).

Several mouse models exist with mutations in *Dysf* (11). The SJL/J mouse harbors a splice site mutation that results in a deletion corresponding to human exon 45 (12). SJL/J mice have long served as a model for autoimmune diseases, such as experimental allergic encephalomyelitis and myositis. The development of lymphomas is typically observed in older age. Therefore, it has been discussed whether other genetic disorders, apart from dysferlin deficiency, might play a role in SJL/J, and more defined models were engineered (13, 14). A mouse with a 12-kb deletion at the 3' end leading to complete loss of dysferlin was designed. In this study the observation of a defective membrane repair mechanism in dysferlin deficiency has been made (8). The A/J mouse has a unique ETn retrotransposon insertion within intron 4 (15). For another targeted disruption of dysferlin, the highly conserved C2E domain was replaced by a neomycin gene, resulting in a $Dysf^{-/-}$ mouse (15). All these mice develop progressive muscular dystrophy after 2 mo of age. Interestingly, all mice also display different degrees of inflammatory changes in skeletal muscle.

Current Therapies

To date, there is no known treatment, medicine, or surgery that will cure muscular dystrophy, or stop the muscles from weakening. The goal of treatment is to prevent deformity and allow the child to function as independently as possible.

Samsung Bioepis Exhibit No. 1002
Page 1652

Since muscular dystrophy is a life-long condition that is not correctable, management includes focusing on preventing or minimizing deformities and maximizing the child's functional ability at home and in the community.

Clinically, many patients with a muscular dystrophy show improvement with prednisone treatment, although they will not be cured (Fenichel et al., Arch Neurol. 1991 Jun;48(6):575-9; Griggs et al., Arch Neurol. 1991 Apr;48(4):383-8). Dysferlin-deficiency was recently recognized as a cause of late-onset dystrophy with substantial inflammation in muscle. Corticosteroid usage by these patients may result in nonrecoverable loss of strength. There has thus been a long felt need for new approaches and better methods to control muscular dystrophy associated with dysferlin-deficiency.

Khurana and Davies reviewed the various pharmaceutical strategies for muscular dystrophy to date. Nature Reviews 2:379-390 (May 2003). Current therapeutic approaches generally utilize drugs or molecules in an attempt to improve the phenotype by, for example, decreasing inflammation, improving calcium homeostasis, upregulating compensatory protein such as utrophin, increasing muscle progenitor proliferation or commitment, and increasing muscle strength. Specific agents that have been used in patients or in clinical or preclinical studies include corticosteroids, calcium ionophores and/or blockers of the sarcoplasmic reticulum calcium reuptake pump, mast-cell stabilizers such as cromoglycate, clenbutarol (a non-steroid b2 adrenoreceptor agonist), creatine or creatine monohydrate, and gentamicin.

Methods and Compositions

As discussed above, the present invention relates to a method for treating muscular dystrophy associated with dysferlin-deficiency by the administration of an agent capable of inhibiting complement (for example, by inhibiting the formation of MAC) to a patient in need of such treatment. In particular embodiments, the agent inhibits the formation of the MAC by inhibiting the cleavage of C5 into C5a and C5b or the formation of C3 and/or C5 convertases.

In certain embodiments, a complement inhibitor may be a small molecule (up to 6,000 Da in molecular weight), a nucleic acid or nucleic acid analog, a

Samsung Bioepis Exhibit No. 1002
Page 1653

peptidomimetic, or a macromolecule that is not a nucleic acid or a protein. These agents include, but are not limited to, small organic molecules, RNA aptamers, L-RNA aptamers, Spiegelmers, antisense compounds, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

In certain embodiments, complement inhibitor may be an antibody capable of inhibiting complement, such as an antibody that can block the formation of MAC. For example, an antibody complement inhibitor may include an anti-C5 antibody. Such anti-C5 antibodies may directly interact with C5, C5a, and/or C5b, so as to inhibit the formation of and/or physiologic function of C5a and/or C5b.

The concentration and/or physiologic activity of C5a and C5b in a body fluid can be measured by methods well known in the art. For C5a such methods include chemotaxis assays, RIAs, or ELISAs (see, for example, Ward and Zvaifler, J Clin Invest. 1971 Mar;50(3):606-16.; Wurzner, et al., Complement Inflamm. 8:328-340, 1991). For C5b, hemolytic assays or assays for soluble C5b-9 as discussed herein can be used. Other assays known in the art can also be used. Using assays of these or other suitable types, candidate antibodies capable of inhibiting complement such as anti-C5 antibodies, now known or subsequently identified, can be screened in order to 1) identify compounds that are useful in the practice of the invention and 2) determine the appropriate dosage levels of such compounds.

An antibody capable of inhibiting complement such as an anti-C5 antibody affecting C5a is preferably used at concentrations providing substantial reduction (i.e., reduction by at least about 25%) in the C5a levels present in at least one blood-derived fluid of the patient, e.g., blood, plasma, or serum, following activation of complement within the fluid.

Similarly, an antibody capable of inhibiting complement such as an anti-C5 antibody affecting C5b is preferably used at concentrations providing substantial reduction (i.e., reduction by at least about 25% as compared to that in the absence of the anti-C5 antibody) in the C5b levels present in at least one blood-derived fluid of the patient following activation of complement within the fluid. In the case of C5b, such concentrations can be conveniently determined by measuring the cell-lysing ability (e.g., hemolytic activity) of complement present in the fluid or the levels of soluble C5b-9 present in the fluid. Accordingly, a specific concentration for an

Samsung Bioepis Exhibit No. 1002
Page 1654

antibody that affects C5b is one that results in a substantial reduction (i.e., a reduction by at least about 25%) in the cell-lysing ability of the complement present in at least one of the patient's blood-derived fluids. Reductions of the cell-lysing ability of complement present in the patient's body fluids can be measured by methods well known in the art such as, for example, by a conventional hemolytic assay such as the hemolysis assay described by Kabat and Mayer (eds), "Experimental Immunochemistry, 2d Edition", 135-240, Springfield, IL, CC Thomas (1961), pages 135-139, or a conventional variation of that assay such as the chicken erythrocyte hemolysis method described below.

Specific antibodies capable of inhibiting complement such as an anti-C5 antibody are relatively specific, and do not block the functions of early complement components. In particular, such specific agents will not substantially impair the opsonization functions associated with complement component C3b, which functions provide a means for clearance of foreign particles and substances from the body.

C3b is generated by the cleavage of C3, which is carried out by classical and/or alternative C3 convertases, and results in the generation of both C3a and C3b. Therefore, in order not to impair the opsonization functions associated with C3b, specific antibodies capable of inhibiting complement such as an anti-C5 antibody do not substantially interfere with the cleavage of complement component C3 in a body fluid of the patient (e.g., serum) into C3a and C3b. Such interference with the cleavage of C3 can be detected by measuring body fluid levels of C3a and/or C3b, which are produced in equimolar ratios by the actions of the C3 convertases. Such measurements are informative because C3a and C3b levels will be reduced (compared to a matched sample without the antibody capable of inhibiting complement such as an anti-C5 antibody) if cleavage is interfered with by an antibody capable of inhibiting complement such as an anti-C5 antibody.

In practice, the quantitative measurement of such cleavage is generally more accurate when carried out by the measurement of body fluid C3a levels rather than of body fluid C3b levels, since C3a remains in the fluid phase whereas C3b is rapidly cleared. C3a levels in a body fluid can be measured by methods well known in the art such as, for example, by using a commercially available C3a EIA kit, e.g.,

Samsung Bioepis Exhibit No. 1002
Page 1655

that sold by Quidel Corporation, San Diego, Calif., according to the manufacturer's specifications. Particularly specific antibodies capable of inhibiting complement such as an anti-C5 antibody produce essentially no reduction in body fluid C3a levels following complement activation when tested in such assays.

Certain antibodies of the disclosure will prevent the cleavage of C5 to form C5a and C5b, thus preventing the generation of the anaphylatoxic activity associated with C5a and preventing the assembly of the membrane attack complex associated with C5b. As discussed above, in a particular embodiment, these anti-C5 antibodies will not impair the opsonization function associated with the action of C3b.

A specific method of inhibiting complement activity is to use a monoclonal antibody which binds to complement C5 and prevents C5 from being cleaved. This prevents the formation of both C5a and C5b-9 while at the same time allowing the formation of C3a and C3b which are beneficial to the recipient. Such antibodies that are specific to human complement are known (U.S. Patent 6,355,245). These antibodies disclosed in U.S. Patent 6,355,245 include both a whole or full-length antibody (now named eculizumab) and a single-chain antibody (now named pexelizumab). A similar antibody against mouse C5 is called BB5.1 (Frei Y, et al. (1987). *Mol. Cell. Probes* 1:141-149). BB5.1 was utilized in the experiments set forth below. Antibodies to inhibit complement activity need not be monoclonal antibodies. They can be, e.g., polyclonal antibodies. They may additionally be antibody fragments. An antibody fragment includes, but is not limited to, an Fab, F(ab'), $F(ab')_2$, a single-chain antibody, a domain antibody, and an Fv. Furthermore, it is well known by those of skill in the art that antibodies can be humanized (Jones PT, et al. (1986). *Nature* 321:522-525), chimerized, or deimmunized. An antibody may also comprise a mutated Fc portion, such that the mutant Fc does not activate complement. The antibodies to be used in the present invention may be any of these. Antibody analogs or mimics can also be used in the present invention, such as those described in U.S. Patent Application Publication No. 20050255548.

In certain embodiments, the disclosure provides a method of treating muscular dystrophy associated with dysferlin-deficiency in a mammal comprising administering to said mammal a therapeutically effective amount of a CD55 agonist.

Samsung Bioepis Exhibit No. 1002
Page 1656

In certain embodiments, the mammal is a human. In certain embodiments, the CD55 agonist increases the expression and/or activity of CD55. In certain embodiments, the CD55 agonist comprises an agonistic ligand of CD97. In certain embodiments, the CD55 agonist comprises an agent that inhibits the association of C4b and C3b. In particular embodiments, the CD55 agonist blocks the formation of C3 or C5 convertases. CD55 activity can be measured by conventional methods, such as for example by measuring decay dissociation of the C4b2a enzyme, as described in Medof et al., J Exp Med. 160(5):1558-78 (1984).

Therapeutic Agents: Antibodies and Other Agents

The disclosure provides various therapeutic agents. A therapeutic agent of the disclosure can be administered to a patient in need thereof as a single therapy. Alternatively, a therapeutic agent of the disclosure can be administered to a patient in need thereof in the form of combination therapy or adjuvant therapy. Such combination or adjuvant therapy further includes one or more other agents, such as other therapeutic agents (e.g., drugs or biologics) or nutritional agents (e.g., nutraceuticals or dietary supplements). As described herein, such combination therapy may include either simultaneous or sequential dosing or administration of the various agents as desired.

In certain embodiments, a therapeutic agent comprises an antibody or antibody fragment to C5 (or an anti-C5 antibody or fragment thereof). In certain embodiments, an anti-C5 antibody or fragment thereof binds C5 and inhibits C5 activity. For example, an anti-C5 antibody or its fragment of the disclosure binds C5 and blocks the cleavage of C5 to form C5a and C5b. In certain embodiments, an anti-C5 antibody or fragment thereof binds C5 and results in a more rapid clearance of C5 from the plasma than will occur in the absence of the anti-C5 antibody or fragment thereof.

In certain embodiments, the CD55 agonist includes a polypeptide, a polypeptide analog, a nucleic acid, a nucleic acid analog, or a small molecule, or any combination thereof. In certain embodiments, the polypeptide comprises an amino acid sequence of greater than 90% sequence identity to the amino acid sequence of a soluble portion of a naturally occurring CD55 protein. In certain embodiments, the

Samsung Bioepis Exhibit No. 1002
Page 1657

nucleic acid comprises a polynucleotide sequence of greater than 90% sequence identity to the nucleotide sequence of a naturally occurring CD55 mRNA. "Percent (%) nucleic acid or amino acid sequence identity" is defined as the percentage of nucleotides or amino acids in a candidate sequence that are identical with the nucleotides or amino acids in a reference sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. Appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared can be determined by known methods.

A CD55 peptide agonist may alternatively include an anti-CD55 agonist antibody, such as an antibody that binds CD55 and increases an activity and/or expression level (for example by increasing stability or half-life of CD55) of CD55. A CD55 peptide agonist may alternatively include an anti-CD97 agonist antibody. A CD55 peptide agonist may also include an antagonistic antibody that is specific to C4b or C3b and capable of blocking the formation of the C3 and/or C5 convertase.

"Small molecule" as used herein, is meant to refer to an agent, which has a molecular weight of less than about 6 kD and most preferably less than about 2.5 kD. Many pharmaceutical companies have extensive libraries of chemical and/or biological mixtures comprising arrays of small molecules, often fungal, bacterial, or algal extracts, which can be screened with any of the assays of the application. This application contemplates using, among other things, small chemical libraries, peptide libraries, or collections of natural products. Tan et al. described a library with over two million synthetic compounds that is compatible with miniaturized cell-based assays (J. Am. Chem. Soc. 120, 8565-8566, 1998). It is within the scope of this application that such a library may be used to screen for agents of the invention. There are numerous commercially available compound libraries, such as the Chembridge DIVERSet. Libraries are also available from academic investigators, such as the Diversity set from the NCI developmental therapeutics

Samsung Bioepis Exhibit No. 1002
Page 1658

program. Rational drug design may also be employed. For example, the interaction interface of CD55 may be targeted when designing a compound. Rational drug design can also be achieved based on known compounds, e.g., a known agonist of CD55.

Peptidomimetics can be compounds in which at least a portion of a subject polypeptide of the disclosure (such as for example, a polypeptide comprising an amino acid sequence of greater than 90% sequence identity to the amino acid sequence of a soluble portion of a naturally occurring CD55 protein) is modified, and the three dimensional structure of the peptidomimetic remains substantially the same as that of the subject polypeptide. Peptidomimetics may be analogues of a subject polypeptide of the disclosure that are, themselves, polypeptides containing one or more substitutions or other modifications within the subject polypeptide sequence. Alternatively, at least a portion of the subject polypeptide sequence may be replaced with a nonpeptide structure, such that the three-dimensional structure of the subject polypeptide is substantially retained. In other words, one, two or three amino acid residues within the subject polypeptide sequence may be replaced by a non-peptide structure. In addition, other peptide portions of the subject polypeptide may, but need not, be replaced with a non-peptide structure. Peptidomimetics (both peptide and non-peptidyl analogues) may have improved properties (e.g., decreased proteolysis, increased retention or increased bioavailability). Peptidomimetics generally have improved oral availability, which makes them especially suited to treatment of disorders in a human or animal. It should be noted that peptidomimetics may or may not have similar two-dimensional chemical structures, but share common three-dimensional structural features and geometry. Each peptidomimetic may further have one or more unique additional binding elements.

Nucleic acid analogs may include modified subject nucleic acid of the disclosure (such as for example, a nucleic acid comprising a polynucleotide sequence of greater than 90% sequence identity to the polynucleotide sequence of a naturally occurring CD55 gene). Various well-known modifications to nucleic acid molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3'

Samsung Bioepis Exhibit No. 1002
Page 1659

ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

In specific embodiments, a therapeutic agent of the disclosure comprises an antibody or antibody fragment. Antibodies and fragments thereof may be made by any conventional method, such as those methods described herein.

Antibodies are found in multiple forms, e.g., IgA, IgG, IgM, etc. Additionally, antibodies can be engineered in numerous ways. They can be made as single-chain antibodies (including small modular immunopharmaceuticals or SMIPs™), Fab and $F(ab')_2$ fragments, etc. Antibodies can be humanized, chimerized, deimmunized, or fully human. Numerous publications set forth the many types of antibodies and the methods of engineering such antibodies. For example, see U.S. Patent Nos. 6,355,245; 6,180,370; 5,693,762; 6,407,213; 6,548,640; 5,565,332; 5,225,539; 6,103,889; and 5,260,203.

This invention provides fragments of anti-C5 antibodies or CD55 agonist antibodies, which may comprise a portion of an intact antibody, preferably the antigen-binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies (Zapata et al., *Protein Eng.*1995; 8(10): 1057-1062); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment of an antibody yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" usually refers to the minimum antibody fragment that contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an

Samsung Bioepis Exhibit No. 1002
Page 1660

antigen) has the ability to recognize and bind antigen, although likely at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments that have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

This disclosure also provides monoclonal anti-C5 antibodies or CD55 agonist antibodies. A monoclonal antibody can be obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are often synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. Monoclonal antibodies may also be produced in transfected cells, such as CHO cells and NS0 cells. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies and does not require production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present disclosure may be made by the hybridoma method first described by Kohler et al., *Nature* 1975; 256:495, or may be made by recombinant DNA methods (*see, e.g.*, U.S. Patent Nos. 4,816,567 and 6,331,415). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature* 1991; 352:624-628 and Marks et al., *J. Mol. Biol.*1991; 222:581-597, for example.

Samsung Bioepis Exhibit No. 1002
Page 1661

General methods for the immunization of animals (in this case with C5, C5a, and/or C5b), isolation of antibody producing cells, fusion of such cells with immortal cells (e.g., myeloma cells) to generate hybridomas secreting monoclonal antibodies, screening of hybridoma supernatants for reactivity of secreted monoclonal antibodies with a desired antigen (in this case the immunogen or a molecule containing the immunogen), the preparation of quantities of such antibodies in hybridoma supernatants or ascites fluids, and for the purification and storage of such monoclonal antibodies, can be found in numerous publications. These include: Coligan, et al., eds. Current Protocols In Immunology, John Wiley & Sons, New York, 1992; Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988; Liddell and Cryer, A Practical Guide To Monoclonal Antibodies, John Wiley & Sons, Chichester, West Sussex, England, 1991; Montz, et al., Cellular Immunol. 127:337-351, 1990; Wurzner, et al., Complement Inflamm. 8:328-340, 1991; and Mollnes, et al., Scand. J. Immunol. 28:307-312, 1988.

A description of the preparation of a mouse anti-human-C5 monoclonal antibody with specific binding characteristics is presented in U.S. Patent Application Publication No. 20050226870. Wurzner, et al., Complement Inflamm. 8:328-340, 1991, describe the preparation of other suitable mouse anti-human-C5 monoclonal antibodies referred to as N19-8 and N20-9.

Other antibodies specifically contemplated are "oligoclonal" antibodies. As used herein, the term "oligoclonal" antibodies" refers to a predetermined mixture of distinct monoclonal antibodies. *See, e.g.*, PCT publication WO 95/20401; U.S. Patent Nos. 5,789,208 and 6,335,163. In one embodiment, oligoclonal antibodies consisting of a predetermined mixture of antibodies against one or more epitopes are generated in a single cell. In other embodiments, oligoclonal antibodies comprise a plurality of heavy chains capable of pairing with a common light chain to generate antibodies with multiple specificities (*e.g.,* PCT publication WO 04/009618). Oligoclonal antibodies are particularly useful when it is desired to target multiple epitopes on a single target molecule (*e.g.,* C5 or CD55). In view of the assays and epitopes disclosed herein, those skilled in the art can generate or select antibodies or mixture of antibodies that are applicable for an intended purpose and desired need.

Samsung Bioepis Exhibit No. 1002
Page 1662

In certain embodiments that include a humanized and/or chimeric antibody, one or more of the CDRs are derived from an anti-human C5 antibody or CD55 agonist antibody. In a specific embodiment, all of the CDRs are derived from an anti-human C5 antibody or CD55 agonistic antibody. In another specific embodiment, the CDRs from more than one anti-human C5 antibody or CD55 agonist antibody are mixed and matched in a chimeric antibody. For instance, a chimeric antibody may comprise a CDR1 from the light chain of a first anti-human C5 antibody or CD55 agonist antibody combined with CDR2 and CDR3 from the light chain of a second anti-human C5 antibody or CD55 agonist antibody, and the CDRs from the heavy chain may be derived from a third anti-human C5 antibody or CD55 agonist antibody. Further, the framework regions may be derived from one of the same anti-human C5 antibodies or CD55 agonist antibodies, from one or more different antibodies, such as a human antibody, or from a humanized antibody. Human or humanized antibodies are specific for administration to human patients.

"Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains that enables the scFv to form the desired structure for antigen binding. For a review of scFv see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore, eds. (Springer-Verlag: New York, 1994), pp. 269-315.

SMIPs are a class of single-chain peptide engineered to include a target binding region, effector domain (CH2 and CH3 domains). *See, e.g.*, U.S. Patent Application Publication No. 20050238646. The target binding region may be derived from the variable region or CDRs of an antibody, e.g., an anti-C5 antibody or CD55 agonist antibody of the invention. Alternatively, the target binding region is derived from a protein that binds C5 or CD55.

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on

Samsung Bioepis Exhibit No. 1002
Page 1663

the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., Proc. Natl. Acad. Sci. USA, 90: 6444-6448 (1993).

An "isolated" antibody is one that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In specific embodiments, the antibody will be purified to greater than 95% by weight of antibody as determined by the Lowry method, or greater than 99% by weight, to a degree that complies with applicable regulatory requirements for administration to human patients (e.g., substantially pyrogen-free), to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells, since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step, for example, an affinity chromatography step, an ion (anion or cation) exchange chromatography step, or a hydrophobic interaction chromatography step.

It is well known that the binding to a molecule (or a pathogen) of antibodies with an Fc region assists in the processing and clearance of the molecule (or pathogen). The Fc portions of antibodies are recognized by specialized receptors expressed by immune effector cells. The Fc portions of IgG1 and IgG3 antibodies are recognized by Fc receptors present on the surface of phagocytic cells such as macrophages and neutrophils, which can thereby bind and engulf the molecules or pathogens coated with antibodies of these isotypes (C. A. Janeway et al., Immunobiology 5th edition, page 147, Garland Publishing (New York, 2001)).

In certain embodiments, single chain antibodies, and chimeric, humanized or primatized (CDR-grafted) antibodies, as well as chimeric or CDR-grafted single

Samsung Bioepis Exhibit No. 1002
Page 1664

chain antibodies, comprising portions derived from different species, are also encompassed by the present disclosure as antigen-binding fragments of an antibody. The various portions of these antibodies can be joined together chemically by conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques. For example, nucleic acids encoding a chimeric or humanized chain can be expressed to produce a contiguous protein. See, e.g., U.S. Pat. Nos. 4,816,567 and 6,331,415; U.S. Pat. No. 4,816,397; European Patent No. 0,120,694; WO 86/01533; European Patent No. 0,194,276 B1; U.S. Pat. No. 5,225,539; and European Patent No. 0,239,400 B1. See also, Newman, R. et al., BioTechnology, 10: 1455-1460 (1992), regarding primatized antibody. See, e.g., Ladner et al., U.S. Pat. No. 4,946,778; and Bird, R. E. et al., Science, 242: 423-426 (1988)), regarding single chain antibodies.

In addition, functional fragments of antibodies, including fragments of chimeric, humanized, primatized or single chain antibodies, can also be produced. Functional fragments of the subject antibodies retain at least one binding function and/or modulation function of the full-length antibody from which they are derived. Preferred functional fragments retain an antigen-binding function of a corresponding full-length antibody (such as for example, ability of anti-C5 antibody to bind C5 or an anti-CD55 antibody to bind CD55).

Pharmaceutical Formulations and Uses

Methods of administration of therapeutic agents, particularly antibody therapeutics, are well-known to those of skill in the art. The pharmaceutical formulations, dosage forms, and uses described below generally apply to antibody-based therapeutic agents, but are also useful and can be modified, where necessary, for making and using therapeutic agents of the disclosure that are not antibodies.

To achieve the desired therapeutic effect, the anti-C5 antibodies or CD55 agonist antibodies (or fragments thereof) can be administered in a variety of unit dosage forms. The dose will vary according to the particular antibody. For example, different antibodies may have different masses and/or affinities, and thus require different dosage levels. Antibodies prepared as Fab fragments will also require differing dosages than the equivalent intact immunoglobulins, as they are of

Samsung Bioepis Exhibit No. 1002
Page 1665

considerably smaller mass than intact immunoglobulins, and thus require lower dosages to reach the same molar levels in the patient's blood. The dose will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician. Dosage levels of the antibodies for human subjects are generally between about 1 mg per kg and about 100 mg per kg per patient per treatment, and preferably between about 5 mg per kg and about 50 mg per kg per patient per treatment. In terms of plasma concentrations, the antibody concentrations are preferably in the range from about 25 μg/mL to about 500 μg/mL. However, greater amounts may be required for extreme cases and smaller amounts may be sufficient for milder cases.

Administration of the anti-C5 antibodies will generally be performed by an intravascular route, e.g., via intravenous infusion by injection. Other routes of administration may be used if desired but an intravenous route will be the most preferable. Formulations suitable for injection are found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, Pa., 17th ed. (1985). Such formulations must be sterile and non-pyrogenic, and generally will include a pharmaceutically effective carrier, such as saline, buffered (e.g., phosphate buffered) saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions, and the like. The formulations may contain pharmaceutically acceptable auxiliary substances as required, such as, tonicity adjusting agents, wetting agents, bactericidal agents, preservatives, stabilizers, and the like.

Administration of the antibodies capable of inhibiting complement such as an anti-C5 antibody will generally be performed by a parenteral route, typically via injection such as intra-articular or intravascular injection (e.g., intravenous infusion) or intramuscular injection. Other routes of administration, e.g., oral (p.o.), may be used if desired and practicable for the particular antibody capable of inhibiting complement to be administered. Antibodies capable of inhibiting complement such as an anti-C5 antibody can also be administered in a variety of unit dosage forms and their dosages will also vary with the size, potency, and in vivo half-life of the particular antibody capable of inhibiting complement being administered. Doses of antibodies capable of inhibiting complement such as an anti-C5 antibody will also

Samsung Bioepis Exhibit No. 1002
Page 1666

vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician.

In certain embodiments, a typical therapeutic treatment includes a series of doses, which will usually be administered concurrently with the monitoring of clinical endpoints such as deposition of membrane attack complex (MAC) on nonnecrotic muscle fibers, age at reaching Hammersmith score of 30/40, age at becoming wheelchair bound, muscle pain or spasms, etc., with the dosage levels adjusted as needed to achieve the desired clinical outcome. In certain embodiments, treatment is administered in multiple dosages over at least a week. In certain embodiments, treatment is administered in multiple dosages over at least a month. In certain embodiments, treatment is administered in multiple dosages over at least a year. In certain embodiments, treatment is administered in multiple dosages over the remainder of the patient's life.

The frequency of administration may also be adjusted according to various parameters. These include the clinical response, the plasma half-life of the therapeutic of the disclosure, and the levels of the antibody in a body fluid, such as, blood, plasma, serum, or synovial fluid. To guide adjustment of the frequency of administration, levels of the therapeutic of the disclosure in the body fluid may be monitored during the course of treatment.

Alternatively, for therapeutic of the disclosure that affect C5b, levels of the cell-lysing ability of complement present in one or more of the patient's body fluids are monitored to determine if additional doses or higher or lower dosage levels are needed. Such doses are administered as required to maintain at least about a 25% reduction, and preferably about a 50% or greater reduction of the cell-lysing ability of complement present in blood, plasma, or serum. The cell-lysing ability can be measured as percent hemolysis in hemolytic assays of the types described herein. A 10% or 25% or 50% reduction in the cell-lysing ability of complement present in a body fluid after treatment with the antibody capable of inhibiting complement used in the practice of the invention means that the percent hemolysis after treatment is 90, 75, or 50 percent, respectively, of the percent hemolysis before treatment.

Samsung Bioepis Exhibit No. 1002
Page 1667

In yet another alternative, dosage parameters are adjusted as needed to achieve a substantial reduction of C5a levels in blood, plasma, or serum. As discussed above, C5a levels can be measured using the techniques described in Wurzner, et al., Complement Inflamm 8:328-340, 1991. Other protocols of administration can, of course, be used if desired as determined by the physician.

For the treatment of muscular dystrophy associated with dysferlin-deficiency by systemic administration of an antibody capable of inhibiting complement such as an anti-C5 antibody (as opposed to local administration) administration of a large initial dose is specific, i.e., a single initial dose sufficient to yield a substantial reduction, and more preferably an at least about 50% reduction, in the hemolytic activity of the patient's serum. Such a large initial dose is preferably followed by regularly repeated administration of tapered doses as needed to maintain substantial reductions of serum hemolytic titer. In another embodiment, the initial dose is given by both local and systemic routes, followed by repeated systemic administration of tapered doses as described above.

Formulations particularly useful for antibody-based therapeutic agents are also described in U.S. Patent App. Publication Nos. 20030202972, 20040091490 and 20050158316. In certain embodiments, the liquid formulations of the invention are substantially free of surfactant and/or inorganic salts. In another specific embodiment, the liquid formulations have a pH ranging from about 5.0 to about 7.0. In yet another specific embodiment, the liquid formulations comprise histidine at a concentration ranging from about 1 mM to about 100 mM. In still another specific embodiment, the liquid formulations comprise histidine at a concentration ranging from 1 mM to 100 mM. It is also contemplated that the liquid formulations may further comprise one or more excipients such as a saccharide, an amino acid (e.g., arginine, lysine, and methionine) and a polyol. Additional descriptions and methods of preparing and analyzing liquid formulations can be found, for example, in PCT publications WO 03/106644, WO 04/066957, and WO 04/091658.

Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the pharmaceutical compositions of the invention.

Samsung Bioepis Exhibit No. 1002
Page 1668

In certain embodiments, formulations of the subject antibodies are pyrogen-free formulations which are substantially free of endotoxins and/or related pyrogenic substances. Endotoxins include toxins that are confined inside microorganisms and are released when the microorganisms are broken down or die. Pyrogenic substances also include fever-inducing, thermostable substances (glycoproteins) from the outer membrane of bacteria and other microorganisms. Both of these substances can cause fever, hypotension and shock if administered to humans. Due to the potential harmful effects, it is advantageous to remove even low amounts of endotoxins from intravenously administered pharmaceutical drug solutions. The Food & Drug Administration ("FDA") has set an upper limit of 5 endotoxin units (EU) per dose per kilogram body weight in a single one hour period for intravenous drug applications (The United States Pharmacopeial Convention, Pharmacopeial Forum 26 (1):223 (2000)). When therapeutic proteins are administered in amounts of several hundred or thousand milligrams per kilogram body weight, as can be the case with monoclonal antibodies, it is advantageous to remove even trace amounts of endotoxin.

Formulations of the subject antibodies include those suitable for oral, dietary, topical, parenteral (e.g., intravenous, intraarterial, intramuscular, subcutaneous injection), ophthalmologic (e.g., topical or intraocular), inhalation (e.g., intrabronchial, intranasal or oral inhalation, intranasal drops), rectal, and/or intravaginal administration. Other suitable methods of administration can also include rechargeable or biodegradable devices and controlled release polymeric devices. Stents, in particular, may be coated with a controlled release polymer mixed with an agent of the invention. The pharmaceutical compositions of this disclosure can also be administered as part of a combinatorial therapy with other agents (either in the same formulation or in a separate formulation).

The amount of the formulation which will be therapeutically effective can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may

Samsung Bioepis Exhibit No. 1002
Page 1669

be extrapolated from dose-response curves derived from in vitro or animal model test systems. The dosage of the compositions to be administered can be determined by the skilled artisan without undue experimentation in conjunction with standard dose-response studies. Relevant circumstances to be considered in making those determinations include the condition or conditions to be treated, the choice of composition to be administered, the age, weight, and response of the individual patient, and the severity of the patient's symptoms. For example, the actual patient body weight may be used to calculate the dose of the formulations in milliliters (mL) to be administered. There may be no downward adjustment to "ideal" weight. In such a situation, an appropriate dose may be calculated by the following formula:

Dose (mL) = [patient weight (kg) x dose level (mg/kg)/ drug concentration (mg/mL)]

To achieve the desired reductions of body fluid parameters, such anti-C5 antibodies can be administered in a variety of unit dosage forms. The dose will vary according to the particular antibody. For example, different antibodies may have different masses and/or affinities, and thus require different dosage levels. Antibodies prepared as Fab' fragments or single chain antibodies will also require differing dosages than the equivalent native immunoglobulins, as they are of considerably smaller mass than native immunoglobulins, and thus require lower dosages to reach the same molar levels in the patient's blood.

Other therapeutics of the disclosure can also be administered in a variety of unit dosage forms and their dosages will also vary with the size, potency, and *in vivo* half-life of the particular therapeutic being administered.

Doses of therapeutics of the disclosure will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician.

In certain embodiments, a typical therapeutic treatment includes a series of doses, which will usually be administered concurrently with the monitoring of clinical endpoints such as deposition of membrane attack complex (MAC) on nonnecrotic muscle fibers, age at reaching Hammersmith score of 30/40, age at

Samsung Bioepis Exhibit No. 1002
Page 1670

becoming wheelchair bound, muscle pain or spasms, etc., with the dosage levels adjusted as needed to achieve the desired clinical outcome.

The frequency of administration may also be adjusted according to various parameters. These include the clinical response, the plasma half-life of the antibody capable of inhibiting complement, and the levels of the antibody in a body fluid, such as, blood, plasma, serum, or synovial fluid. To guide adjustment of the frequency of administration, levels of the antibody capable of inhibiting complement in the body fluid may be monitored during the course of treatment.

Alternatively, for antibodies capable of inhibiting complement such as an anti-C5 antibody that affects C5b, levels of the cell-lysing ability of complement present in one or more of the patient's body fluids are monitored to determine if additional doses or higher or lower dosage levels are needed. Such doses are administered as required to maintain at least about a 25% reduction, and preferably about a 50% or greater reduction of the cell-lysing ability of complement present in blood, plasma, or serum. The cell-lysing ability can be measured as percent hemolysis in hemolytic assays of the types described herein. A 10% or 25% or 50% reduction in the cell-lysing ability of complement present in a body fluid after treatment with the antibody capable of inhibiting complement used in the practice of the invention means that the percent hemolysis after treatment is 90, 75, or 50 percent, respectively, of the percent hemolysis before treatment.

In yet another alternative, dosage parameters are adjusted as needed to achieve a substantial reduction of C5a levels in blood, plasma, or serum. As discussed above, C5a levels can be measured using the techniques described in Wurzner, et al., Complement Inflamm 8:328-340, 1991. Other protocols of administration can, of course, be used if desired as determined by the physician.

Administration of the therapeutics of the disclosure will generally be performed by a parenteral route, typically via injection such as intra-articular or intravascular injection (e.g., intravenous infusion) or intramuscular injection. Other routes of administration, e.g., oral (p.o.), may be used if desired and practicable for the particular antibody capable of inhibiting complement to be administered.

For the treatment of muscular dystrophy associated with dysferlin-deficiency by systemic administration (as opposed to local administration) administration of a

Samsung Bioepis Exhibit No. 1002
Page 1671

large initial dose is specific, i.e., a single initial dose sufficient to yield a substantial reduction, and more preferably an at least about 50% reduction, in the hemolytic activity of the patient's serum. Such a large initial dose is preferably followed by regularly repeated administration of tapered doses as needed to maintain substantial reductions of serum hemolytic titer. In another embodiment, the initial dose is given by both local and systemic routes, followed by repeated systemic administration of tapered doses as described above.

Formulations suitable for injection, p.o., and other routes of administration are well known in the art and may be found, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, Pa., 17th ed. (1985). Parenteral formulations must be sterile and non-pyrogenic, and generally will include a pharmaceutically effective carrier, such as saline, buffered (e.g., phosphate buffered) saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions, and the like. These formulations may contain pharmaceutically acceptable auxiliary substances as required, such as, tonicity adjusting agents, wetting agents, bactericidal agents, preservatives, stabilizers, and the like.

The formulations of the invention can be distributed as articles of manufacture comprising packaging material and a pharmaceutical agent which comprises the antibody capable of inhibiting complement and a pharmaceutically acceptable carrier as appropriate to the mode of administration. The packaging material will include a label which indicates that the formulation is for use in the treatment of muscular dystrophy associated with dysferlin-deficiency.

Without intending to limit it in any manner, the present invention will be more fully described by the following examples. The methods and materials which are common to various of the examples are as follows.

EXAMPLES

EXAMPLE 1. DAF/CD55 IS DOWN-REGULATED IN DYSFERLIN-DEFICIENT MICE

The GeneChip Murine Genome U74Av2 array was used to compare the gene expression profiles of skeletal and cardiac muscles of SJL/J mice with dysferlin

Samsung Bioepis Exhibit No. 1002
Page 1672

deficiency to those of C57BL/6 control mice. Analysis of gene expression in the nonpooled skeletal muscle of SJL/J vs control mice revealed 291 differentially expressed genes at a threshold of $p < 0.001$.

DAF1 was 5-fold down-regulated in skeletal muscle of SJL/J compared with skeletal muscle of C57/BL6 mice, with a significance of $p = 0.0000009$. In contrast, in left cardiac ventricle, a mild 1.5-fold up-regulation was found. Similar results were observed with DAF2 (8.2-fold down-regulation in skeletal muscle, $p = 0.0027$; 4-fold up-regulation in heart). Therefore, analyzed intraindividually, these two complement inhibitory factors, corresponding to human CD55, are significantly differentially expressed in skeletal muscle and heart. CD59, another well-described inhibitor of complement activation, was not differentially expressed (Table IIhttp://www.jimmunol.org/cgi/content/full/175/9/6219 - TII#TII). There was no significant difference in the expression of complement receptor 1, complement component factor H, or factor H-like 1 in skeletal muscles of dysferlin-deficient and control mice (Table IIhttp://www.jimmunol.org/cgi/content/full/175/9/6219 - TII#TII). The differential expression of DAF1 and DAF2 was validated by TaqMan RT-PCR and revealed a 4-fold down-regulation of DAF1 ($p = 0.005$) and a 2-fold down-regulation of DAF2 ($p = 0.003$; Figure 1http://www.jimmunol.org/cgi/content/full/175/9/6219 - F1#F1). The mild up-regulation of cardiac DAF was confirmed independently for the left and right ventricles (not shown).

In accordance with the results obtained by microarray and TaqMan RT-PCR, the DAF/CD55 protein was absent by immunohistochemical staining of SJL/J quadriceps muscle, but was readily detectable on the sarcolemma of C57BL/6 control muscle (Figure 2http://www.jimmunol.org/cgi/content/full/175/9/6219 - F2#F2, *A* and *D*). A decrease in DAF/CD55 was found in SJL/J mice of all age groups (12, 16, 20, 28, and 32 wk; at least two mice per age group were tested), indicating that CD55 down-regulation is not merely a consequence of age and progressive dystrophic changes in muscle. Skeletal muscle tissues obtained from two additional dysferlin-deficient mouse strains (A/J and $Dysf^{-/-}$) at 16 wk of age also revealed the absence of DAF/CD55 (Figure

Samsung Bioepis Exhibit No. 1002
Page 1673

2http://www.jimmunol.org/cgi/content/full/175/9/6219 - F2#F2, *B* and *C*). Protein expression of DAF/CD55 in SJL/J myocardial tissue was not different from that in C57/BL6 control mice (Figure 2http://www.jimmunol.org/cgi/content/full/175/9/6219 - F2#F2, *E* and *F*).

5

EXAMPLE 2. DAF/CD55 IS DOWN-REGULATED IN LGMD2B PATIENTS

Next, skeletal muscle from four patients with dysferlin-deficient muscular dystrophy (LGMD2B) was studied. The diagnosis of LGMD2B was confirmed by the absence of dysferlin in immunohistochemical staining, in Western blot analysis, and by genomic sequencing of *DYSF* (Table IIIhttp://www.jimmunol.org/cgi/content/full/175/9/6219 - TIII#TIII). All patients had reduced sarcolemmal CD55 expression compared with normal skeletal muscle (Figure 2http://www.jimmunol.org/cgi/content/full/175/9/6219 - F2#F2, *G* and *H*). The degree of down-regulation of CD55 varied between patients, from trace staining to complete absence. Staining for CD46 and CD59, two other complement inhibitory molecules, was normal in all patients and controls (not shown).

The expression of DAF/CD55 in human skeletal muscle was also analyzed at the RNA level by TaqMan analysis. Compared with four control specimens from healthy individuals, DAF/CD55 mRNA in LGMD2B was 2.1-fold reduced (Figure 1http://www.jimmunol.org/cgi/content/full/175/9/6219 - F1#F1, *E* and *F*).

EXAMPLE 3. DYSFERLIN-DEFICIENT HUMAN MYOTUBES ARE SUSCEPTIBLE TO COMPLEMENT ATTACK

Functionally, the absence of CD55 should lead to an increased sensitivity against complement-mediated lysis. Human myotube cultures obtained from normal ($n = 2$) and dysferlin-deficient human skeletal muscle ($n = 3$; at least two independent experiments per patient) were established and exposed to complement-mediated lysis. Lysed and dead cells were identified by PI uptake. Normal human myotubes were resistant to complement-mediated lysis (Figure 3http://www.jimmunol.org/cgi/content/full/175/9/6219 - F3#F3, *A* and *B*). This

Samsung Bioepis Exhibit No. 1002
Page 1674

effect could be partially inhibited by preincubation with anti-CD55 Ab (Figure 3http://www.jimmunol.org/cgi/content/full/175/9/6219 - F3#F3*C*). On the contrary, myoblasts and myotubes obtained from patients with dysferlin deficiency were highly susceptible to complement attack (Figure 3http://www.jimmunol.org/cgi/content/full/175/9/6219 - F3#F3, *A* and *D*). The percentage of lysed cells was not altered by the addition of anti-CD55 mAb (Figure 3http://www.jimmunol.org/cgi/content/full/175/9/6219 - F3#F3*E*).

In immunohistochemistry, the presence of the C5b9 MAC on the surface of nonnecrotic muscle fibers was demonstrated in four of four muscle specimens obtained from dysferlin-deficient patients (Figure 3http://www.jimmunol.org/cgi/content/full/175/9/6219 - F3#F3, *F* and *G*).

EXAMPLE 4. DAF/CD55, MYOSTATIN, AND SMAD

To elucidate possible regulatory mechanisms of DAF/CD55 in dysferlin deficiency, it was concluded that if DAF/CD55 down-regulation in dysferlin deficiency only plays a role in skeletal muscle, but not in heart, there should be genes that 1) are differentially expressed in dysferlin-deficient skeletal muscle and cardiac tissue and 2) regulate DAF/CD55. Indeed, within the microarray data obtained from dysferlin-deficient SJL/J mice, a small group of differentially expressed and potentially regulatory genes was identified: myostatin, SMAD2, SMAD3, SMAD4, cardiac ankyrin repeat protein (CARP), and early growth response 1 (EGR1). Therefore, the expression of these genes was quantified in skeletal and cardiac tissues by TaqMan RT-PCR in SJL/J mice and also in skeletal muscle from patients with dysferlin-deficient muscular dystrophy. In both mice and patients, compared with controls, myostatin, SMAD3, and SMAD4 were significantly down-regulated in skeletal muscle (Figure 4http://www.jimmunol.org/cgi/content/full/175/9/6219 - F4#F4*A*). In the heart, SMAD and myostatin were not differentially expressed in SJL/J and C57BL/6 mice (not shown). On the protein level, because of the availability of Abs, only phosphorylated SMAD2 was investigated, and it could be shown to also be markedly reduced in LGMD2B (Figure 4http://www.jimmunol.org/cgi/content/full/175/9/6219

Samsung Bioepis Exhibit No. 1002
Page 1675

- F4#F4*B*) compared with normal controls (Figure 4http://www.jimmunol.org/cgi/content/full/175/9/6219 - F4#F4*C*). CARP and EGR1 were strikingly up-regulated in skeletal muscle (Figure 4http://www.jimmunol.org/cgi/content/full/175/9/6219 - F4#F4*A*) and were reduced in heart (down-regulation of 2.5- and 4-fold in left and right ventricles, respectively).

EXAMPLE 5. SMAD BINDING SITE IN DAF/CD55 PROMOTER

To investigate whether any of these identified, differentially expressed, regulatory genes might influence DAF/CD55 expression, the DAF/CD55 promoter sequence (16) was analyzed for transcription factor binding sites using the MATInspector program (Genomatix) (17). This analysis revealed a binding site for the SMAD complex, GTCTgggct (SEQ ID NO: 49) (18, 19, 20), indicating that SMAD might influence DAF/CD55 expression. Among the 291 differentially expressed genes in skeletal muscle, other SMAD-regulated genes that are known to have significance for muscular dystrophies could not be identified.

EXAMPLE 6. ANTI-C5 ANTIBODY REDUCES SYMPTOMS IN DYSFERLIN-DEFICIENT MICE

To investigate the efficacy of complement inhibition *in vivo*, dysferlin-deficient mice were treated with an anti-murine C5 antibody. The myopathological changes in SJL/J skeletal muscle was reduced by selective blockade of terminal complement with the anti-C5 antibody.

EXAMPLE 7. MATERIALS AND METHODS

Mice

Female SJL/J mice and C57BL/6 mice were purchased from Charles River Laboratories. The microarray experiments were performed in mice 32–34 wk of age. At this age, SJL/J mice showed marked histological signs of muscular dystrophy. Lymphomas were not detected. Muscle sections for immunohistochemistry were obtained from SJL/J mice at 12, 16, 20, 28, and 32 wk of age. For each age group, three mice were examined. Muscle sections from A/J and *Dysf*$^{-/-}$ mice were obtained at 16 wk of age. All experiments were approved by local committees.

Samsung Bioepis Exhibit No. 1002
Page 1676

Total RNA preparation

RNA was extracted from mouse right quadriceps muscle, the left and right ventricles of mouse heart, and human skeletal muscle using TRIzol reagent (Invitrogen Life Technologies). Total RNA was treated by deoxyribonuclease I (Invitrogen Life Technologies) and was purified using the RNeasy Mini Kit (Qiagen).

Microarray experiments

Nonpooled microarray experiments were performed with cRNA prepared from quadriceps muscles and left ventricles of five SJL/J and five C57BL/6 mice using GeneChip Murine Genome U74Av2 (Affymetrix). Eight micrograms of RNA was transcribed in double-stranded cDNA using a cDNA Synthesis System (Roche). cRNA was produced by MEGAscript High Yield Transcription Kit (Ambion) and was labeled with biotin-11-CTP and biotin-16-UTP nucleotides (PerkinElmer). Arrays were hybridized with 16 μg of fragmentized biotinylated cRNA at 45°C and 60 rpm for 16 h in a GeneChip Hybridization Oven 640 (Affymetrix), washed and stained on GeneChip Fluidics Station 400, and scanned in GeneArray scanner 2500 (Affymetrix).

Microarray data analysis

The resulting signals were processed using Affymetrix MicroArray Suite 5.0 software (MAS5.0) with a target intensity of 200. After standard data quality checks, the MAS5.0 expression signal values of each dataset were used for statistical analysis. Probe sets showing an absent call throughout all comparison groups were removed. A Nalimov test with a threshold of $p < 0.001$ was used to exclude outliers. Student's t test (unpaired, two-tailed assumed unequal variance) was used to check the differences between two selected experimental groups.

Quantitative real-time RT-PCR (TaqMan)

cDNA was synthesized from 5 μg of total RNA using PowerScript reverse transcriptase (BD Clontech) and an oligo$(dT)_{18}$ primer. Real-time PCR experiments were performed using TaqMan chemistry on ABI PRISM 7700 Sequence Detection

Samsung Bioepis Exhibit No. 1002
Page 1677

System (Applied Biosystems). Each reaction was performed in a singleplex format and contained TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM forward and reverse primers, and 200 nM TaqMan probe (BioTez). An annealing/extension temperature of 58°C and 40 cycles were used. Primer/probe sets were designed using Primer Express 1.5 software (Applied Biosystems; Table Ihttp://www.jimmunol.org/cgi/content/full/175/9/6219 - TI#TI). For every sample, three independent runs in triplicate were performed, and the relative change in gene expression was quantified by the comparative threshold cycle method (21). Unpaired two-tail unequal variance *t* test with a significance threshold of $p < 0.05$ was used to compare the individual changes in threshold cycle values of the control and experimental group.

Patients

Patients were followed in the Neuromuscular Unit of Department of Neurology, Charité University Hospital (Berlin, Germany). Genomic sequencing of *DYSF* was performed if LGMD2B was suspected clinically and by immunohistochemistry and/or Western blotting. Patients included in this study gave their written informed consent. All studies were performed according to Declaration of Helsinki principles.

Immunohistochemistry

Murine DAF was detected with polyclonal rat anti-mouse Ab (MDI) (22); human CD55 was detected with SM1141PS (Acris Antibodies). Anti-human C5b9 mAb (DakoCytomation) was applied for MAC detection. Anti-phospho-MADR2 mAb against phosphorylated SMAD2 was obtained from EMD Biosciences. Double-immunofluorescent staining for SMAD protein (FITC) and nuclear membrane protein lamin A/C (Novocastra; Cy3) were examined using a two-photon microscope (Leica).

Complement attack assay

Myoblast/myotube cultures and complement attack assays were performed according to published protocols (23, 24). Myoblasts were grown in SMG-Medium (Promo

Samsung Bioepis Exhibit No. 1002
Page 1678

Cell) supplemented with Promo Cell Supplement Mix, gentamicin (40 μg/ml; Invitrogen Life Technologies), 2 mM glutamine, and 10% FCS. Myoblasts were transferred on 96-well plates and grown to near confluence. Differentiation into myotubes was induced with DMEM containing 2% heat-inactivated horse serum. For complement attack assays, wells were incubated in sexplicate for 30 min with normal human serum diluted 1/5 and 1/20 in Veronal buffer (Hollborn & Söhne) containing 1% BSA. Half the wells were preincubated with anti-human CD55 mAb (5 μg/ml). Propidium iodide (PI; 0.5 μg/ml) was added to assess killing. The total number of myotubes was compared with PI-positive cells using a fluorescent tissue culture microscope (Leica) and by FACS analysis.

Samsung Bioepis Exhibit No. 1002
Page 1679

Table I. Primer and probe sequences used for TaqMan RT-PCR

Gene Product	Forward Primer	Reverse Primer	Probe	Ref. Sequence
Mouse				
PBGD	(SEQ ID NO.1) GCACGATCCTGAAACTCTGC	(SEQ ID NO.9)TCCTTCCAGGTGCCTCAGAA	(SEQ ID NO.17)FAM-TCGCTGCATTGCTGAAAGGGCT-TAMRA	NM_013551
DAF1	(SEQ ID NO.2)GTACAGGAACCCCCTCAACG	(SEQ ID NO.10)TGAGGAGTTGGTTGGTCTCC	(SEQ ID NO.18)FAM-CAGAAACCCACACAGAAAGTGTTCCAAAT-TAMRA	NM_010016
DAF2	(SEQ ID NO.3)ACAGGAATCCCCTCAACGC	(SEQ ID NO.11)CTGAGGAGTTGGTTGGTCTCC	(SEQ ID NO.19)FAM-CAGAAACCCACACAGAAAGTGTTCCAAATCC-TAMRA	NM_007827
Myostatin	(SEQ ID NO.4)AGGTGACAGACACACCCAAGA	(SEQ ID NO.12)GATTCCGTGGAGTGCTCATC	(SEQ ID NO.20)FAM-TCCCGAGAGACTTTGGGCTTGACTG-TAMRA	NM_010834
CARP	(SEQ ID NO.5)GGACTGGTCATTACGAGTGCG	(SEQ ID NO.13)CCTTGGCATTGAGATCAGCC	(SEQ ID NO.21)FAM-TGAGCACCTCATCGCCTGCG-TAMRA	NM_013468
SMAD2	(SEQ ID NO.6)CCCATTCCTGTTCTGGTTCA	(SEQ ID NO.14)AGCCAGCAGTGCAACTTTTT	(SEQ ID NO.22)FAM-AGCAGTACAGCAGAATGACGTCGTGC-TAMRA	NM_010754
SMAD3	(SEQ ID NO.7)GGGCCTACTGTCCAATGTCA	(SEQ ID NO.15)CCCAATGTGTCGCCTTGTA	(SEQ ID NO.23)FAM-CCGGAATGCAGCGCGTGGAAC-TAMRA	NM_016769
SMAD4	(SEQ ID NO.8)CTGGACAGAGAAGCTGGCC	(SEQ ID NO.16)ACGCGCTTGGGTAGATCTT	(SEQ ID NO.24)FAM-AGCACCTGGCGACGCTGTTCA-TAMRA	NM_008540
Human				
β2MG	(SEQ ID NO.25)ACTGAAAAAGATGAGTATGCCTGC	(SEQ ID NO.33)CATCTTCAAACCTCCATGATGCT	(SEQ ID NO.41)FAM-TGAACCATGTGACTTTGTCACAGCCCA-TAMRA	NM_004048
DAF/CD55	(SEQ ID NO.26)AAGTAATCTTTGGCTGTAAGGCA	(SEQ ID NO.34)TTCACCAGCATGTTTTTACCTTTAA	(SEQ ID NO.42)FAM-TTTCATCTTTCCTTCGGGTTGGCAAA-TAMRA	NM_000574
Myostatin	(SEQ ID NO.27)TGCTGTAACCTTCCCAGGAC	(SEQ ID NO.35)GGTGTGTCTGTTACCTTGACCTC	(SEQ ID NO.43)FAM-AGGAGAAGATGGGCTGAATCCGTTTTT-TAMRA	NM_005259
EGR1	(SEQ ID NO.28)TTCACGTCTTGGTGCCTTTT	(SEQ ID NO.36)CCCTCACAATTGCACATGTCA	(SEQ ID NO.44)FAM-TGATGCGCCTTGCTGATGGC-TAMRA	NM_001964
CARP	(SEQ ID NO.29)AAGTTGCTCAGCAGCGCT	(SEQ ID NO.37)TGCTCCGCGCACTCATAGT	(SEQ ID NO.45)FAM-CATGTGGCGGTGAGGACTGGC-TAMRA	NM_014391
SMAD2	(SEQ ID NO.30)TGTTTTAGTGCCCTGCTGC	(SEQ ID NO.38)GCTCACAAGATGGGTAGTGGA	(SEQ ID NO.46)FAM-CTTCCAGACTTTGTGCTGTCCAGTAATTATGTC-TAMRA	NM_005901
SMAD3	(SEQ ID NO.31)GGGCACGCCAGTTCTGAA	(SEQ ID NO.39)TTGGTGTTTCTGGATGCTGA	(SEQ ID NO.47)FAM-TTGGTGGAGGGTGTAGTGGCTTTTTGG-TAMRA	NM_005902
SMAD4	(SEQ ID NO.32)CAGCCGTGGCAGGAAAC	(SEQ ID NO.40)GCTGACAGACTGATAGCTGGAGC	(SEQ ID NO.48)FAM-TCCCTGGCCCAGGATCAGTAGGTGGA-TAMRA	NM_005359

Samsung Bioepis Exhibit No. 1002
Page 1680

Table II. *mRNA expression of regulatory proteins of the complement system in SJL/J mice*

	Skeletal Muscle			Heart (LV)[a]			
	Fold change	Direction[a]	*p* Value	Fold change	Direction	*p* Value	Accession No.
Decay accelerating factor 1	–5.2	Down	0.0000009	1.5	Up	0.000003	NM_010016
Decay accelerating factor 2	–8.2	Down	0.0027	4	Up	0.0018	NM_007827
C1 inhibitor	1.3	NC	0.11	1.8	Up	0.005	NM_009776
Complement receptor 1	1.5	NC	0.08	1.6	Up	0.0012	NM_010740
Complement component factor h	1.9	NC	0.23	2.1	Up	0.00002	NM_009888
Complement component factor H-like 1	1.3	NC	0.06	2.1	Up	0.0046	NM_015780
CD59a Ag (protectin)	1.7	NC	0.21	–0.9	NC	0.34	NM_007652

[a] LV, Left ventricle; NC, not significantly changed.

Samsung Bioepis Exhibit No. 1002
Page 1681

Table III. *Patients with limb girdle muscular dystrophy 2B*[a]

Patient	Gene	Age	Sex	Alleles	Mutation
1	*DYSF*	52	M	Homozygous	c.4022T > C
2	*DYSF*	43	F	Heterozygous	c.855 + 1delG
				Heterozygous	c.895G > A
3	*DYSF*	39	M	Heterozygous	c.855 + 1delG
				Heterozygous	c.895G > A
4	*DYSF*	32	F	Heterozygous	c.1448C > A
				Heterozygous	c.6350T > A

[a] The mutations are submitted to the Leiden Muscular Dystrophy Database (http://dmd.nl). The MIAME-compliant microarray data are available at (www.ncbi.nlm.nih.gov/geo) under accession no. GSE2507.

INCORPORATION BY REFERENCE

All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

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4. Walport, M. J. 2001. Complement: first of two parts. *N. Engl. J. Med. 344:1058-1066.*

Samsung Bioepis Exhibit No. 1002
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Samsung Bioepis Exhibit No. 1002
Page 1683

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EQUIVALENTS

While specific embodiments of the subject inventions are explicitly disclosed herein, the above specification is illustrative and not restrictive. Many variations of the inventions will become apparent to those skilled in the art upon review of this specification and the claims below. The full scope of the inventions should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

Samsung Bioepis Exhibit No. 1002
Page 1684

CLAIMS:

1. A method of treating muscular dystrophy associated with dysferlin-deficiency in a mammal comprising administering to said mammal a therapeutically effective amount of an anti-C5 antibody.

2. The method of claim 1, wherein said mammal is a human.

3. The method of claim 1, wherein said antibody is a whole antibody or an antibody fragment.

4. The method of claim 3, wherein said whole antibody or antibody fragment is the group consisting of a polyclonal antibody, a monoclonal antibody or antibody fragment, a diabody, a chimerized or chimeric antibody or antibody fragment, a humanized antibody or antibody fragment, a deimmunized human antibody or antibody fragment, a fully human antibody or antibody fragment, a single chain antibody, an Fv, an Fab, an Fab', and an $F(ab')_2$.

5. The method of claim 1, wherein said antibody is pexelizumab.

6. The method of claim 1, wherein said antibody is eculizumab.

7. The method of claim 1, wherein said antibody is administered chronically to said mammal.

8. The method of claim 1, wherein said antibody is administered systemically to said mammal.

9. The method of claim 1, wherein said agent is administered locally to said mammal.

10. A method of treating muscular dystrophy associated with dysferlin-deficiency in a mammal comprising administering to said mammal a therapeutically effective amount of a CD55 agonist.

11. The method of claim 10, wherein said mammal is a human.

12. The method of claim 10, wherein said CD55 agonist increases the expression and/or activity of CD55.

Samsung Bioepis Exhibit No. 1002
Page 1685

13. The method of claim 10, wherein said CD55 agonist is selected from: a polypeptide, a polypeptide analog, a nucleic acid, a nucleic acid analog, and a small molecule.

14. The method of claim 10, wherein said CD55 agonist comprises an agonistic ligand of CD97.

15. The method of claim 10, wherein said CD55 agonist comprises an agent that prevents the association of C4b and C3b.

16. The method of claim 13, wherein said polypeptide comprises an amino acid sequence of greater than 90% sequence identity to the amino acid sequence of a soluble portion of a naturally occurring CD55 protein.

17. The method of claim 13, wherein said nucleic acid comprises a polynucleotide sequence of greater than 90% sequence identity to the nucleotide sequence of a naturally occurring CD55 mRNA.

18. The method of claim 13, wherein said polypeptide is an antibody.

19. The method of claim 18, wherein said antibody is a whole antibody or an antibody fragment.

20. The method of claim 19, wherein said whole antibody or antibody fragment is the group consisting of a polyclonal antibody, a monoclonal antibody or antibody fragment, a diabody, a chimerized or chimeric antibody or antibody fragment, a humanized antibody or antibody fragment, a deimmunized human antibody or antibody fragment, a fully human antibody or antibody fragment, a single chain antibody, an Fv, an Fab, an Fab', and an $F(ab')_2$.

21. The method of claim 18, wherein said antibody is an agonistic antibody that specifically binds CD55 and increases CD55 activity.

22. Use of an anti-C5 antibody in the manufacture of a medicament or medicament package for the treatment of muscular dystrophy associated with dysferlin-deficiency in a mammal.

Samsung Bioepis Exhibit No. 1002
Page 1686

23. Use of a CD55 agonist in the manufacture of a medicament or medicament package for the treatment of muscular dystrophy associated with dysferlin-deficiency in a mammal.

Samsung Bioepis Exhibit No. 1002
Page 1687

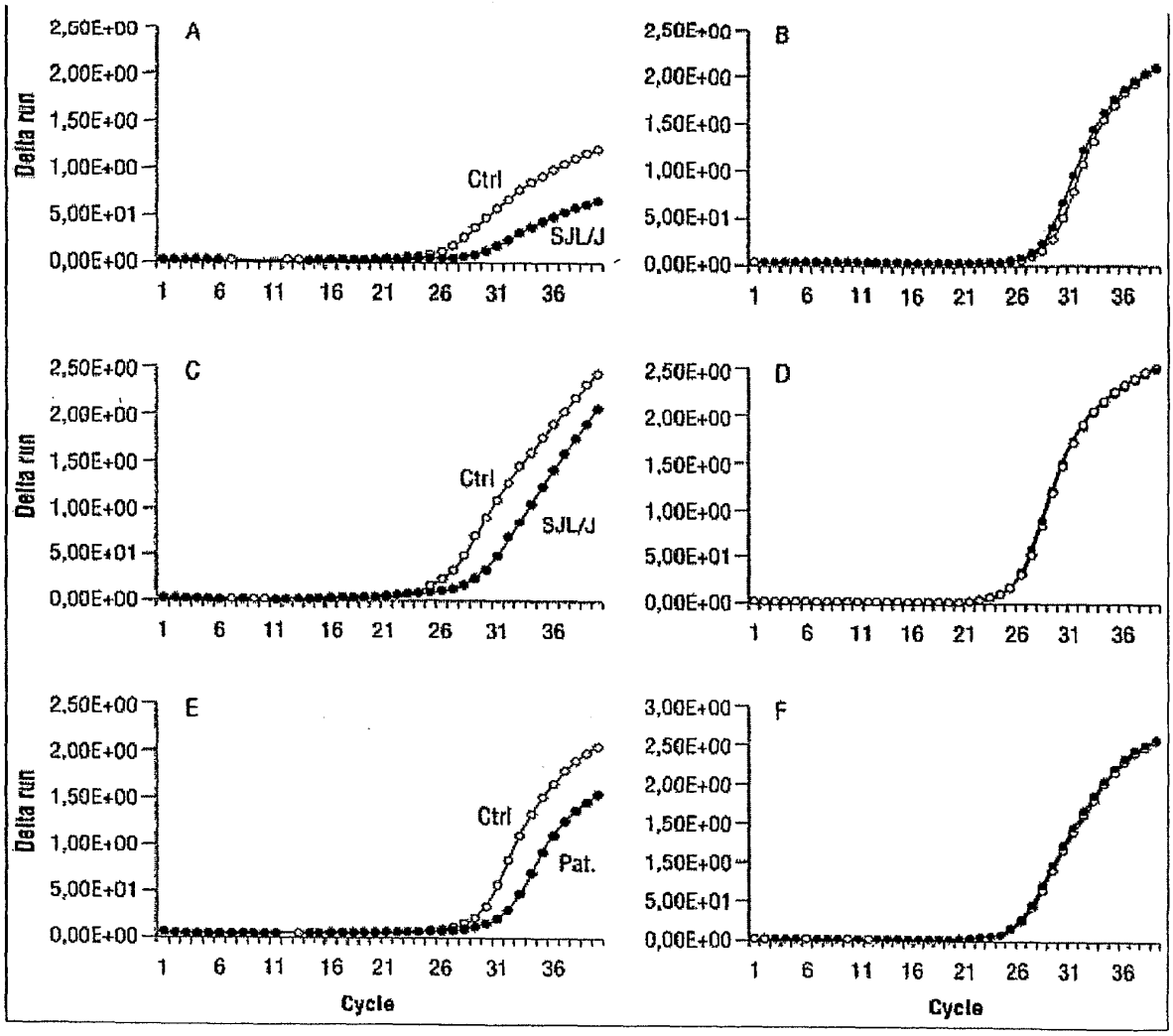


FIGURE 1. TaqMan RT-PCR amplification plots of DAF/CD55.

Samsung Bioepis Exhibit No. 1002
Page 1688

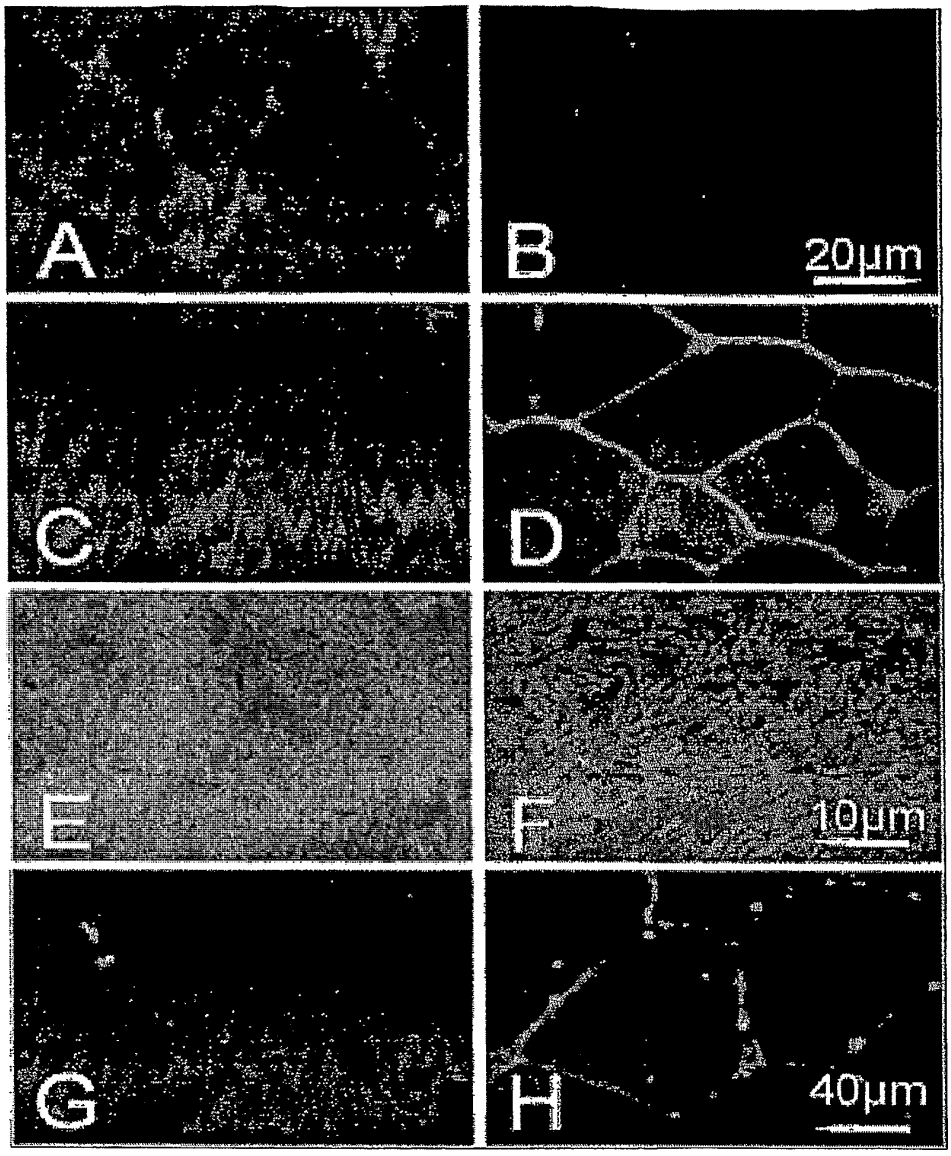


FIGURE 2. DAF/CD55 protein expression in dysferlin deficiency.

Samsung Bioepis Exhibit No. 1002
Page 1689

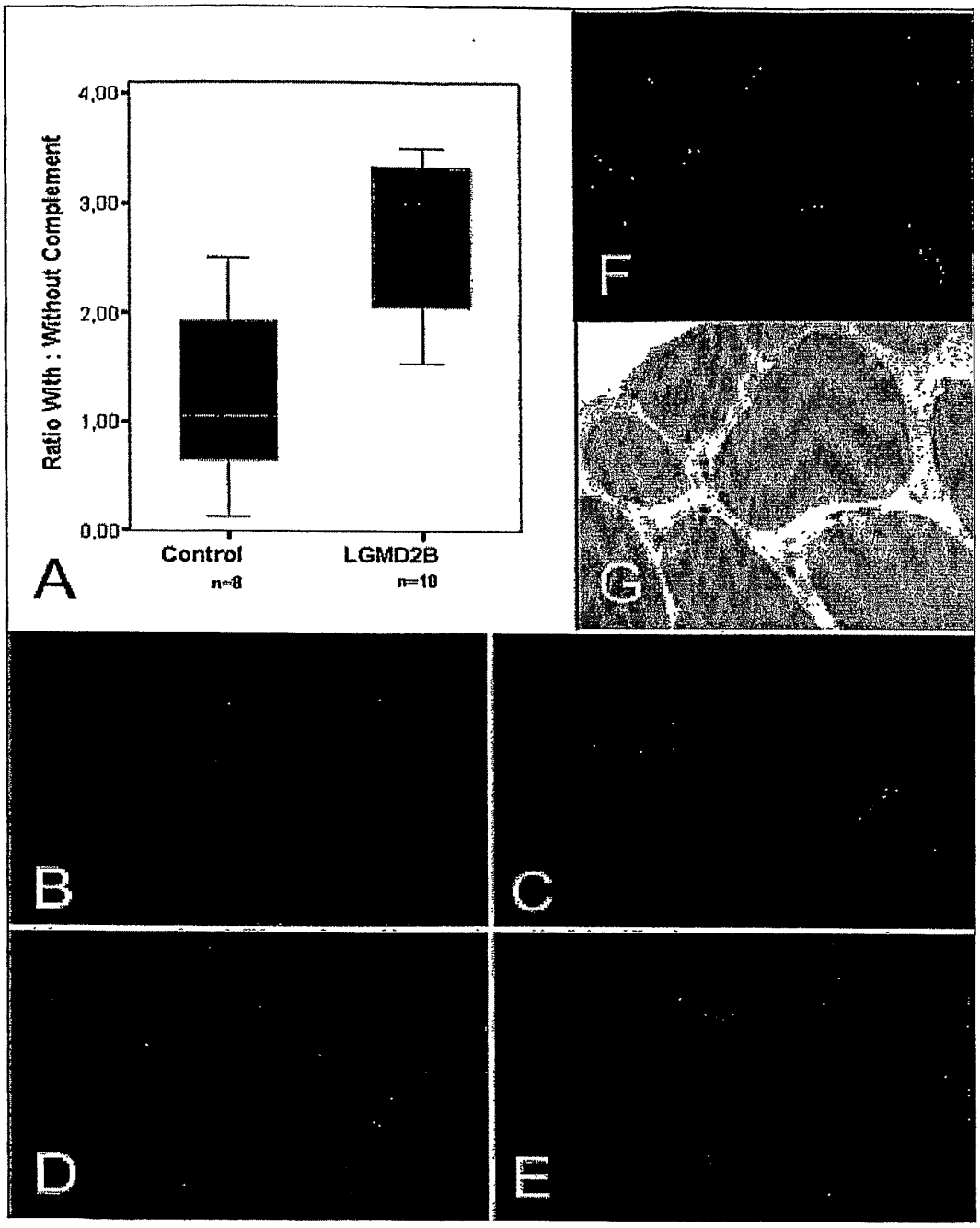


FIGURE 3. Complement lysis assay and binding of C5b9-MAC to nonnecrotic muscle cells.

Samsung Bioepis Exhibit No. 1002
Page 1690

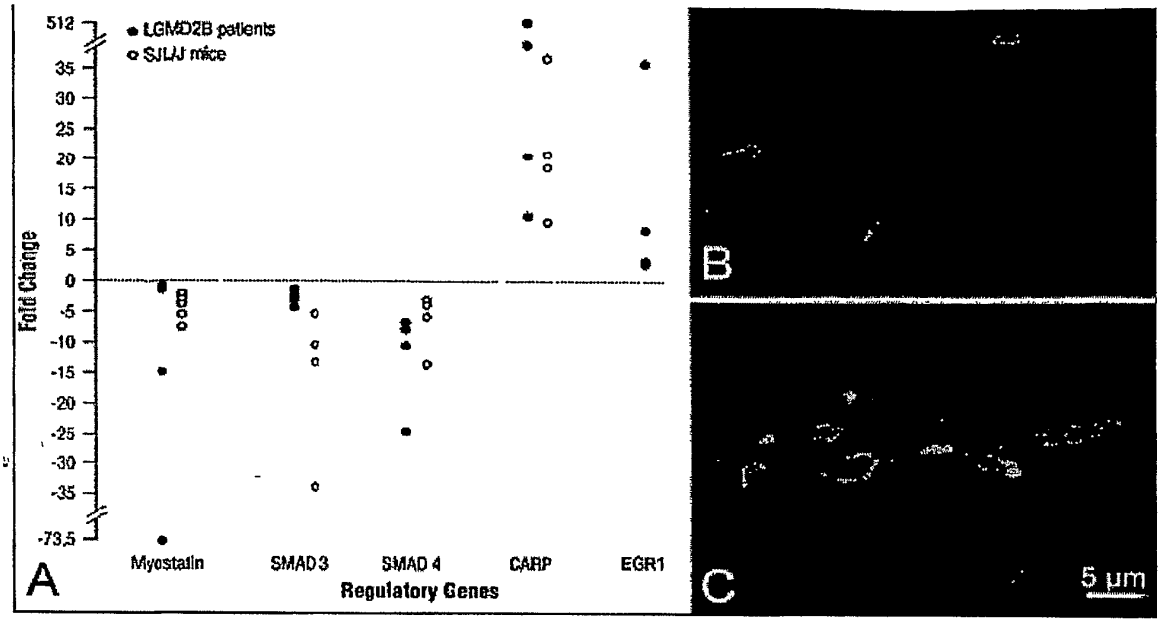


FIGURE 4. Expression of regulatory factors in skeletal muscle of dysferlin-deficient patients and SJL/J mice (aged 20–30 wk).

Samsung Bioepis Exhibit No. 1002
Page 1691

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2006/016980

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/28 A61K38/17 A61P21/00 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WENZEL KATRIN ET AL: "Increased susceptibility to complement attack due to down-regulation of decay-accelerating factor/CD55 in dysferlin-deficient muscular dystrophy" JOURNAL OF IMMUNOLOGY, vol. 175, no. 9, November 2005 (2005-11), pages 6219-6225, XP002426426 ISSN: 0022-1767 the whole document	1-13, 16-23
A	US 6 355 245 B1 (EVANS MARK J [US] ET AL) 12 March 2002 (2002-03-12) column 7, line 1 - column 9, line 4 claims 1-23	1-9,22

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 March 2007	11/04/2007

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ulbrecht, Matthias

Form PCT/ISA/210 (second sheet) (April 2005)

Samsung Bioepis Exhibit No. 1002
Page 1692

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WHISS P A: "PEXELIZUMAB" CURRENT OPINION IN INVESTIGATIONAL DRUGS, PHARMAPRESS, US, vol. 3, no. 6, June 2002 (2002-06), pages 870-877, XP009045357 ISSN: 1472-4472 the whole document	1-9,22
A	YAZDANBAKHSH KARINA: "DEVELOPMENT OF COMPLEMENT THERAPEUTICS FOR INHIBITION OF IMMUNE-MEDIATED RED CELL DESTRUCTION" TRANSFUSION, AMERICAN ASSOCIATION OF BLOOD BANKS, BETHESDA, MD, US, vol. 45, no. 2 SUPPL S, August 2005 (2005-08), pages 122S-129S, XP008073567 ISSN: 0041-1132 page 125S, left-hand column, paragraph 2 - page 126S, left-hand column, paragraph 1	1-9,22
A	CONFALONIERI PAOLO ET AL: "Muscle inflammation and MHC class I up-regulation in muscular dystrophy with lack of dysferlin: An immunopathological study." JOURNAL OF NEUROIMMUNOLOGY, vol. 142, no. 1-2, September 2003 (2003-09), pages 130-136, XP002426427 ISSN: 0165-5728 abstract page 134, left-hand column, paragraph 2 page 135, left-hand column, paragraph 3	1-23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2006/016980

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-21 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 14, 15 (compeletely)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Although claims 1-21 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box II.2

Claims Nos.: 14, 15 (compeletely)

Claims 14 and 15 refer to, respectively, an agonist ligand of CD97 and an agent that prevents association of C4b and C3b. There is no indication in the application as to any structural features of such compounds neither is any specific instance thereof disclosed. It is thus undeterminable which compounds are actually covered by these functional definitions (Art. 6 PCT) and consequently no search could be performed on said claims.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2006/016980

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(54) **METHODS AND COMPOSITIONS FOR THE TREATMENT OF ANTIBODY MEDIATED NEUROPATHIES**

VERFAHREN UND ZUSAMMENSETZUNGEN ZUR BEHANDLUNG ANTIKÖRPER-VERMITTELTER NERVENLEIDEN

PROCÉDÉS ET COMPOSITIONS DE TRAITEMENT DE NEUROPATHIES INDUITES PAR DES ANTIBIOTIQUES

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(73) Proprietor: **ALEXION PHARMACEUTICALS, INC.**
Cheshire, CT 06410 (US)

(72) Inventors:
- **HALSTEAD, Susan**
  Glasgow G42 8NA (GB)
- **WILLISON, Hugh**
  Glasgow G61 4AJ (GB)
- **ROTHER, Russell, P.**
  Prospect, CT 06721 (US)

(74) Representative: **Vossius & Partner**
Siebertstrasse 4
81675 München (DE)

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EP 2 061 810 B1

Description

FIELD OF THE INVENTION

[0001] This application provides compositions for the treatment of antibody mediated neuropathies that activate complement to induce nerve injury. In specific embodiments, the application relates to the use of complement cascade inhibitors as therapeutic agents to treat antibody mediated neuropathies.

BACKGROUND OF THE INVENTION

[0002] Neuropathy is a generic term used to describe diseases of the peripheral nervous system. There are about 200 known different causes of peripheral neuropathies. Although most neuropathies affect all three major classes of nerve fibers to varying degrees, some diseases involve only one or two, and are thus said to be purely or predominantly motor, sensory, or autonomic neuropathies.

[0003] Guillain-Barré syndrome (GBS) is an acute illness involving the peripheral nervous system that usually occurs two to three weeks after a flu-like disease or other infection, including *Campylobacter* enteritis. It is mostly a motor neuropathy, meaning that its symptoms are largely related to the involvement of the motor nerves. Despite the primarily motor nature of the disease, the earliest symptoms may be numbness and tingling felt in the lower extremities followed shortly by weakness of the distal muscles of the lower extremities. The danger occurs when the weakness involves the muscles of respiration. GBS is associated in a proportion of cases with antiganglioside autoantibodies (AGAs) to a wide range of specific glycolipid structures, and also with antibodies to other nerve components including myelin proteins and glycosaminoglycans in some cases, and in other GBS cases, antibodies are presumed to be present but have yet to be formally identified.

[0004] Miller Fisher syndrome (MFS) is a variant of Guillain-Barré syndrome, accounting for 5-10% of cases. In one survey, the annual incidence has been estimated at 0.09 per 100,000 population. MFS is characterized by the acute onset of ophthalmoplegia, ataxia and areflexia. Anti-GQ1b ganglioside antibodies are the serological hallmark of MFS. The antibodies may arise in some cases through molecular mimicry with *Campylobacter* lipopolysaccharides. It is now widely accepted that well over 90% of patients with MFS have anti-GQ1b IgG antibodies during the acute phase of the illness. Equally significant is the complete absence of anti-GQ1b IgG antibodies from normal and other disease control groups, indicative of a high level of specificity for this disease association. The antibody titers peak at clinical presentation, decaying rapidly with the course of clinical recovery. Anti-GQ1b IgG antibody titers are also elevated in the acute phase sera of some patients with Guillain-Barré syndrome with ophthalmoplegia. The anti-GQ1b IgG antibody marker also identifies a cluster of closely related syndromes, often considered *formes frustes* of MFS, that have in common the presence of external ophthalmoplegia or cerebellar-like ataxia.

[0005] The current treatment for GBS, including AGA-mediated cases is intravenous immunoglobulin (IVIg), plasma exchange, or a combination of both. Although some neuropathies like GBS are usually self-limiting illnesses, intensive therapeutic intervention is often needed. Some individuals are left with residual deficits. Other neuropathies are chronic and only symptoms such as pain are treated. There is a great need for additional treatments to improve symptoms and decrease recovery time of antibody mediated neuropathies.

[0006] WO 2005/002513 discloses agents that bind and/or inhibit classical complement C1 subcomponent, C1q and methods of their use in the treatment of autoimmune diseases and cancer.

[0007] Halstead et al. 2005 (Annals of Neurology (58), 203 - 210) discloses that APT070 abrogates nerve terminal injury in rodent models of Miller Fisher syndrome, by reduction of intermediate C3 convertase as well as by inhibition of C5 convertase. APT070 is a soluble form of complement receptor 1 (sCR1) protein that binds to C3b and C4b and thereby inhibits, among other things, opsonisation by host phagocytes (e.g., macrophages) of objects to which C3b and C4b are bound.

[0008] US 2005/004031 discloses small molecule inhibitors of one or more of C5a receptor, C1r, C1q, C1s, and MASP-2 and the use of these small molecule inhibitors in treating neurodegenerative diseases

SUMMARY OF THE INVENTION

[0009] Compositions for treating patients suffering from antibody-mediated neuropathies are presented as characterised in the appended claims. .

[0010] In a number of neuropathies antibodies to specific components of nerves have been identified. Some of these diseases are mediated by anti-ganglioside and anti-glycolipid autoantibodies such as the Miller Fisher variant of Guillain-Barré syndrome. Antibodies to a wide range of glycolipids including GM1, GM1(NeuGc), GM1b, GalNAc-GM1b, GD1a, GalNAc-GD1a, GD1b, 9-*O*-acetyl GD1b, GD3, GT1a, GT1b, GQ1b, GQ1ba, LM1, galactocerebroside and SGPG have been reported in papers on inflammatory neuropathies, as case reports and in larger series.

[0011] Antibodies to other nerve components including proteins and glycoproteins have also been reported to be causative in neuropathies. For example, monoclonal IgM against myelin-associated glycoprotein causes anti-MAG IgM paraproteinemic neuropathy (Willison and Yuki, Brain, 2002, 125, 2591-2625). In a proportion of neuropathy cases, antibodies are presumed to be present owing to similar pathological patterns and treatment responses as seen in antibody-associated cases, but the specificity of the presumed antibody has yet to be formally

Samsung Bioepis Exhibit No. 1002
Page 1698

identified. Also described herein is that the antibody mediated neuropathy may be one or more of the following: acute motor axonal neuropathy, acute inflammatory demyelinating polyneuropathy, Bickerstaff's brainstem encephalitis, acute ophthalmoparesis, ataxic Guillain-Barré syndrome, pharyngeal-cervical-brachial weakness, chronic neuropathy syndromes with anti-glycolipid antibodies, anti-MAG IgM paraproteinemic neuropathy, chronic sensory ataxic neuropathy with anti-disialosyl antibodies, IgM, IgG and IgA paraproteinemic neuropathy, motor neuropathy with anti-GM1 and anti-GM2 antibodies, chronic inflammatory demyelinating neuropathy (CIDP), multifocal motor neuropathy (MMN), and multifocal acquired demyelinating sensory and motor neuropathy (MADSAM).

[0012] In some neuropathies the activation of complement may be antibody-independent, for example, in hereditary amyloid neuropathy. In this disease, early classical pathway activation markers, C1q and C4, have been detected in amyloid deposits in the absence of detectable antibody (Hafer-Macko et al., J Peripher Nerv Syst. 2000 Sep;5(3):131-9; incorporated herein by reference). This suggests that antibody-independent activation of the classical pathway can occur and that complement cascade inhibitors may be effective in treating antibody-independent, complement mediated neuropathies.

[0013] An antibody of the application inhibits activation of the classical pathway, the alternative pathway or the lectin complement pathway. In certain embodiments, said antibody is an antibody against any member of the group comprising the complement components C5 or C5b. In certain embodiments, said antibody is an antibody against C5. In certain embodiments, said antibody is an inhibitor of C5 cleavage.

[0014] Also described herein is a complement cascade inhibitor selected from: a polypeptide, a polypeptide analog, a peptidomimetic, an antibody, a nucleic acid, an RNAi construct, a nucleic acid analog, and a small molecule.

[0015] In certain embodiments, an antibody of the application is a whole antibody or an antibody fragment. In certain embodiments, said whole antibody or antibody fragment is selected from the group consisting of: a polyclonal antibody, a monoclonal antibody or antibody fragment, a diabody, a chimerized or chimeric antibody or antibody fragment, a humanized antibody or antibody fragment, a deimmunized human antibody or antibody fragment, a fully human antibody or antibody fragment, a single chain antibody, an Fv, an Fab, an Fab', or an $F(ab')_2$.

[0016] In certain embodiments, an antibody of the application inhibits activation of the classical pathway, the alternative pathway or the lectin complement pathway. In certain embodiments, said antibody is an antibody against any member of the group comprising the complement components C5 or C5b. In certain embodiments, said antibody is an antibody against C5. In certain embodiments, said antibody is an inhibitor of C5 cleavage.

[0017] In certain embodiments, an antibody of the application is eculizumab. In certain embodiments, said antibody is pexelizumab. In certain embodiments, said treatment with eculizumab is chronic. In certain embodiments, said treatment with pexelizumab comprises treating an acute episode.

[0018] In certain embodiments, a mammal of the application is to be treated by administering said antibody intravenously. In certain embodiments, said antibody is to be administered systemically to said mammal. In certain embodiments, said agent is to be administered locally to said mammal.

[0019] In certain embodiments, treatments of the application result in decreased neural injury in a mammal of the application.

[0020] In certain embodiments, a mammal of the application is to be treated to inhibit or decrease the amount and/or extent of an undesirable physiological condition resulting from excess nerve injury due to antibody dependent neuropathy. In certain embodiments, said undesirable physiological condition is selected from the group consisting of ophthalmoplegia, ataxia, areflexia, abnormal muscle coordination, paralysis of the eye muscles, aching pain in the muscles, absence of the tendon reflexes, numbness and tingling felt in the lower extremities, weakness of the distal muscles of the lower extremities, a footdrop, weakness involving the entire lower extremities, weakness involving the upper extremities, weakness in the muscles of respiration, and death.

[0021] In certain embodiments, the antibody for use in accordance with the application results in decreased membrane attack complex (MAC) formation at presynaptic motor axons.

[0022] In certain embodiments, the antibody for use in accordance with the application results in restoration of a normal frequency of miniature endplate potentials.

[0023] In certain embodiments, the antibody for use in accordance with the application results in restoration of synaptic transmission at the neuromuscular junctions.

[0024] In certain embodiments, the antibody for use in accordance with the application results in decreased loss of terminal integrity at the neuromuscular junctions.

[0025] In another embodiment, an anti-C5 antibody for use in treating Guillain-Barré syndrome in a mammal is to be administered to the mammal in a therapeutically effective amount . In another embodiment, an anti-C5 antibody for use in treating the Miller Fisher variant of Guillain-Barré syndrome in a mamal is to be administered to the mammal in a therapeutically effective amount.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026]

FIGURES 1A-1F show an immunohistological demonstration of the protective effect of eculizumab against MAC deposition and terminal motor axonopathy at NMJs in the in *vitro* MFS model. Mouse hemi-

Samsung Bioepis Exhibit No. 1002
Page 1699

diaphragm preparations were pre-incubated with anti-GQ1b ganglioside mAb CGM3 (50μg/ml) and subsequently treated with 40% normal human serum (NHS) with either added eculizumab (anti-human C5 neutralizing mAb; 100μg/ml) or non-specific isotype-matched control mAb (100 μg(ml), and compared with untreated tissue. FIG. 1A shows tissues treated with NHS/eculizumab showed intense C3c staining at neuromuscular junctions (NMJs), similar to that of NHS/control mAb-treated tissues (p=0.12). FIG. 1B shows that membrane attack complex (MAC) staining was not observed in the presence of eculizumab (intensity comparable to baseline, p=0.92), indicating complete inhibition of terminal complement. FIG. 1C shows that axonal integrity was preserved in the presence of eculizumab, as assessed by the presence of neurofilament (NF) signal, comparable to baseline in untreated controls (p=0.80). FIGs. 4D, 4E and 4F are illustrative immunofluorescent images of NMJs in whole-mount muscle demonstrating the protective effect of eculizumab against MAC deposition (FIG. 4D), perisynaptic Schwann cell (pSC) damage (FIG. 4E) and terminal motor axonal damage (FIG. 4F), as compared to NHS/control mAb treatment. Postsynaptic nicotinic acetylcholine receptor (nAChR; purple) staining was used to delineate the NMJ. Intermediate complement protein C3c (FIG. 4D) remains abundantly deposited at NMJs. *p<0.01, different from untreated; #p<0.01, different from control mAb-treated; scale bar = 20 μm.

FIGURES 2A-2G show eculizumab protects against electrophysiological and functional defects induced at NMJs in the *in vitro* MFS model. Mouse hemi-diaphragm preparations were pre-incubated with anti-GQ1b ganglioside mAb CGM3 (50 μg/ml) and subsequently treated with 40% normal human serum (NHS) with either added 100 μg/ml eculizumab (anti-human C5 neutralizing mAb) or non-specific isotype-matched control mAb. FIG. 2A shows spontaneous uniquantal acetylcholine release, measured as MEPP frequency, at the NMJ. Eculizumab prevented the induction of a high MEPP frequency (p<0.001, n=5 muscles). FIG. 2B shows representative 1 s duration traces obtained during incubation with NHS, either with added eculizumab (upper trace) or control mAb (lower trace). FIG. 2C shows asynchronous twitching of muscle fibers induced by NHS was largely prevented by eculizumab (p<0.01, n= 5 muscles). FIG. 2D shows that eculizumab completely prevented the occurrence of 'silent' NMJs (i.e. without detectable synaptic electrophysiological signals). For FIGs. 2E, 2F and 2G hemi-diaphragm muscle-nerve preparations were pre-incubated with 200 μg/ml CGM3 and the protective effect of several concentrations of eculizumab (0-100 μg/ml, n=2-5 muscles per concentration) added to the NHS was observed in muscle contraction force recording experiments. FIG. 2E shows examples of the contraction profiles observed at 0, 30, 60 and 90 min after the start of the NHS incubation with either no eculizumab added or 6 or 100 μg/ml eculizumab added. Each contraction was elicited by supramaximal electric stimulation of the phrenic nerve for 3 s at 40 Hz. FIG. 2F shows development of the contraction loss during NHS incubation with the various concentrations of eculizumab added. FIG. 2G shows the concentration-effect relationship of eculizumab and the protection against loss of contraction after 90 min after the start of the NHS incubation. A Boltzmann sigmoidal curve is fitted through the data points, yielding an EC_{50} of 7.1 μg/ml. Error bars in FIGs. 2A, 2D, 2F and 2G represent S.E.M.

FIGURES 3A-3B show eculizumab dose response curve *in vivo*. FIG. 3A shows that an increasing dose of eculizumab results in a dose-dependent reduction in MAC deposition at the NMJ. FIG. 3B shows the neurofilament signal and demonstrates preservation of axonal integrity at all doses of eculizumab investigated when compared to PBS treated baseline control.

FIGURES 4A-4H show that respiratory paralysis in the *in vivo* MFS model is prevented by eculizumab due to inhibition of presynaptic block of neuromuscular transmission in the diaphragm. Mice (n=6) were intraperitoneally injected with 1.5 mg anti-GQ1b ganglioside mAb CGM3 and, 16 h later, with 0.5 ml 100% normal human serum (NHS) as complement source. A dose of 200 μg eculizumab or control mAb was injected in the tail vein shortly before the NHS injection. FIG. 4A shows grip-strength analysis at 2h post-NHS injection. Eculizumab prevented the loss of pulling force observed in the control mAb group (p<0.01). FIG. 4B shows average tidal volume and FIG. 4C shows breathing rate measured with whole-body plethysmography before NHS injection and during the 2nd, 4th and 6th hour post-NHS injection. The development of breathing difficulty was prevented by eculizumab. FIG. 4D shows examples of 1 s traces of plethysmography signals obtained in eculizumab- and control mAb-treated mice. FIG. 4E shows twitch force and FIG. 4F shows tetanic force elicited in the dissected diaphragm muscle by single and 40 Hz electrical nerve stimulation, respectively. Eculizumab completely prevented the severe paralysis observed in muscles from control mAb-treated mice (p<0.01). FIG. 4G shows that eculizumab almost completely prevented the occurrence of'silent' NMJs (i.e. without detectable synaptic electrophysiological signals) in these mice (p<0.001). FIG. 4H shows thirty superimposed representative traces of 1 s duration obtained in NMJs of muscle from eculizumab-treated (upper traces) or control mAb-treated (lower traces) mice.

Samsung Bioepis Exhibit No. 1002
Page 1700

Error bars in the bar graphs of these figures represent S.E.M.

FIGURES 5A-5F show a morphological analysis of NMJs of diaphragm tissue from *in vivo* MFS mice. Mice were passively immunized with 1.5 mg anti-GQ1b ganglioside mAb, CGM3, followed 16 h later with concomitant injections of 0.5 ml 100% normal human serum (intraperitoneal) and eculizumab or control mAb (intravenous; 200μg). FIGs. 5A and 5B show that C3c and membrane attack complex (MAC) deposition is reduced at neuromuscular junctions (NMJs) of both eculizumab- and control mAb-treated mice ($p < 0.01$). FIG. 5C shows that neurofilament (NF) signal was significantly greater at NMJs of muscles from eculizumab-treated mice, compared with control ($p<0.01$). FIGs. 5D and 5E show illustrative immunofluorescent images of NMJs in whole mount muscle. Postsynaptic nicotinic acetylcholine receptors (nAChR; purple) staining was used to delineate NMJs. FIG. 5D further shows C3c and MAC deposition. FIG. 5E further shows NF integrity and MAC deposition. FIG. 5F is an electron micrograph of eculizumab-protected nerve terminals with tightly packed synaptic vesicle and electron dense mitochondria with parallel cristae. At NMJs from control mAb-treated mice, nerve terminals are electron lucent with sparse synaptic vesicles and swollen mitochondria. #$p<0.01$, different from control mAb-treated. Scale bars in FIGs. 5D and 5E = 20 μm; and in FIG. 5F = 1 μm.

DETAILED DESCRIPTION OF THE INVENTION

Overview

[0027] It is here proposed that treatment of an antibody mediated neuropathy patient with a complement inhibitor will attenuate neural injury. For example, the antibody mediated neuropathy may be an AGA mediated neuropathy. Inhibitors of members of the complement cascade include, for example, antibodies to components such as C5, C5b, C6, C7, C8, and C9. In particular embodiments, this application contemplates the use of the anti-C5 antibody eculizumab (a whole antibody) which is known to inhibit cleavage of C5 into C5a and C5b. Treatment of patients with such inhibitors thereby decreases complement mediated neural injury. Eculizumab has been used in clinical studies and found to be well tolerated with minimal side effects. See U.S. Patent 6,355,245 and Hillmen et al., New Engl. J. Med. 350:552-559 (2004), the contents of which are specifically incorporated herein by reference.

The Complement System

[0028] The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of cellular and viral pathogens. There are at least 25 complement proteins, which are found as a complex collection of plasma proteins and membrane cofactors. The plasma proteins (which are also found in most other body fluids, such as lymph, bone marrow, synovial fluid, and cerebrospinal fluid) make up about 10% of the globulins in vertebrate serum. Complement components achieve their immune defensive functions by interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions.

[0029] The complement cascade progresses via the classical pathway or the alternative pathway. These pathways share many components and, while they differ in their early steps, both converge and share the same terminal complement components responsible for the destruction of target cells and viruses.

[0030] The classical complement pathway is typically initiated by antibody recognition of and binding to an antigenic site on a target cell. This surface bound antibody subsequently reacts with the first component of complement, C1. The C1 thus bound undergoes a set of autocatalytic reactions that result in, inter alia, the induction of C1 proteolytic activity acting on complement components C2 and C4.

[0031] This activated C1 cleaves C2 and C4 into C2a, C2b, C4a, and C4b. The function of C2b is poorly understood. C2a and C4b combine to form the C4b,2a complex, which is an active protease known as classical C3 convertase. C4b,2a acts to cleave C3 into C3a and C3b. C3a and C4a are both relatively weak anaphylatoxins that may induce degranulation of mast cells, resulting in the release of histamine and other mediators of inflammation.

[0032] C3b has multiple functions. As opsonin, it binds to bacteria, viruses and other cells and particles and tags them for removal from the circulation. C3b can also form a complex with C4b,C2a to produce C4b,2a,3b, or classical C5 convertase, which cleaves C5 into C5a (another anaphylatoxin) and C5b. Alternative C5 convertase is C3b,Bb,C3b and performs the same function. C5b combines with C6 yielding C5b,6, and this complex combines with C7 to form the ternary complex C5b,6,7. The C5b,6,7 complex binds C8 at the surface of a cell membrane. Upon binding of C9, the complete membrane attack complex (MAC) is formed (C5b-9) which mediates the lysis of foreign cells, microorganisms, and viruses.

[0033] Further discussions of the classical complement pathway, as well as a detailed description of the alternative pathway of complement activation, can be found in numerous publications including, for example, Muller-Eberhard, Annu Rev Biochem. 1988;57:321-47.

Inhibitors of the Complement Cascade

[0034] Also described herein is that a complement inhibitor may be a small molecule (up to 6,000 Da in mo-

Samsung Bioepis Exhibit No. 1002
Page 1701

lecular weight), a nucleic acid or nucleic acid analog, a peptidomimetic, or a macromolecule that is not a nucleic acid or a protein. These agents include, but are not limited to, small organic molecules, RNA aptamers, L-RNA aptamers, Spiegelmers, antisense compounds, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

[0035] Also described herein is that a complement inhibitor may be a protein or protein fragment. Proteins are known which inhibit the complement cascade, including CD59, CD55, CD46 and other inhibitors of C8 and C9 (see, e.g., U.S. Patent 6,100,443). Proteins known as complement receptors and which bind complement are also known (see, Published PCT Patent Application WO 92/10205 and U.S. Patent 6,057,131). Use of soluble forms of complement receptors, e.g., soluble CR1, can inhibit the consequences of complement activation such as neutrophil oxidative burst, complement mediated neural injury, and C3a and C5a production. Those of skill in the art recognize the above as some, but not all, of the known methods of inhibiting complement and its activation.

[0036] In certain embodiments, a complement inhibitor may be an antibody capable of inhibiting complement, such as an antibody that can block the formation of MAC. For example, an antibody complement inhibitor may include an anti-C5 antibody. Such anti-C5 antibodies may directly interact with C5 and/or C5b, so as to inhibit the formation of and/or physiologic function of C5b.

[0037] Suitable anti-C5 antibodies are known to those of skill in the art. Antibodies can be made to individual components of activated complement (see, e.g., U.S. Patent 6,534,058; published U.S. patent application US 2003/0129187; and U.S. Patent 5,660,825). U.S. Patent 6,355,245 teaches an antibody which binds to C5 and inhibits cleavage into C5a and C5b thereby decreasing the formation not only of C5a but also the downstream complement components.

[0038] The concentration and/or physiologic activity of C5a and C5b in a body fluid can be measured by methods well known in the art. For C5a such methods include chemotaxis assays, RIAs, or ELISAs (see, for example, Ward and Zvaifler, J Clin Invest. 1971 Mar;50(3):606-16; Wurzner, et al., Complement Inflamm. 8:328-340, 1991). For C5b, hemolytic assays or assays for soluble C5b-9 as discussed herein can be used. Other assays known in the art can also be used. Using assays of these or other suitable types, candidate antibodies capable of inhibiting complement such as anti-C5 antibodies, now known or subsequently identified, can be screened in order to 1) identify compounds that are useful in the practice of the application and 2) determine the appropriate dosage levels of such compounds.

[0039] An antibody capable of inhibiting complement such as an anti-C5 antibody affecting C5b is preferably used at concentrations providing substantial reduction (i.e., reduction by at least about 25% as compared to that in the absence of the anti-C5 antibody) in the C5b levels present in at least one blood-derived fluid of the patient following activation of complement within the fluid. Such concentrations can be conveniently determined by measuring the cell-lysing ability (e.g., hemolytic activity) of complement present in the fluid or the levels of soluble C5b-9 present in the fluid. Accordingly, a specific concentration for an antibody that affects C5b is one that results in a substantial reduction (i.e., a reduction by at least about 25%) in the cell-lysing ability of the complement present in at least one of the patient's blood-derived fluids. Reductions of the cell-lysing ability of complement present in the patient's body fluids can be measured by methods well known in the art such as, for example, by a conventional hemolytic assay such as the hemolysis assay described by Kabat and Mayer (eds.), "Experimental Immunochemistry, 2d Edition", 135-240, Springfield, IL, CC Thomas (1961), pages 135-139, or a conventional variation of that assay such as the chicken erythrocyte hemolysis method described below.

[0040] Specific antibodies capable of inhibiting complement, such as an anti-C5 antibody, are relatively specific and do not block the functions of early complement components. In particular, such specific agents will not substantially impair the opsonization functions associated with complement component C3b, which functions provide a means for clearance of foreign particles and substances from the body.

[0041] C3b is generated by the cleavage of C3, which is carried out by classical and/or alternative C3 convertases and results in the generation of both C3a and C3b. Therefore, in order not to impair the opsonization functions associated with C3b, specific antibodies capable of inhibiting complement downstream of C3, such as an anti-C5 antibody, do not substantially interfere with the cleavage of complement component C3 in a body fluid of the patient (e.g., serum) into C3a and C3b. Such interference with the cleavage of C3 can be detected by measuring body fluid levels of C3a and/or C3b, which are produced in equimolar ratios by the actions of the C3 convertases. Such measurements are informative because C3a and C3b levels will be reduced (compared to a matched sample without the antibody capable of inhibiting complement such as an anti-C5 antibody) if cleavage is interfered with by an antibody capable of inhibiting complement.

[0042] In practice, the quantitative measurement of such cleavage is generally more accurate when carried out by the measurement of body fluid C3a levels rather than of body fluid C3b levels, since C3a remains in the fluid phase whereas C3b is rapidly cleared. C3a levels in a body fluid can be measured by methods well known in the art such as, for example, by using a commercially available C3a EIA kit, e.g., that sold by Quidel Corporation, San Diego, Calif., according to the manufacturer's specifications. Particularly specific antibodies capable of inhibiting complement such as an anti-C5 antibody produce essentially no reduction in body fluid C3a levels following complement activation when tested in such as-

Samsung Bioepis Exhibit No. 1002
Page 1702

says.

[0043] Certain antibodies of the disclosure will prevent the cleavage of C5 to form C5a and C5b, thus preventing the generation of the anaphylatoxic activity associated with C5a and preventing the assembly of the membrane attack complex associated with C5b. As discussed above, in a particular embodiment, these anti-C5 antibodies will not impair the opsonization function associated with the action of C3b.

[0044] A preferred method of inhibiting complement activity is to use a monoclonal antibody which binds to complement C5 and inhibits cleavage. This decreases the formation of both C5a and C5b while at the same time allowing the formation of C3a and C3b which are beneficial to the recipient. Such antibodies which are specific to human complement are known (U.S. Patent 6,355,245). These antibodies disclosed in U.S. Patent 6,355,245 include a preferred whole antibody (now named eculizumab). A similar antibody against mouse C5 is called BB5.1 (Frei et al., Mol. Cell. Probes. 1:141-149 (1987)). Antibodies to inhibit complement activity need not be monoclonal antibodies. They can be, e.g., polyclonal antibodies. They may additionally be antibody fragments. An antibody fragment includes, but is not limited to, an Fab, F(ab'), $F(ab')_2$, single-chain antibody, and Fv. Furthermore, it is well known by those of skill in the art that antibodies can be humanized (Jones et al., Nature 321:522-5 (1986)), chimerized, or deimmunized. The antibodies to be used in the present disclosure may be any of these. It is preferable to use humanized antibodies.

[0045] In specific embodiments, a therapeutic agent of the disclosure comprises an antibody or antibody fragment. Antibodies and fragments thereof may be made by any conventional method, such as those methods described herein. Antibodies are found in multiple forms, e.g., IgA, IgG, IgM, etc. Additionally, antibodies can be engineered in numerous ways. They can be made as single-chain antibodies (including small modular immunopharmaceuticals or SMIPS™), Fab and $F(ab')_2$ fragments, etc. Antibodies can be humanized, chimerized, deimmunized, or fully human. Numerous publications set forth the many types of antibodies and the methods of engineering such antibodies. For example, see U.S. Patent Nos. 6,355,245; 6,180,370; 5,693,762; 6,407,213; 6,548,640; 5,565,332; 5,225,539; 6,103,889; and 5,260,203.

[0046] This invention provides fragments of anti-C5 antibodies, which may comprise a portion of an intact antibody, preferably the antigen-binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies (Zapata et al., Protein Eng. 8:1057-1062 (1995)); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

[0047] Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment of an antibody yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

[0048] "Fv" refers to the minimum antibody fragment that contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although likely at a lower affinity than the entire binding site.

[0049] The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments that have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

[0050] "Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains that enables the scFv to form the desired structure for antigen binding. For a review of scFv see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore, eds. (Springer-Verlag: New York, 1994), pp. 269-315.

[0051] SMIPs are a class of single-chain peptide engineered to include a target binding region, effector domain (CH2 and CH3 domains). *See, e.g.,* U.S. Patent Application Publication No. 20050238646. The target binding region may be derived from the variable region or CDRs of an antibody, e.g., an anti-C5 antibody of the application. Alternatively, the target binding region is derived from a protein that binds C5.

[0052] The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger

Samsung Bioepis Exhibit No. 1002
Page 1703

et al., Proc. Natl. Acad. Sci. USA, 90: 6444-6448 (1993).

[0053] It is well known that the binding to a molecule (or a pathogen) of antibodies with an Fc region assists in the processing and clearance of the molecule (or pathogen). The Fc portions of antibodies are recognized by specialized receptors expressed by immune effector cells. The Fc portions of IgG1 and IgG3 antibodies are recognized by Fc receptors present on the surface of phagocytic cells such as macrophages and neutrophils, which can thereby bind and engulf the molecules or pathogens coated with antibodies of these isotypes (C. A. Janeway et al., Immunobiology 5th edition, page 147, Garland Publishing (New York, 2001)).

[0054] This disclosure also provides monoclonal anti-C5 antibodies. A monoclonal antibody can be obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are often synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. Monoclonal antibodies may also be produced in transfected cells, such as CHO cells and NS0 cells. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies and does not require production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present disclosure may be made by the hybridoma method first described by Kohler et al., Nature 256:495-497 (1975), or may be made by recombinant DNA methods (*see, e.g.,* U.S. Patent Nos. 4,816,567 and 6,331,415). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., Nature 352:624-628 (1991) and Marks et al., J. Mol. Biol. 222:581-597 (1991), for example.

[0055] A description of the preparation of a mouse anti-human-C5 monoclonal antibody with specific binding characteristics is presented in U.S. Patent Application Publication No. 20050226870. Wurzner et al., Complement Inflamm. 8:328-340 (1991), describe the preparation of other mouse anti-human-C5 monoclonal antibodies referred to as N19-8 and N20-9.

[0056] Other antibodies specifically contemplated are "oligoclonal" antibodies. As used herein, the term "oligoclonal" antibodies" refers to a predetermined mixture of distinct monoclonal antibodies. *See, e.g.,* PCT publication WO 95/20401; U.S. Patent Nos. 5,789,208 and 6,335,163. In one embodiment, oligoclonal antibodies consisting of a predetermined mixture of antibodies against one or more epitopes are generated in a single cell. In other embodiments, oligoclonal antibodies comprise a plurality of heavy chains capable of pairing with a common light chain to generate antibodies with multiple specificities (*e.g.*, PCT publication WO 04/009618). Oligoclonal antibodies are particularly useful when it is desired to target multiple epitopes on a single target molecule (*e.g.*, C5). In view of the assays and epitopes disclosed herein, those skilled in the art can generate or select antibodies or mixtures of antibodies that are applicable for an intended purpose and desired need.

[0057] In certain embodiments that include a humanized and/or chimeric antibody, one or more of the CDRs are derived from an anti-human C5 antibody. In a specific embodiment, all of the CDRs are derived from an anti-human C5 antibody. In another specific embodiment, the CDRs from more than one anti-human C5 antibody are mixed and matched in a chimeric antibody. For instance, a chimeric antibody may comprise a CDR1 from the light chain of a first anti-human C5 antibody combined with CDR2 and CDR3 from the light chain of a second anti-human C5 antibody, and the CDRs from the heavy chain may be derived from a third anti-human C5 antibody. Further, the framework regions may be derived from one of the same anti-human C5 antibodies, from one or more different antibodies, such as a human antibody, or from a humanized antibody. Human or humanized antibodies are specific for administration to human patients.

[0058] In certain embodiments, single chain antibodies, and chimeric, humanized or primatized (CDR-grafted) antibodies, as well as chimeric or CDR-grafted single chain antibodies, comprising portions derived from different species, are also encompassed by the present disclosure as antigen-binding fragments of an antibody. The various portions of these antibodies can be joined together chemically by conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques. For example, nucleic acids encoding a chimeric or humanized chain can be expressed to produce a contiguous protein. See, e.g., U.S. Pat. Nos. 4,816,567 and 6,331,415; U.S. Pat. No. 4,816,397; European Patent No. 0,120,694; WO 86/01533; European Patent No. 0,194,276 B1; U.S. Pat. No. 5,225,539; and European Patent No. 0,239,400 B1. See also, Newman et al., BioTechnology 10:1455-1460 (1992), regarding primatized antibody. See, e.g., Ladner et al., U.S. Pat. No. 4,946,778; and Bird et al., Science 242:423-426 (1988), regarding single chain antibodies.

[0059] In addition, functional fragments of antibodies, including fragments of chimeric, humanized, primatized or single chain antibodies, can also be produced. Functional fragments of the subject antibodies retain at least one binding function and/or modulation function of the full-length antibody from which they are derived. Preferred functional fragments retain an antigen-binding function of a corresponding full-length antibody (such as for example, ability of anti-C5 antibody to bind C5).

[0060] General methods for the immunization of ani-

Samsung Bioepis Exhibit No. 1002
Page 1704

mals (in this case with C5 and/or C5b, etc.), isolation of antibody producing cells, fusion of such cells with immortal cells (e.g., myeloma cells) to generate hybridomas secreting monoclonal antibodies, screening of hybridoma supernatants for reactivity of secreted monoclonal antibodies with a desired antigen (in this case the immunogen or a molecule containing the immunogen), the preparation of quantities of such antibodies in hybridoma supernatants or ascites fluids, and for the purification and storage of such monoclonal antibodies, can be found in numerous publications. These include: Coligan, et al., eds. Current Protocols In Immunology, John Wiley & Sons, New York, 1992; Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988; Liddell and Cryer, A Practical Guide To Monoclonal Antibodies, John Wiley & Sons, Chichester, West Sussex, England, 1991; Montz et al., Cellular Immunol. 127:337-351 (1990); Wurzner et al., Complement Inflamm. 8:328-340 (1991); and Mollnes et al., Scand. J. Immunol. 28:307-312 (1988).

Pharmaceutical Formulations and Uses

[0061] Methods of administration of small molecules, proteins, and nucleic acids are well-known to those of skill in the art. Methods of administration of antibodies are well-known to those of skill in the art. To achieve the desired inhibition, the antibodies can be administered in a variety of unit dosage forms. The dose will vary according to the particular antibody. For example, different antibodies may have different masses and/or affinities, and thus require different dosage levels. Antibodies prepared as Fab fragments will also require differing dosages than the equivalent intact immunoglobulins, as they are of considerably smaller mass than intact immunoglobulins, and thus require lower dosages to reach the same molar levels in the patient's blood. The dose will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician. Dosage levels of the antibodies for human subjects are generally between about 1 mg per kg and about 100 mg per kg per patient per treatment, and preferably between about 5 mg per kg and about 50 mg per kg per patient per treatment. In terms of plasma concentrations, the antibody concentrations are preferably in the range from about 25 μg/mL to about 500 μg/mL. However, greater amounts may be required for extreme cases and smaller amounts may be sufficient for milder cases.

[0062] Administration of the anti-C5 antibodies will generally be performed by an intravascular route, e.g., via intravenous infusion by injection. Other routes of administration may be used if desired but an intravenous route will be the most preferable. Formulations suitable for injection are found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, Pa., 17th ed. (1985). Such formulations must be sterile and non-pyrogenic, and generally will include a pharmaceutically effective carrier, such as saline, buffered (e.g., phosphate buffered) saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions, and the like. The formulations may contain pharmaceutically acceptable auxiliary substances as required, such as, tonicity adjusting agents, wetting agents, bactericidal agents, preservatives, stabilizers, and the like.

[0063] Administration of the antibodies capable of inhibiting complement such as an anti-C5 antibody will generally be performed by a parenteral route, typically via injection such as intra-articular or intravascular injection (e.g., intravenous infusion) or intramuscular injection. Other routes of administration, e.g., oral (p.o.), may be used if desired and practicable for the particular antibody capable of inhibiting complement to be administered. Antibodies capable of inhibiting complement such as an anti-C5 antibody can also be administered in a variety of unit dosage forms and their dosages will also vary with the size, potency, and in vivo half-life of the particular antibody capable of inhibiting complement being administered. Doses of antibodies capable of inhibiting complement such as an anti-C5 antibody will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician.

[0064] In certain embodiments, a typical therapeutic treatment includes a series of doses, which will usually be administered concurrently with the monitoring of clinical endpoints with the dosage levels adjusted as needed to achieve the desired clinical outcome. In certain embodiments, treatment is administered in multiple dosages over at least a week. In certain embodiments, treatment is administered in multiple dosages over at least a month. In certain embodiments, treatment is administered in multiple dosages over at least a year. In certain embodiments, treatment is administered in multiple dosages over the remainder of the patient's life.

[0065] The frequency of administration may also be adjusted according to various parameters. These include the clinical response, the plasma half-life of the therapeutic of the disclosure, and the levels of the antibody in a body fluid, such as, blood, plasma, serum, or synovial fluid. To guide adjustment of the frequency of administration, levels of the therapeutic of the disclosure in the body fluid may be monitored during the course of treatment.

[0066] In certain embodiments, the frequency of administration may be adjusted according to an assay measuring cell-lysing ability of complement present in one or more of the patient's body fluids. The cell-lysing ability can be measured as percent hemolysis in hemolytic assays of the types described herein. A 10% or 25% or 50% reduction in the cell-lysing ability of complement present in a body fluid after treatment with the antibody capable of inhibiting complement used in the practice of the application means that the percent hemolysis after

Samsung Bioepis Exhibit No. 1002
Page 1705

treatment is 90, 75, or 50 percent, respectively, of the percent hemolysis before treatment.

[0067] For the treatment of antibody mediated neuropathies by systemic administration of an antibody capable of inhibiting complement such as an anti-C5 antibody (as opposed to local administration), administration of a large initial dose is specific, i.e., a single initial dose sufficient to yield a substantial reduction, and more preferably an at least about 50% reduction, in the hemolytic activity of the patient's serum. Such a large initial dose is preferably followed by regularly repeated administration of tapered doses as needed to maintain substantial reductions of serum hemolytic titer. In another embodiment, the initial dose is given by both local and systemic routes, followed by repeated systemic administration of tapered doses as described above. For administration of an anti-C5 antibody to humans, see, e.g., Hillmen et al., N. Engl. J. Med. 350:552-559 (2004). The levels of antibody administered in Hillmen et al. are based on half-life of the antibody and are relevant to administration of an anti-C5 antibody for other indications such as for treating neuropathies.

[0068] Formulations particularly useful for antibody-based therapeutic agents are also described in U.S. Patent App. Publication Nos. 20030202972, 20040091490 and 20050158316. In certain embodiments, the liquid formulations of the application are substantially free of surfactant and/or inorganic salts. In another specific embodiment, the liquid formulations have a pH ranging from about 5.0 to about 7.0. In yet another specific embodiment, the liquid formulations comprise histidine at a concentration ranging from about 1 mM to about 100 mM. It is also contemplated that the liquid formulations may further comprise one or more excipients such as a saccharide, an amino acid (e.g., arginine, lysine, and methionine) and a polyol. Additional descriptions and methods of preparing and analyzing liquid formulations can be found, for example, in PCT publications WO 03/106644, WO 04/066957, and WO 04/091658.

[0069] Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the pharmaceutical compositions of the application.

[0070] In certain embodiments, formulations of the subject antibodies are pyrogen-free formulations which are substantially free of endotoxins and/or related pyrogenic substances. Endotoxins include toxins that are confined inside microorganisms and are released when the microorganisms are broken down or die. Pyrogenic substances also include fever-inducing, thermostable substances (glycoproteins) from the outer membrane of bacteria and other microorganisms. Both of these substances can cause fever, hypotension and shock if administered to humans. Due to the potential harmful effects, it is advantageous to remove even low amounts of endotoxins from intravenously administered pharmaceutical drug solutions. The Food & Drug Administration ("FDA") has set an upper limit of 5 endotoxin units (EU) per dose per kilogram body weight in a single one hour period for intravenous drug applications (The United States Pharmacopeial Convention, Pharmacopeial Forum 26 (1):223 (2000)). When therapeutic proteins are administered in amounts of several hundred or thousand milligrams per kilogram body weight, as can be the case with monoclonal antibodies, it is advantageous to remove even trace amounts of endotoxin.

[0071] Formulations of the subject antibodies include those suitable for oral, dietary, topical, parenteral (e.g., intravenous, intraarterial, intramuscular, subcutaneous injection), ophthalmologic (e.g., topical or intraocular), inhalation (e.g., intrabronchial, intranasal or oral inhalation, intranasal drops), rectal, and/or intravaginal administration. Other suitable methods of administration can also include rechargeable or biodegradable devices and controlled release polymeric devices. Stents, in particular, may be coated with a controlled release polymer mixed with an agent of the application. The pharmaceutical compositions of this disclosure can also be administered as part of a combinatorial therapy with other agents (either in the same formulation or in a separate formulation).

[0072] The amount of the formulation which will be therapeutically effective can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems. The dosage of the compositions to be administered can be determined by the skilled artisan without undue experimentation in conjunction with standard dose-response studies. Relevant circumstances to be considered in making those determinations include the condition or conditions to be treated, the choice of composition to be administered, the age, weight, and response of the individual patient, and the severity of the patient's symptoms. For example, the actual patient body weight may be used to calculate the dose of the formulations in milliliters (mL) to be administered. There may be no downward adjustment to "ideal" weight. In such a situation, an appropriate dose may be calculated by the following formula: Dose (mL) = [patient weight (kg) x dose level (mg/kg)/ drug concentration (mg/mL)]

[0073] To achieve the desired treatment results, anti-C5 antibodies can be administered in a variety of unit dosage forms. The dose will vary according to the particular antibody. For example, different antibodies may have different masses and/or affinities, and thus require different dosage levels. Antibodies prepared as Fab' fragments or single chain antibodies will also require differing dosages than the equivalent native immunoglobulins, as

Samsung Bioepis Exhibit No. 1002
Page 1706

they are of considerably smaller mass than native immunoglobulins, and thus require lower dosages to reach the same molar levels in the patient's blood.

[0074] Other therapeutics of the disclosure can also be administered in a variety of unit dosage forms and their dosages will also vary with the size, potency, and *in vivo* half-life of the particular therapeutic being administered.

[0075] Doses of therapeutics of the disclosure will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician.

[0076] The formulations of the application can be distributed as articles of manufacture comprising packaging material and a pharmaceutical agent which comprises the antibody capable of inhibiting complement and a pharmaceutically acceptable carrier as appropriate to the mode of administration. The packaging material may include a label which indicates that the formulation is for use in the treatment of antibody mediated neuropathies. Although antibodies are preferred, especially anti-C5 antibodies which have already been shown to be safe and effective at decreasing the accumulation of downstream complement components in persons, the use of other complement inhibitors is also contemplated by this disclosure. The pharmaceutical formulations and uses of the disclosure may be combined with any known complement inhibitors or antibody mediated neuropathy treatments known in the art (supra).

[0077] Treatments of the application may be used in combination with other treatments of the application or known treatments for antibody mediated neuropathies. Treatments of the application may be co-administered with other treatments that treat symptoms of antibody mediated neuropathies. Administration of the therapeutics of the disclosure will generally be performed by a parenteral route, typically via injection such as intra-articular or intravascular injection (e.g., intravenous infusion) or intramuscular injection. Other routes of administration, e.g., oral (p.o.), may be used if desired and practicable for the particular antibody capable of inhibiting complement to be administered.

[0078] In case of conflict, the present application, including any definitions herein, will control.

[0079] The present methods are described with reference to the following Examples, which are offered by way of illustration and are not intended to limit the disclosure in any manner. Standard techniques well known in the art or the techniques specifically described below are utilized.

EXAMPLES

EXAMPLE 1. ECULIZUMAB PREVENTS COMPLEMENT-MEDIATED STRUCTURAL AND FUNCTIONAL LESIONS IN THE IN VITRO MFS MODEL

[0080] In the *in vitro* MFS model, treatment of diaphragm nerve-muscle preparations with CGM3 anti-GQ1b mAb and normal human serum (NHS), in the presence of an irrelevant isotype matched mAb (100 μg/ml), that served as a negative control for eculizumab, induced complement activation at NMJs. This was morphologically evidenced by C3c and MAC deposition (FIGs. 1A, 1B, 1D, 1E and 1F), as well as damaged perisynaptic Schwann cells (pSCs) and motor nerve terminals shown by ethidium homodimer-1 (EthD-1) staining and loss of neurofilament (NF) staining, respectively (FIGs. 1C, 1E and 1F). These features were identical to those reported before (Goodyear et al. J. Clin. Invest 104:697-708 (1999); Halstead et al. Brain 127:2109-2123 (2004); O'Hanlon et al. Brain 124:893-906 (2001)). The addition of 100 μg/ml eculizumab to the NHS as complement source completely prevented MAC deposition (FIGs. 1B, 1D, 1E and 1F; $p<0.001$) and terminal axonal NF loss (FIGs. 1C and 1F; $p<0.001$), while the deposition of the earlier complement component C3c was not affected (FIGs. 1A and 1D; $p= 0.2$). Furthermore, damage to pSCs was abolished as only 2% of the 1439 NMJs investigated had one or more EthD-1 positive nuclei, compared with 33% of 1529 NMJs investigated in control mAb-treated tissue (FIG. 1E; $p<0.001$).

[0081] These immunohistological observations paralleled the electrophysiological and functional observations. At CGM3 pre-incubated NMJs, NHS (40%) in the presence of the control mAb induced a large increase in the frequency of miniature endplate potentials (MEPPs, the postsynaptic events arising from spontaneous release of single acetylcholine quanta from the presynaptic motor nerve terminal), as previously observed (Goodyear et al. J. Clin. Invest 104:697-708 (1999)). This effect was almost completely prevented by 100 μg/ml eculizumab. The MEPP frequency was about 0.7 s^{-1} before and after 50 μg/ml CGM3 incubation and rose to 34.0 ± 4.6 s^{-1} in the presence of NHS with 100 μg/ml control mAb added. By contrast the MEPP frequency was only 3.1 ± 1.3 s^{-1} in the presence of NHS with 100 μg/ml eculizumab added (FIGs. 2A and 2B; n=5 muscles; $p<0.001$).

[0082] Eculizumab did not alter the muscle fiber resting membrane potential or MEPP amplitude, rise- and decay times (data not shown). As described before (Goodyear et al. J. Clin. Invest 104:697-708 (1999); Plomp et al. Ann. Neurol. 45:189-199 (1999)), asynchronous twitching of individual muscle fiber occurs during NHS incubation (median visual score 4; n= 5 muscles). Eculizumab prevented such high grade of twitching with only a median score of 1 being observed (FIG. 2C; n=5 muscles, $p<0.01$, Mann-Whitney test), which equals the low basal levels observed in the observation periods before and

Samsung Bioepis Exhibit No. 1002
Page 1707

after CGM3 incubation.

[0083] The final result of anti-CGM3/complement-mediated presynaptic damage is block of synaptic transmission at the NMJ due to the inability to release acetylcholine (Goodyear et al. J. Clin. Invest 104:697-708 (1999)). Eculizumab completely prevented this effect. No 'silent' NMJs (i.e. absence of MEPPs and nerve-stimulation evoked muscle action potentials) were encountered, whereas in the control mAb condition 19 ± 2.6 % of the total number of NMJs sampled were observed to be silenced (FIG. 2D; n=5 muscles). Furthermore, we quantified the protective effect of eculizumab on muscle paralysis in the *in vitro* MFS model. Eculizumab inhibited loss of nerve stimulation-induced contraction force of hemi-diaphragm preparations in a concentration dependent manner following 200 μg/ml CGM3 and 33% NHS treatment (FIGs. 2E and 2F). In the control experiments without eculizumab, NHS induced almost complete loss of contraction force within 90 min. Addition of 3 or 6 μg/ml eculizumab to the NHS slowed down the rate of contraction force loss considerably (50% loss was observed at 39 and 60 min, respectively, compared to 27 min in the control condition; FIG. 2F). Higher eculizumab concentrations of 9 and 12 μg/ml were almost completely protective (>90% of the initial contraction left after 90 min) while 25, 50 and 100 μg/ml completely prevented any CGM3/NHS-induced loss of contraction. A Boltzmann sigmoidal curve fitted through the obtained concentration-effect data points yielded an EC_{50} of 7.1 μg/ml for eculizumab under these conditions (FIG. 2G).

[0084] These immunohistological and functional analyses show that eculizumab efficiently prevents complement-mediated pathophysiological effects in the *in vitro* MFS model.

EXAMPLE 2. ECULIZUMAB DOSE RESPONSE CURVE *IN VIVO*.

[0085] Next the *in vivo* benefits of eculizumab in this murine model of Guillain-Barre syndrome were examined. Mice were passively immunized with anti-ganglioside antibody or PBS (as a negative control) followed by concomitant injections of NHS (intraperitoneal) and eculizumab (intravenous) at 0, 50, 100, 200, and 400 μg/mouse in PBS. Complement C3 was richly deposited at the NMJ of all mice receiving anti-ganglioside antibody. An increasing dose of eculizumab results in a dose-dependent reduction in MAC deposition at the NMJ (FIG. 3A). Examination of the neurofilament signal demonstrates preservation of axonal integrity at all doses of eculizumab investigated when compared to PBS treated baseline control. (*n*=3 for each dose) (FIG. 3B).

EXAMPLE 3. ECULIZUMAB PROTECTS AGAINST NEUROPATHY AND RESPIRATORY PARALYSIS IN AN IN VIVO MFS MODEL

[0086] In order to determine the *in vivo* efficacy of eculizumab, we generated an *in vivo* MFS mouse model through intraperitoneal injection of CGM3 anti-GQ1b antibody and NHS as complement source. At 16 h after the CGM3 injection, a dose of 200 μg eculizumab or control mAb was administered systemically, via the tail vein, followed by intraperitoneal injection of 0.5 ml NHS). The protocol of different injection routes of eculizumab and NHS was applied to avoid the immediate inhibition of C5 in the NHS by eculizumab within the confinement of the peritoneal cavity. Mice treated with CGM3, NHS and control mAb (n=10) developed a general weak appearance, in some cases a low-back posture, invaginated abdominal flanks and breathing difficulties (panting) within 2 h following the NHS injection. Intravenous eculizumab injection completely prevented the development of these symptoms (n=11). We quantified weakness with grip-strength measurement in CGM3 pre-treated mice, before and 2h after injection of control mAb or eculizumab and NHS (FIG. 4A). Control group mice (n=5) pulled 56.0 ± 5.7 g just before the control mAb and NHS injection, while the eculizumab group mice (n=5) pulled 54.3 ± 8.4 g (p= 0.87) at this stage. The pulling force 2h after control mAb/NHS injection was 22% lower (43.7 ± 4.5 g; p<0.005). Eculizumab ameliorated such an effect (8% reduction; p=0.07). Respiratory disturbance was assessed with whole-body plethysmography continuously after mAb/NHS injection for a period of 6h (FIGs. 4B and 4C; n=5 mice per group). The tidal volume before CGM3 injection was similar in both groups (0.21 ± 0.01 and 0.22 ± 0.03 ml in the control mAb and eculizumab group, respectively). A reduction of approximately 50% (p<0.001) was observed in the control mAb group at 4 and 6 h after NHS injection. Such a decrease was largely prevented by eculizumab (only 17% reduction at these time points, FIG. 4B). Similarly, respiration rate was depressed in the control mAb group by approximately 30% (p<0.01) at 4 and 6 h post-NHS injection. In the eculizumab group, this reduction was only 7% (FIG. 4C). Both treatment groups showed an equal initial reduction of about 40% of the respiration rate when measured 2 h post mAb/NHS, compared to the rate before CGM3 injection, which apparently was due to the intraperitoneal injection regimen. Example traces of the respiration signals obtained are shown in FIG. 4D.

[0087] The hemi-diaphragm phrenic nerve preparations were dissected from in *vivo* CGM3/NHS treated mice at 4 h following NHS injection and performed electrophysiological measurements at NMJs, muscle contraction experiments, and detailed immunohistological analyses. Visual inspection indicated that only a very small part of the muscles from control mAb-treated mice contracted upon supramaximal electrical stimulation of the phrenic nerve, as compared to a complete contraction of muscles obtained from eculizumab-treated mice. In *in vitro* contraction experiments in tissues harvested from passive immunizations, this effect was quantified (FIGs. 4E and 4F). Twitch and tetanic (40Hz) tension were 0.07 ± 0.06 and 0.92 ± 0.61 g, respectively, in the control

Samsung Bioepis Exhibit No. 1002
Page 1708

mAb group (n=4), while in the eculizumab group (n=4) these were 1.36 ± 0.29 and 9.26 ± 0.65 g, respectively, equalling the values of muscles from non-NHS treated age-matched mice (data not shown). With electrophysiological analysis we tested whether paralysis was caused by NMJ dysfunction. In dissected muscles from the control mAb group 66 ± 7% of the sampled NMJs had been 'silenced', i.e. there were no detectable MEPPs and no muscle action potentials upon nerve stimulations. In the eculizumab group only 3 ± 2% had been 'silenced' (FIGs. 4G and 4H; $p<0.001$). Immunohistological analysis showed that CGM3 was deposited equally at NMJs of the two treatment groups (data not shown). C3c deposits were identified at NMJs of both groups, albeit with a somewhat less intensity in the eculizumab group (FIGs. 5A and 5D; $p<0.001$). MAC was clearly deposited at NMJs of muscles from control mAb-treated mice but virtually absent in the eculizumab-treated group (FIGs. 5B, 5D and 5E). Eculizumab prevented loss of terminal integrity at NMJs of control-mAb-treated mice, as evidenced by the NF signal intensity and pattern overlying the endplate region, i.e. terminal axonal branching clearly remaining intact (FIGs. 5C and 5E). In addition, electron microscopy showed the presence of a well-preserved presynaptic ultrastructure at the NMJ of eculizumab-treated mice in comparison with that of control-mAb-treated mice which showed characteristic terminal swelling, synaptic vesicle depletion and swollen mitochondria (FIG. 5F) (O'Hanlon et al. Brain 124:893-906 (2001)). These electrophysiological, functional and immunohistological data show that complement-mediated terminal motor neuropathy occurs in our *in vivo* MFS model and that diaphragm paralysis contributes to the observed respiratory deficits. Most importantly, *in vivo* eculizumab treatment effectively prevented these *in vivo* neuropathological defects.

EXAMPLE 4. METHODS

Mice

[0088] Male Balb/c mice (3-6 weeks old, 10-25 g) were obtained from Harlan (UK or NL). In some muscle contraction experiments (see below) male and female GM2/GD2-synthase *null*-mutant mice (Bullens et al. J. Neurosci. 22:6876-6884 (2002)) were used at 12-22 weeks of age (19-37 g). All animal experiments were carried out in accordance to UK Home Office guidelines (UK PPL60/3096), Dutch law and Glasgow and Leiden University guidelines.

Monoclonal antibodies and normal human serum

[0089] The IgM anti-GQ1b ganglioside mAb, CGM3 was derived from mice inoculated with a GT1a-bearing *Campylobacter jejuni* lipooligosaccharide (Goodyear et al. J. Clin. Invest 104:697-708 (1999)). CGM3 reacts with gangliosides GQ1b, GD3 and GT1a that all share the terminal disialylgalactose structure. Previous studies have shown that CGM3 has similar ganglioside specificity and induced identical complement-dependent pathogenic effects as human MFS sera (Goodyear et al. J. Clin. Invest 104:697-708 (1999)). CGM3 concentration was measured using quantitative ELISA (Bethyl Laboratories, Texas, USA). Normal human serum (NHS) was taken from a single donor stock that had been freshly frozen and stored in multiple aliquots at -70°C to preserve complement activity. Prior to experimental use, CGM3 and NHS were dialyzed for 24 h at 4°C against Ringer's solution (116 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM NaH_2PO_4, 23 mM $NaHCO_3$, 11 mM glucose, pH 7.4), pre-gassed with 95% O_2 /5% CO_2. The humanized anti-human C5 mAb eculizumab and a non-specific isotype-matched control mAb ALXN3300 were obtained from Alexion Pharmaceuticals (Cheshire, USA) and stored at 4°C

In vitro MFS model

[0090] The *in vitro* model for MFS using CGM3 and NHS has been described before (Goodyear et al. J. Clin. Invest 104:697-708 (1999); O'Hanlon et al. Brain 124:893-906 (2001)). In short, mouse hemi-diaphragms with phrenic nerve attached (or triangularis sterni muscle in some cases for illustrative NMJ immunohistology) were dissected and mounted in Ringer's medium at room temperature (20-22 °C). Untreated small control sections were removed from each muscle preparation prior to any incubations and snap frozen on dry ice for subsequent baseline immunohistological analysis. Muscles were incubated with CGM3 (50 μg/ml) for 2-2.5 h at 32°C, then for 30 min at 4°C and then equilibrated for 10 mins at room temperature, rinsed in Ringer's medium and subsequently exposed to 33 or 40% NHS in Ringer's medium for 1 h at room temperature. Eculizumab (100 μg/ml) or the control mAb (100 μg/ml) was mixed with NHS 10 min prior to the incubation of the muscle preparation. *In vitro* electrophysiological measurements at the NMJ (see below) and scoring of muscle fiber twitchings, a hallmark of uncontrolled transmitter release at NMJs in this model (Jacobs et al. Muscle Nerve 25:549-558 (2002); O'Hanlon et al. Brain 124:893-906 (2001)), were performed before and after CGM3 incubation, and during the l h NHS incubation. Muscle strips were frozen on dry ice and stored at -20°C, both before and after incubation with NHS. NMJs and terminal motor axons were assessed for levels of IgM, C3c, MAC, NF and for pSC viability (see below).

In vivo MFS model

[0091] Balb/c mice (3-4 weeks old, 10-15 g) were injected intraperitoneally with 1.5 mg CGM3, followed 16 h later by an intraperitoneal injection of 0.5 ml 100% NHS. Mice were observed for a further 4-6 h and analyzed with whole-body plethysmography (see below) and grip-

Samsung Bioepis Exhibit No. 1002
Page 1709

strength testing as described before (Kaja et al. Eur J. Neurosci 25:2009-2020 (2007)). The mice were then killed by CO_2 asphyxiation and hemi-diaphragm muscle tissue was dissected and analyzed in electrophysiological and muscle contraction experiments (see below) or further processed for immunohistological analyses (see below). The effect of eculizumab on the *in vivo* symptoms and the subsequently analyzed electrophysiological, functional and histological lesions was tested in this model by injecting 200 μg eculizumab or control mAb in the tail vein, shortly before intraperitoneal NHS injection. Initial dose-range finding experiments (data not shown) indicated that eculizumab at doses of >50 μg/mouse protected against neuropathy.

Plethysmography

[0092] Non-invasive whole-body plethysmography (EMMS, Hants, UK) was employed to demonstrate changes in breathing parameters in *in vivo* experiments. Baseline data were collected prior to onset of experiment. Mice were injected with CGM3 followed by NHS with eculizumab or control mAb 16 h later (protocol as above) and monitored continuously for 6 h. Flow derived parameters of breath frequency and tidal volume were collected from 25 accepted breaths and averaged over 1 h periods.

In vitro electrophysiological analysis of NMJs and visual scoring of muscle contractility

[0093] Left and right hemi-diaphragms with their phrenic nerve attached were dissected and pinned out in a silicone rubber-lined dish in 1.5 ml Ringer's medium at room temperature. Intracellular recordings of miniature endplate potentials (MEPPs, the postsynaptic events arising from spontaneous release of single acetylcholine quanta from the presynaptic motor nerve terminal) were made at NMJs at 20-22°C. Muscle fibers were impaled near the NMJ with a 10-20 MΩ glass micro-electrode filled with 3 M KCl, connected to a GeneClamp 500B amplifier (Axon Instruments/Molecular Devices, Union City, CA, USA) for amplifying and filtering (10 kHz low-pass) of the signal. Signals were digitized, stored and analyzed (off-line) using a Digidata 1322A interface, Clampex 9.2 and Clampfit 9.2 programs (all from Axon Instruments/Molecular Devices), Mini Analysis 6.0 (Synaptosoft, Fort Lee, USA) and routines programmed in Matlab (The MathWorks Inc., Natick, MA, USA).

[0094] The occurrence of spontaneous asynchronous fiber twitches that are induced by a high MEPP frequency resulting from CGM3/complement-mediated presynaptic damage (Plomp et al. Ann. Neurol. 45:189-199 (1999)) was scored visually every 5 min (O'Hanlon et al. Brain 124:893-906 (2001)). The tissue scored 0 for no activity, 1 for the twitching of <10 fibers, 2 for a small amount of twitching across the tissue, 3 for a moderate amount and 4 for an extensive amount. The average score during each measuring period was calculated. The final result of CGM3/complement-mediated presynaptic damage is a silencing of synaptic transmission at the NMJ. The number of 'silent' NMJs (i.e. without detectable MEPPs and occurrence of phrenic nerve-stimulation evoked muscle action potentials) was expressed as percentage of the total number of NMJs sampled (8-41) in a muscle under each experimental condition.

Muscle contraction experiments

[0095] The protective efficacy of eculizumab on *in vitro* CGM3/NHS-induced loss of nerve stimulation-evoked contraction force of the hemi-diaphragm preparation was tested. We used diaphragm from GM2/GD2-synthase null-mutant mice because earlier studies showed a somewhat higher sensitivity of muscle of this strain to CGM3/NHS-induced loss of contraction (Bullens et al. J. Neurosci. 22:6876-6884 (2002)). Left and right phrenic nerve/hemi-diaphragm preparations that had been pre-incubated with 200 μg/ml CGM3 for 3 h at 32 °C were mounted in a silicone-rubber lined dish containing 2.25 ml Ringer's medium at room temperature (20-22 °C), continuously bubbled with 95% O_2 / 5% CO_2. The ribcage side of the muscle was thoroughly fixed to the bottom of the dish by multiple small pins and the central tendon was connected via a metal hook and a string to a force transducer that was connected to an amplifier and digitizing equipment, as described earlier (Kaja et al. Eur J. Neurosci 25:2009-2020 (2007)). Supramaximal stimuli (usually ~10 V) of 100 μs duration were delivered every 5 min for 3 s at 40 Hz from a Master-8 programmable stimulator (AMPI, Jerusalem, Israel). Basic tension was adjusted with a vernier control to obtain maximal stimulated tetanic contraction force (usually about 10 g). Stability of the elicited contraction was monitored for 30-50 min. Subsequently, the medium was replaced by NHS (33%) to which eculizumab (3, 6, 9, 12, 25, 50 or 100 μg/ml) had been added and admixed for ~10 min prior to addition and the effect was monitored for 100-200 min, under continuous gentle bubbling with 95% O_2 / 5% CO_2. In the control experiments without added eculizumab, 100 μg/ml of the control mAb was added to the NHS. The amplitude of contractions was cursor-measured off-line in the Clampfit 9.0 program (Axon Instruments/Molecular Devices, Union City, USA), at 2 s after the start of each nerve stimulation train.

[0096] Tetanic and twitch contraction forces were also measured upon *in vitro* nerve stimulation of left hemi-diaphragm muscles that were dissected from mice that had been subjected to the *in vivo* MFS protocol.

Immunohistological analyses

[0097] Unfixed hemi-diaphragm sections were mounted in Lipshaw's M-1 mounting medium (Pittsburgh, PA, USA), and longitudinal cryostat sections (8-20 μm) were cut onto 3-aminopropyltriethoxysilane coated slides, air-dried, then stored at -20°C. To localize NMJs, TRITC and

Samsung Bioepis Exhibit No. 1002
Page 1710

bodipy-labelled α-bungarotoxin (α-BTx, diluted 1/750 to 1.3 μg/ml; Molecular Probes, Eugene, Oregon) were used. The intermediate complement component C3c was detected by incubation with FITC-labelled rabbit anti-C3c (1/300; Dako, Ely, UK) for 1 h at 4°C. MAC was detected using mouse anti-human C5b-9 (1/50; Dako) followed by FITC conjugated goat anti-mouse IgG (1/300), both for 1 h at 4°C.

[0098] For NF staining, sections of unfixed tissue were pre-incubated for 1 h at 4°C with TRITC-conjugated α-BTx, rinsed, immersed in ethanol at -20°C for 20 min, then incubated overnight at room temperature with the rabbit polyclonal serum 1211 (1/750; reactive with phosphorylated NF; Affiniti Research Products Ltd. Exeter, UK) followed by FITC conjugated goat anti-rabbit IgG (1/300; Southern Biotechnology Associates) for 3 h at 4°C. All detection antibodies were diluted in phosphate-buffered saline (PBS).

[0099] pSC viability was assessed using EthD-1, a membrane impermeant dye that labels with red fluorescence the nucleic acids of membrane-permeabilized cells (Molecular Probes, Eugene, Oregon, USA). In brief, nerve-muscle preparations were exposed to Ringer's containing 2 μM EthD-1. The tissue was incubated in the dark at room temperature for 1 h, rinsed in Ringer's solution and frozen for immunohistology. NMJs were identified in 15 μm cryostat sections by staining with bodipy conjugated α-BTx (1.3 μg/ml), and the percentage of NMJs with EthD-1 positive nuclei at NMJs was calculated.

[0100] For illustrations, whole mount triangularis stemi muscles were incubated with CGM3 (50 μg/ml) and fluorochrome-conjugated α-BTx (TRITC/FITC/CY5) (2 μg/ml; 1:500), followed by NHS plus eculizumab or control mAb. Preparations were incubated with fluorescently conjugated α-BTx and various combinations of the following: mouse anti-C5b-9 (1:40; Dako), anti-C3c-FITC (1:200; Dako), or EthD-1 (2 μM) in Ringer's medium for 1 h at room temperature, rinsed in Ringer's medium, followed by fixation for 20 min in 4% formaldehyde in PBS. Unreactive aldehyde groups were quenched, by incubating with 0.1 M glycine for 10 min. The antibodies were then reapplied in PBS and agitated overnight at room temperature. For staining of intracellular antigens, muscle was fixed in 4% formaldehyde for 20 min followed by 10 min in 0.1M glycine and then incubated in a permeabilizing solution of 0.5% Triton-X100 in PBS for 30 min at RT, and either rabbit anti-S100 (1:150; Dako) or rabbit anti-NF (1:150; Chemicon, Hampshire, UK) diluted in permeabilizing solution applied overnight at room temperature. Tissue was rinsed in PBS and where necessary incubated in the following fluorescently conjugated antibodies diluted 1:300; anti-rabbit IgG-FITC, anti-mouse IgG-FITC or -TRITC and agitated for 7 h at RT in the dark. Tissue was rinsed in PBS and mounted in Citifluor mounting medium (Citifluor Products, Canterbury, UK).

Image acquisition, quantitation and statistical analysis

[0101] Digital images were captured using both Zeiss Pascal confocal laser scanning microscope and Zeiss Axio Imager Z1 with ApoTome. Image-analysis measurements were made using Scion Image (Scion Corporation, Frederick, Maryland, USA) image analysis software. For quantitative analysis of IgM, C3c, MAC and NF, 3 staining runs of each marker were performed on tissue from at least 3 individual hemi-diaphragms, and quantified as previously described (O'Hanlon et al. Brain 124:893-906 (2001)). All studies were observer blinded. For immunohistological analysis of non-parametric data, statistical comparisons were made using Mann-Whitney Test employing a 1% level of significance. For comparison of EthD-1 positive pSC at the NMJ, chi-squared test was used at 1% level of significance.

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Samsung Bioepis Exhibit No. 1002
Page 1711

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Samsung Bioepis Exhibit No. 1002
Page 1712

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Claims

1. An antibody for use in the treatment of Guillain-Barré syndrome in a mammal, wherein said antibody binds to:

   (i) complement component C5 and inhibits the cleavage of C5 into fragments C5a and C5b; or
   (ii) C5b,
   wherein said antibody inhibits the formation of the membrane attack complex.

2. The antibody for use according to claim 1, wherein said mammal has the Miller Fisher variant of Guillain-Barré syndrome.

3. The antibody for use according to claim 1 or 2, wherein said antibody is a whole antibody or an antibody fragment.

4. The antibody for use according to claim 3, wherein said whole antibody or antibody fragment is selected from the group consisting of: a polyclonal antibody, a monoclonal antibody or antibody fragment, a diabody, a chimerized or chimeric antibody or antibody fragment, a humanized antibody or antibody fragment, a deimmunized human antibody or antibody fragment, a fully human antibody or antibody fragment, a single chain antibody, an Fv antibody fragment, an Fab antibody fragment, an Fab' antibody fragment, and an $F(ab')_2$ antibody fragment.

5. The antibody for use according to claim 1 or 2, wherein said antibody is eculizumab.

6. The antibody for use according to claim 1 or 2, wherein said antibody is pexelizumab.

7. The antibody for use according to any one of claims

Samsung Bioepis Exhibit No. 1002
Page 1713

1 to 6, wherein the mammal is a human.

Patentansprüche

1. Antikörper zur Verwendung bei der Behandlung von Guillain-Barré-Syndrom bei einem Säuger, wobei der Antikörper an:

   (i) Komplement-Komponente C5 bindet und die Spaltung von C5 in die Fragmente C5a und C5b hemmt; oder
   (ii) C5b bindet,
   wobei der Antikörper die Ausbildung des Membranangriffskomplexes hemmt.

2. Antikörper zur Verwendung nach Anspruch 1, wobei der Säuger die Miller-Fisher-Variante von Guillain-Barré-Syndrom aufweist.

3. Antikörper zur Verwendung nach Anspruch 1 oder 2, wobei es sich bei dem Antikörper um einen intakten Antikörper oder ein Antikörperfragment handelt.

4. Antikörper zur Verwendung nach Anspruch 3, wobei der intakte Antikörper oder das Antikörperfragment aus der aus einem polyklonalen Antikörper, einem monoklonalen Antikörper oder Antikörperfragment, einem Diabody, einem chimärisierten oder chimären Antikörper oder Antikörperfragment, einem humanisierten Antikörper oder Antikörperfragment, einem deimmunisierten menschlichen Antikörper oder Antikörperfragment, einem komplett menschlichen Antikörper oder Antikörperfragment, einem Einzelkette-Antikörper, einem Fv-Antikörperfragment, einem Fab-Antikörperfragment, einem Fab'-Antikörperfragment und einem $F(ab')_2$-Antikörperfragment bestehenden Gruppe ausgewählt ist.

5. Antikörper zur Verwendung nach Anspruch 1 oder 2, wobei es sich bei dem Antikörper um Eculizumab handelt.

6. Antikörper zur Verwendung nach Anspruch 1 oder 2, wobei es sich bei dem Antikörper um Pexelizumab handelt.

7. Antikörper zur Verwendung nach einem der Ansprüche 1 bis 6, wobei es sich bei dem Säuger um einen Menschen handelt.

Revendications

1. Anticorps pour utilisation dans le traitement du syndrome de Guillain-Barré chez un mammifère, ledit anticorps se liant à :

   (i) le composant du complément C5 et inhibant le clivage de C5 en fragments C5a et C5b ; ou
   (ii) C5b,
   ledit anticorps inhibant la formation du complexe d'attaque membranaire.

2. Anticorps pour utilisation selon la revendication 1, ledit mammifère ayant le variant de Miller-Fisher du syndrome de Guillain-Barré.

3. Anticorps pour utilisation selon la revendication 1 ou 2, ledit anticorps étant un anticorps total ou un fragment d'anticorps.

4. Anticorps pour utilisation selon la revendication 3, ledit anticorps total ou fragment d'anticorps étant choisi dans le groupe constitué de : un anticorps polyclonal, un anticorps monoclonal ou un fragment d'anticorps monoclonal, un dianticorps, un anticorps ou fragment d'anticorps chimérisé ou chimérique, un anticorps ou fragment d'anticorps humanisé, un anticorps ou fragment d'anticorps humain désimmunisé, un anticorps ou fragment d'anticorps totalement humain, un anticorps monocaténaire, un fragment d'anticorps Fv, un fragment d'anticorps Fab, un fragment d'anticorps Fab', et un fragment d'anticorps $F(ab')_2$.

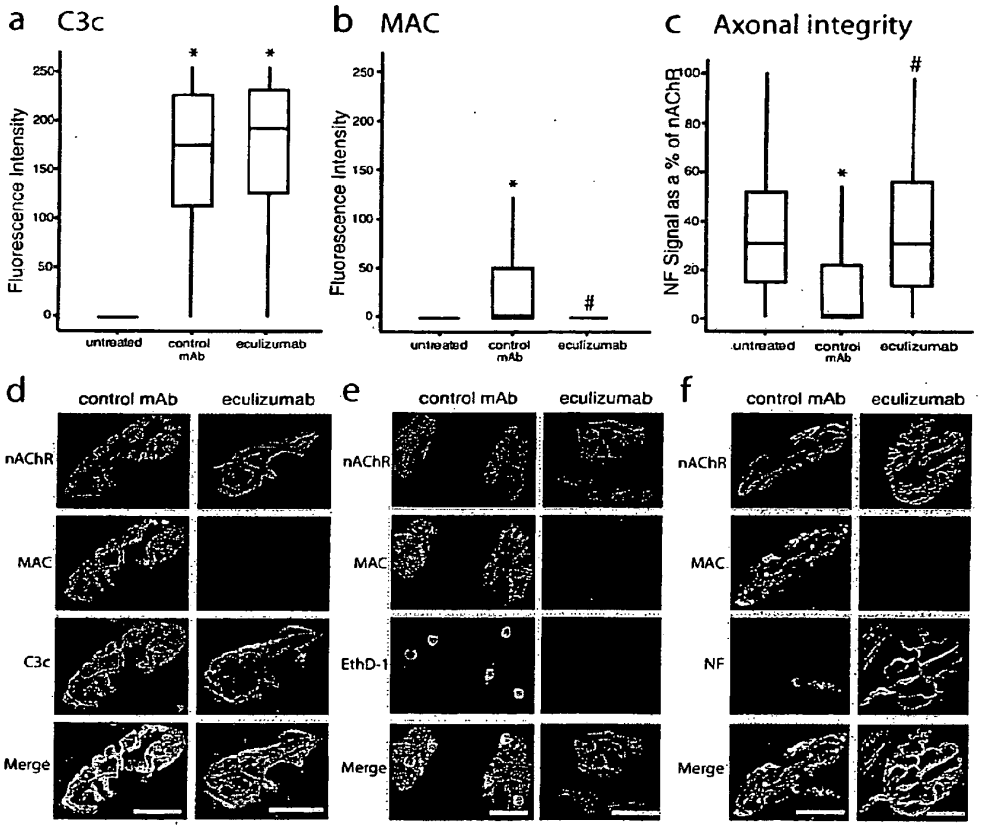
5. Anticorps pour utilisation selon la revendication 1 ou 2, ledit anticorps étant l'éculizumab.

6. Anticorps pour utilisation selon la revendication 1 ou 2, ledit anticorps étant le pexélizumab.

7. Anticorps pour utilisation selon l'une quelconque des revendications 1 à 6, le mammifère étant un humain.

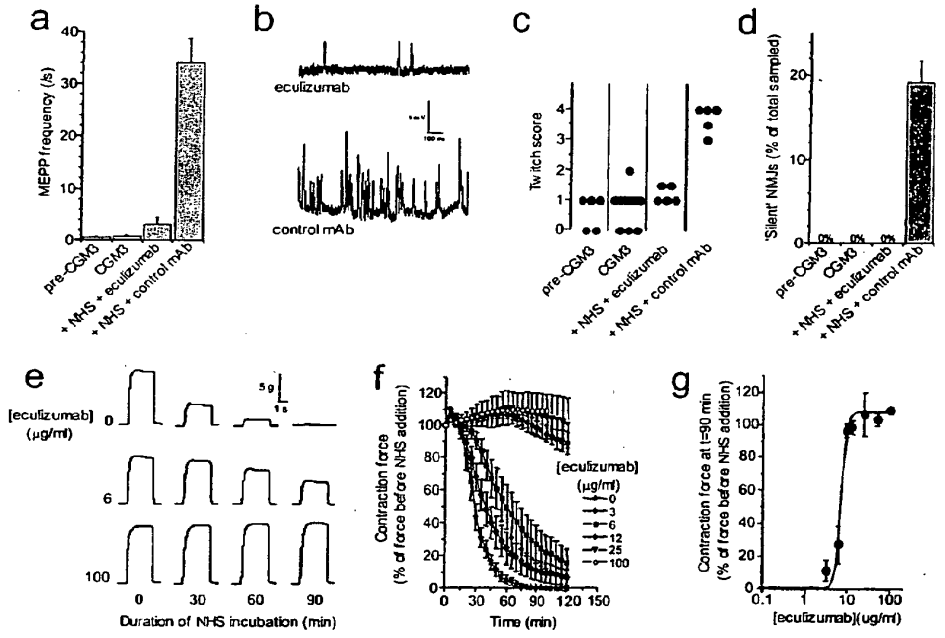
Samsung Bioepis Exhibit No. 1002
Page 1714

Figure 1



Samsung Bioepis Exhibit No. 1002
Page 1715

Figure 2



Samsung Bioepis Exhibit No. 1002
Page 1716

Figure 3A

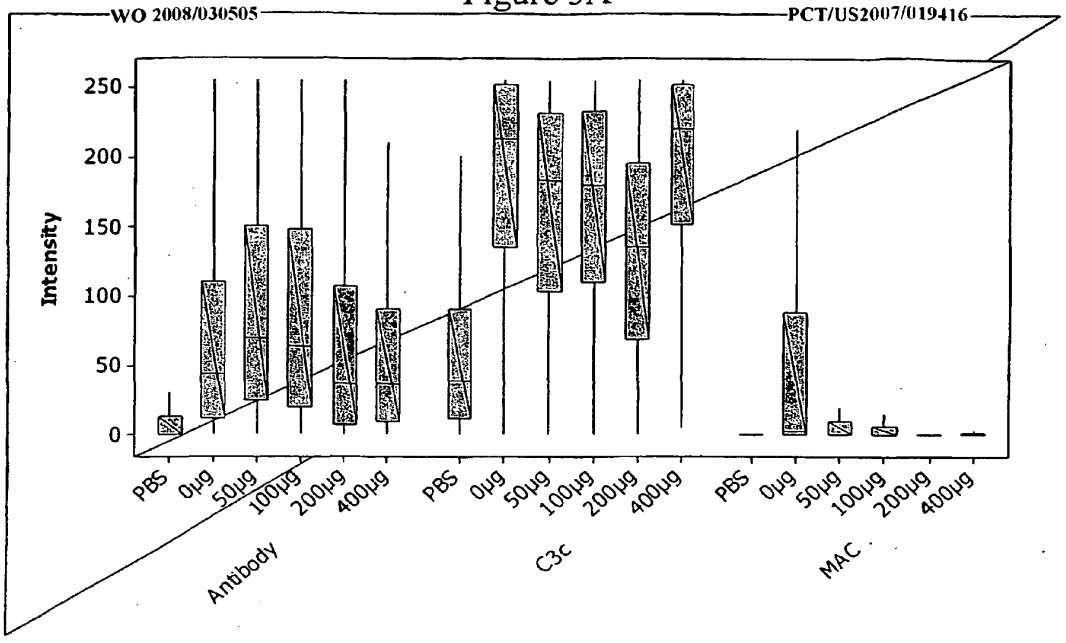
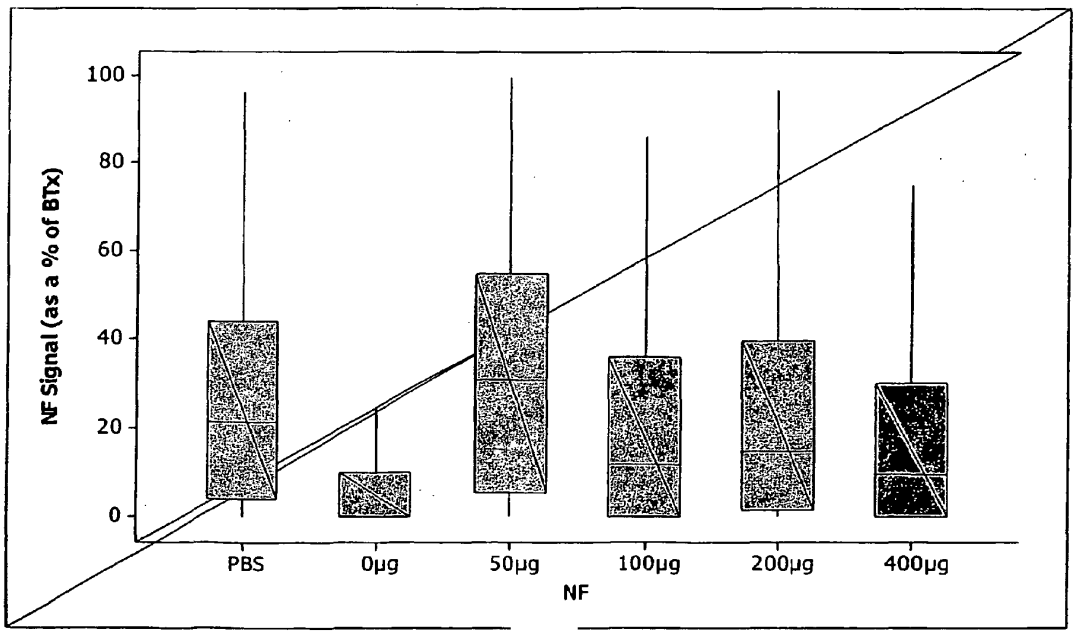
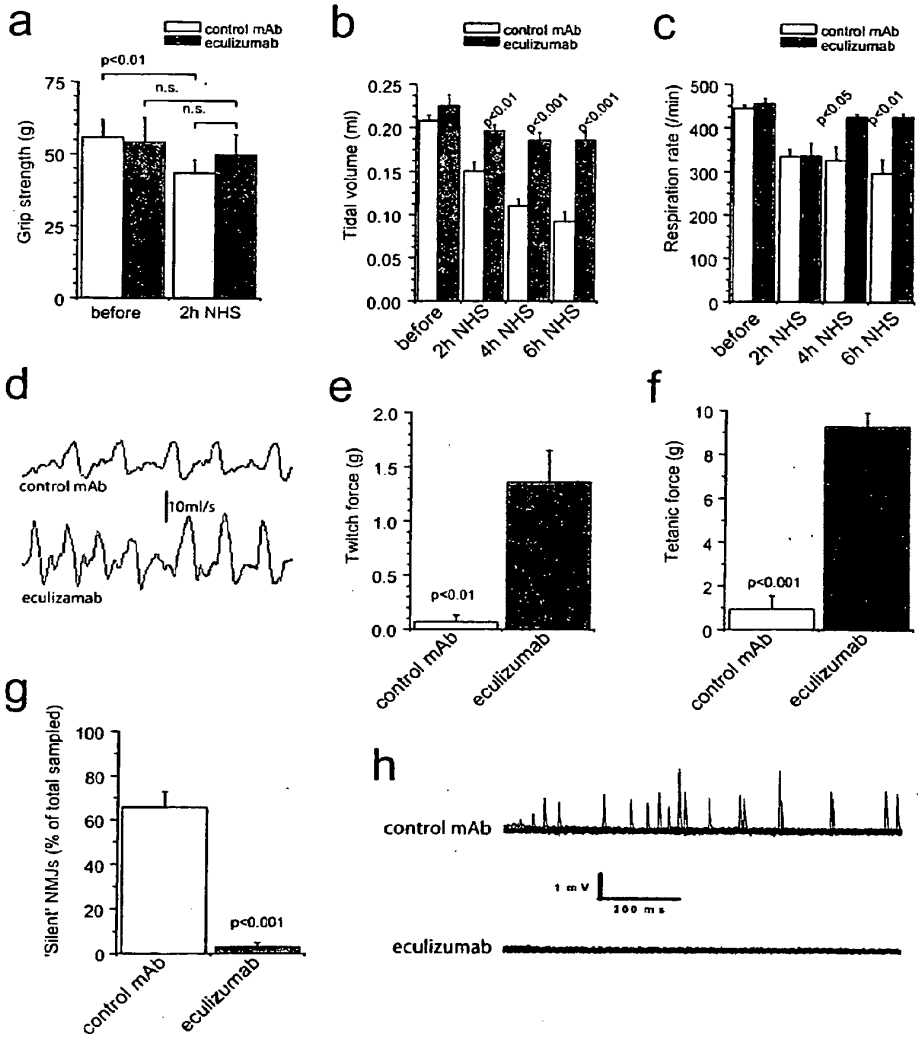


Figure 3B



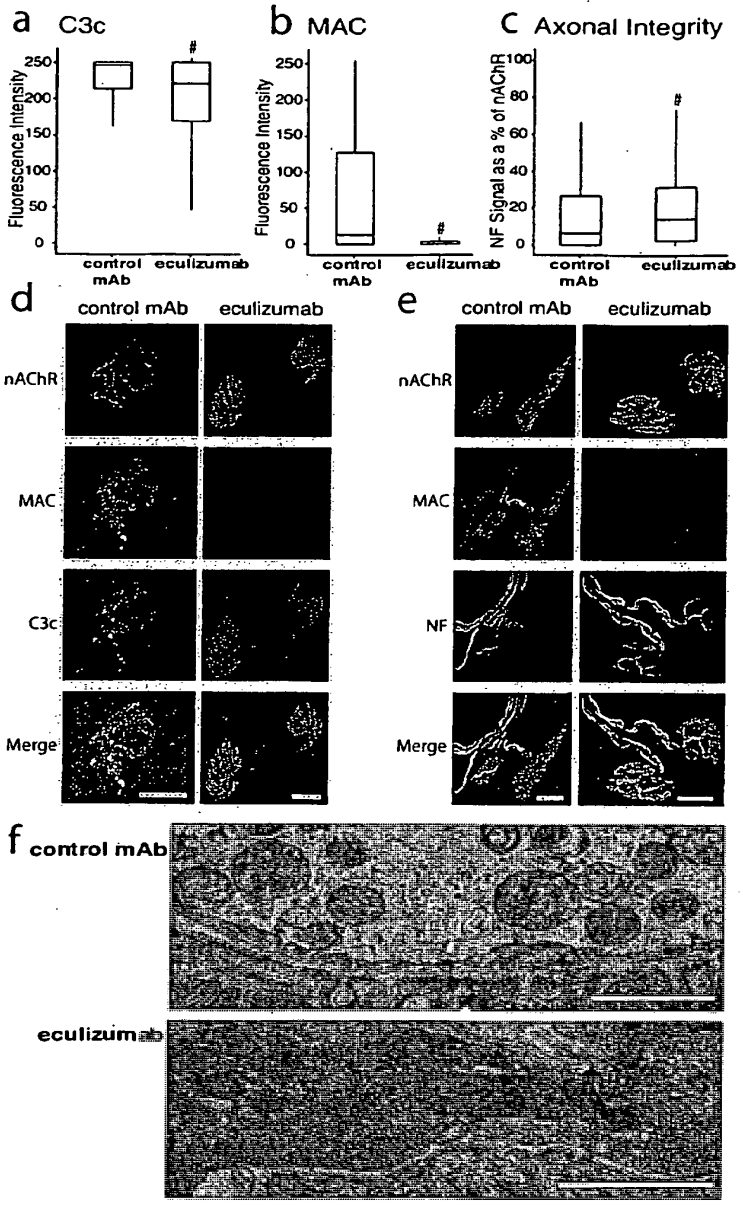
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Page 1717

Figure 4



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Page 1718

Figure 5



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Page 1719

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(71) Applicant *(for all designated States except US)*: **ALEXION PHARMACEUTICALS, INC.** [US/US]; 352 Knotter Drive, Cheshire, CT 06410 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BELL, Leonard** [US/US]; 59 Tumblebrook Road, Woodbridge, CT 06525 (US). **ROTHER, Russell, P.** [US/US]; 25 Blue Trail Drive, Prospect, CT 06712 (US).

(74) Agents: **VARMA, Anita** et al.; Ropes & Gray LLP, One International Place, Boston, MA 02110 (US).

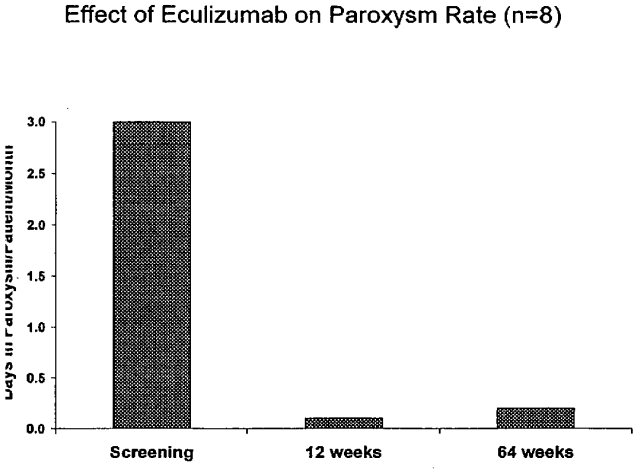
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(54) Title: METHODS OF TREATING HEMOLYTIC ANEMIA

(57) Abstract: Paroxysmal nocturnal hemoglobinuria or other hemolytic diseases are treated using a compound which binds to or otherwise blocks the generation and/or the activity of one or more complement components, such as, for example, a complement-inhibiting antibody.



Bars represent the paroxysm rates (number of paroxysms per patient per month) during the screening period (pre-eculizumab treatment), and during the first 12 weeks and the entire 64 weeks of eculizumab treatment. Three patients were not included in the analysis as either pretreatment urine scores were inadvertently not collected (2 patients) or an iron chelating agent that resulted in artificially colored urine was administered during the extension study (1 patient).

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METHODS OF TREATING HEMOLYTIC ANEMIA

RELATED APPLICATIONS

This application claims the benefit of U.S. Application No. 11/595,118, filed on November 8, 2006, the entire disclosure of which is incorporated herein by reference.

BACKGROUND

1. Technical Field

This disclosure relates to a method of treating a hemolytic disease such as, for example, paroxysmal nocturnal hemoglobinuria ("PNH"), by administering a compound which binds to, or otherwise blocks, the generation and/or activity of one or more complement components.

2. Background of Related Art

Paroxysmal nocturnal hemoglobinuria ("PNH") is an uncommon blood disorder wherein red blood cells are compromised and are thus destroyed more rapidly than normal red blood cells. PNH results from a mutation of bone marrow cells resulting in the generation of abnormal blood cells. More specifically, PNH is believed to be a disorder of hematopoietic stem cells, which give rise to distinct populations of mature blood cells. The basis of the disease appears to be somatic mutations leading to the inability to synthesize the glycosyl-phosphatidylinositol ("GPI") anchor that is responsible for attaching proteins to cell membranes. The mutated gene, PIG-A (phosphatidylinositol glycan class A) resides in the X chromosome and can have several different mutations, varying from deletions to point mutations.

PNH causes a sensitivity to complement-mediated destruction and this sensitivity occurs in the cell membrane. PNH cells are deficient in a number of proteins, particularly essential complement-regulating surface proteins. These complement-regulating surface proteins include the decay-accelerating factor ("DAF") or CD55 and membrane inhibitor of reactive lysis ("MIRL") or CD59.

Samsung Bioepis Exhibit No. 1002
Page 1726

PNH is characterized by hemolytic anemia (a decreased number of red blood cells) and hemoglobinuria (excess hemoglobin in the urine). PNH-afflicted individuals are known to have paroxysms, which are defined here as an exacerbation of hemolysis with dark-colored urine. Hemolytic anemia is due to intravascular destruction of red blood cells by complement components. Other known symptoms include dysphagia, fatigue, erectile dysfunction, thrombosis, recurrent abdominal pain, pulmonary hypertension, and an overall poor quality of life.

Hemolysis resulting from intravascular destruction of red blood cells causes local and systemic nitric oxide (NO) deficiency through the release of free hemoglobin. Free hemoglobin is a very efficient scavenger of NO, due in part to the accessibility of NO in the non-erythrocyte compartment and a 10^6 times greater affinity of the heme moiety for NO than that for oxygen. The occurrence of intravascular hemolysis often generates sufficient free hemoglobin to completely deplete haptoglobin. Once the capacity of this hemoglobin scavenging protein is exceeded, consumption of endogenous NO ensues. For example, in a setting of intravascular hemolysis such as PNH, where LDH levels routinely exceed the upper limit of the normal range and commonly reach levels of 2-3 times their normal levels, free hemoglobin would likely obtain concentrations of 0.8-1.6 g/L. Since haptoglobin can only bind somewhere between 0.7 to 1.5 g/L of hemoglobin depending on the haptoglobin allotype, a large excess of free hemoglobin would be generated. Once the capacity of hemoglobin reabsorption by the kidney proximal tubules is exceeded, hemoglobinuria ensues. The release of free hemoglobin during intravascular hemolysis results in excessive consumption of NO with subsequent enhanced smooth muscle contraction, vasoconstriction and platelet activation and aggregation. PNH-related morbidities associated with NO scavenging by hemoglobin include abdominal pain, erectile dysfunction, esophageal spasm, and thrombosis.

The laboratory evaluation of hemolysis normally includes hematologic, serologic, and urine tests. Hematologic tests include an examination of the blood smear for morphologic abnormalities of red blood cells (RBCs) (to determine causation), and the measurement of the reticulocyte count in whole blood (to determine bone marrow compensation for RBC loss). Serologic tests include lactate dehydrogenase (LDH; widely performed), and free hemoglobin (not widely

Samsung Bioepis Exhibit No. 1002
Page 1727

performed) as a direct measure of hemolysis. LDH levels can be useful in the diagnosis and monitoring of patients with hemolysis. Other serologic tests include bilirubin or haptoglobin, as measures of breakdown products or scavenging reserve, respectively. Urine tests include bilirubin, hemosiderin, and free hemoglobin, and are generally used to measure gross severity of hemolysis and for differentiation of intravascular vs. extravascular etiologies of hemolysis rather than routine monitoring of hemolysis. Further, RBC numbers, RBC (i.e. cell-bound) hemoglobin, and hematocrit are generally performed to determine the extent of any accompanying anemia rather than as a measure of hemolytic activity per se.

Steroids have been employed as a therapy for hemolytic diseases and may be effective in suppressing hemolysis in some patients, although long term use of steroid therapy carries many negative side effects. Afflicted patients may require blood transfusions, which carry risks of infection. Anti-coagulation therapy may also be required to prevent blood clot formation and can result in hemorrhage. Bone marrow transplantation has been known to cure PNH, however, bone marrow matches are often very difficult to find and mortality rates are high with this procedure.

It would be advantageous to provide a treatment which safely and reliably eliminates and/or limits hemolytic diseases, such as PNH, and their effects.

SUMMARY

In one aspect, the application provides a method of reducing the occurrence of thrombosis in a subject, said method comprising inhibiting complement in said subject. In certain embodiments, the method comprises administering a compound to said subject, wherein the compound is selected from the group consisting of: a) compounds which bind to one or more complement components, b) compounds which block the generation of one or more complement components, and c) compounds which block the activity of one or more complement components.

In certain embodiments, the subject has a paroxysmal nocturnal hemoglobinuria (PNH) granulocyte clone greater than 0.1% of the total granulocyte count. In certain embodiments, the subject has a PNH granulocyte clone greater than 1% of the total granulocyte count. In certain embodiments, the subject has a PNH granulocyte clone greater than 10% of the total granulocyte count. In certain

Samsung Bioepis Exhibit No. 1002
Page 1728

embodiments, the subject has a PNH granulocyte clone greater than 50% of the total granulocyte count.

In certain embodiments, the compound is selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

In certain embodiments, the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

In certain embodiments, the compound inhibits C5b activity. In certain embodiments, the compound inhibits cleavage of C5. In certain embodiments, the compound inhibits terminal complement. In certain embodiments, the compound inhibits C5a activity or inhibits binding of C5a to its receptor.

In certain embodiments, the subject is a human. In certain embodiments, the subject has a history of one or more thrombotic events.

In certain embodiments, the compound is an antibody or antibody fragment. In certain embodiments, the antibody or antibody fragment is selected from the group consisting of a polyclonal antibody, a monoclonal antibody or antibody fragment, a diabody, a chimerized or chimeric antibody or antibody fragment, a humanized antibody or antibody fragment, a deimmunized human antibody or antibody fragment, a fully human antibody or antibody fragment, a single chain antibody, an Fv, an Fab, an Fab', an Fd, and an $F(ab')_2$.

In certain embodiments, the antibody is pexelizumab. In certain embodiments, the antibody is eculizumab.

In certain embodiments, the compound is administered chronically to said subject. In certain embodiments, the compound is administered systemically to said subject. In certain embodiments, the compound is administered locally to said subject.

In certain embodiments, the method reduces rates of thromboembolism by greater than 25%. In certain embodiments, the method reduces rates of thromboembolism by greater than 50%. In certain embodiments, the method reduces

Samsung Bioepis Exhibit No. 1002
Page 1729

rates of thromboembolism by greater than 75%. In certain embodiments, the method reduces rates of thromboembolism by greater than 90%.

In certain embodiments, the method results in at least a 25% reduction in LDH levels. In certain embodiments, the method results in at least a 50% reduction in LDH levels. In certain embodiments, the method results in at least a 75% reduction in LDH levels. In certain embodiments, the method in a subject results in at least a 90% reduction in LDH levels.

In certain embodiments, the method further comprising administering a second compound, wherein said second compound increases hematopoiesis. In certain embodiments, the second compound is selected from the group consisting of steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin (EPO), pegylated EPO, EPO mimetics, Aranesp®, erythropoiesis stimulating agents, antithymocyte globulin (ATG) and antilymphocyte globulin (ALG). In certain embodiments, EPO is administered with an anti-C5 antibody. In certain embodiments, the antibody is pexelizumab. In certain embodiments, the antibody is eculizumab.

In certain embodiments, the method further comprising administering an antithrombotic compound. In certain embodiments, the antithrombotic compound is an anticoagulant. In certain embodiments, the anticoagulant is administered with an anti-C5 antibody. In certain embodiments, the anticoagulant is an antiplatelet agent. In certain embodiments, the antibody is pexelizumab. In certain embodiments, the antibody is eculizumab.

In another aspect, the application provides a method of reducing the occurrence of thrombosis in a subject who has a higher than normal lactate dehydrogenase (LDH) level, said method comprising inhibiting complement in said subject.

In certain embodiments, the method comprises administering a compound to said subject, wherein the compound is selected from the group consisting of: a) compounds which bind to one or more complement components, b) compounds which block the generation of one or more complement components, and c) compounds which block the activity of one or more complement components.

In certain embodiments, the subject has an LDH level greater than the upper limit of normal. In certain embodiments, the subject has an LDH level greater than or

Samsung Bioepis Exhibit No. 1002
Page 1730

equal to 1.5 times the upper limit of normal. In certain embodiments, the subject has an LDH level greater than or equal to 2.5 times the upper limit of normal. In certain embodiments, the subject has an LDH level greater than or equal to 5 times the upper limit of normal. In certain embodiments, the subject has an LDH level greater than or equal to 10 times the upper limit of normal.

In certain embodiments, the compound is selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

In certain embodiments, the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

In certain embodiments, the compound inhibits C5b activity. In certain embodiments, the compound inhibits cleavage of C5. In certain embodiments, the compound inhibits terminal complement. In certain embodiments, the compound inhibits C5a activity or inhibits binding of C5a to its receptor.

In certain embodiments, the subject is a human. In certain embodiments, the subject has a history of one or more thrombotic events.

In certain embodiments, the compound is an antibody or antibody fragment. In certain embodiments, the antibody or antibody fragment is selected from the group consisting of a polyclonal antibody, a monoclonal antibody or antibody fragment, a diabody, a chimerized or chimeric antibody or antibody fragment, a humanized antibody or antibody fragment, a deimmunized human antibody or antibody fragment, a fully human antibody or antibody fragment, a single chain antibody, an Fv, an Fab, an Fab', an Fd, and an $F(ab')_2$.

In certain embodiments, the antibody is pexelizumab. In certain embodiments, the antibody is eculizumab.

In certain embodiments, the compound is administered chronically to said subject. In certain embodiments, the compound is administered systemically to said subject. In certain embodiments, the compound is administered locally to said subject.

Samsung Bioepis Exhibit No. 1002
Page 1731

In certain embodiments, the method reduces rates of thromboembolism by greater than 25%. In certain embodiments, the method reduces rates of thromboembolism by greater than 50%. In certain embodiments, the method reduces rates of thromboembolism by greater than 75%. In certain embodiments, the method reduces rates of thromboembolism by greater than 90%.

In certain embodiments, the method results in at least a 25% reduction in LDH levels. In certain embodiments, the method results in at least a 50% reduction in LDH levels. In certain embodiments, the method results in at least a 75% reduction in LDH levels. In certain embodiments, the method in a subject results in at least a 90% reduction in LDH levels.

In certain embodiments, the method further comprising administering a second compound, wherein said second compound increases hematopoiesis. In certain embodiments, the second compound is selected from the group consisting of steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin (EPO), pegylated EPO, EPO mimetics, Aranesp®, erythropoiesis stimulating agents, antithymocyte globulin (ATG) and antilymphocyte globulin (ALG). In certain embodiments, EPO is administered with an anti-C5 antibody. In certain embodiments, the antibody is pexelizumab. In certain embodiments, the antibody is eculizumab.

In certain embodiments, the method further comprising administering an antithrombotic compound. In certain embodiments, the antithrombotic compound is an anticoagulant. In certain embodiments, the anticoagulant is administered with an anti-C5 antibody. In certain embodiments, the anticoagulant is an antiplatelet agent. In certain embodiments, the antibody is pexelizumab. In certain embodiments, the antibody is eculizumab.

In still another aspect, the application provides a method of reducing the occurrence of thrombosis in a subject who has a PNH granulocyte clone and an LDH level greater than the upper limit of normal, said method comprising inhibiting complement in said subject. In certain embodiments, the method comprises administering a compound to said subject, wherein the compound is selected from the group consisting of: a) compounds which bind to one or more complement components, b) compounds which block the generation of one or more complement

Samsung Bioepis Exhibit No. 1002
Page 1732

components, and c) compounds which block the activity of one or more complement components.

In certain embodiments, the subject has a PNH granulocyte clone greater than 0.1% of the total granulocyte count. In certain embodiments, the subject has a PNH granulocyte clone greater than 0.1% of the total granulocyte count. In certain embodiments, the subject has a PNH granulocyte clone greater than 1% of the total granulocyte count. In certain embodiments, the subject has a PNH granulocyte clone greater than 10% of the total granulocyte count. In certain embodiments, the subject has a PNH granulocyte clone greater than 50% of the total granulocyte count.

In certain embodiments, the compound is selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

In certain embodiments, the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

In certain embodiments, the compound inhibits C5b activity. In certain embodiments, the compound inhibits cleavage of C5. In certain embodiments, the compound inhibits terminal complement. In certain embodiments, the compound inhibits C5a activity or inhibits binding of C5a to its receptor.

In certain embodiments, the subject is a human. In certain embodiments, the subject has a history of one or more thrombotic events.

In certain embodiments, the compound is an antibody or antibody fragment. In certain embodiments, the antibody or antibody fragment is selected from the group consisting of a polyclonal antibody, a monoclonal antibody or antibody fragment, a diabody, a chimerized or chimeric antibody or antibody fragment, a humanized antibody or antibody fragment, a deimmunized human antibody or antibody fragment, a fully human antibody or antibody fragment, a single chain antibody, an Fv, an Fab, an Fab', an Fd, and an $F(ab')_2$.

In certain embodiments, the antibody is pexelizumab. In certain embodiments, the antibody is eculizumab.

Samsung Bioepis Exhibit No. 1002
Page 1733

In certain embodiments, the compound is administered chronically to said subject. In certain embodiments, the compound is administered systemically to said subject. In certain embodiments, the compound is administered locally to said subject.

In certain embodiments, the method reduces rates of thromboembolism by greater than 25%. In certain embodiments, the method reduces rates of thromboembolism by greater than 50%. In certain embodiments, the method reduces rates of thromboembolism by greater than 75%. In certain embodiments, the method reduces rates of thromboembolism by greater than 90%.

In certain embodiments, the method results in at least a 25% reduction in LDH levels. In certain embodiments, the method results in at least a 50% reduction in LDH levels. In certain embodiments, the method results in at least a 75% reduction in LDH levels. In certain embodiments, the method in a subject results in at least a 90% reduction in LDH levels.

In certain embodiments, the method further comprising administering a second compound, wherein said second compound increases hematopoiesis. In certain embodiments, the second compound is selected from the group consisting of steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin (EPO), pegylated EPO, EPO mimetics, Aranesp®, erythropoiesis stimulating agents, antithymocyte globulin (ATG) and antilymphocyte globulin (ALG). In certain embodiments, EPO is administered with an anti-C5 antibody. In certain embodiments, the antibody is pexelizumab. In certain embodiments, the antibody is eculizumab.

In certain embodiments, the method further comprising administering an antithrombotic compound. In certain embodiments, the antithrombotic compound is an anticoagulant. In certain embodiments, the anticoagulant is administered with an anti-C5 antibody. In certain embodiments, the anticoagulant is an antiplatelet agent. In certain embodiments, the antibody is pexelizumab. In certain embodiments, the antibody is eculizumab.

In yet another aspect, the application provides a method of reducing the occurrence of thrombosis in a subject suffering from a lower than normal nitric oxide (NO) level, said method comprising inhibiting complement in said subject. In certain embodiments, the method comprises administering a compound to said subject,

Samsung Bioepis Exhibit No. 1002
Page 1734

wherein the compound is selected from the group consisting of: i) compounds which bind to one or more complement components, ii) compounds which block the generation of one or more complement components, and iii) compounds which block the activity of one or more complement components, wherein said method increases serum nitric oxide (NO) levels.

In certain embodiments, the method increases NO levels by greater than 25%. In certain embodiments, the method increases NO levels by greater than 50%. In certain embodiments, the method increases NO levels by greater than 100%. In certain embodiments, the method increases NO levels by greater than 3 fold.

In certain embodiments, the subject has PNH.

In certain embodiments, the compound is selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

In certain embodiments, the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

In certain embodiments, the compound inhibits C5b activity. In certain embodiments, the compound inhibits cleavage of C5. In certain embodiments, the compound inhibits terminal complement. In certain embodiments, the compound inhibits C5a activity or inhibits binding of C5a to its receptor.

In certain embodiments, the subject is a human. In certain embodiments, the subject has a history of one or more thrombotic events.

In certain embodiments, the compound is an antibody or antibody fragment. In certain embodiments, the antibody or antibody fragment is selected from the group consisting of a polyclonal antibody, a monoclonal antibody or antibody fragment, a diabody, a chimerized or chimeric antibody or antibody fragment, a humanized antibody or antibody fragment, a deimmunized human antibody or antibody fragment, a fully human antibody or antibody fragment, a single chain antibody, an Fv, an Fab, an Fab', an Fd, and an $F(ab')_2$.

In certain embodiments, the antibody is pexelizumab. In certain embodiments, the antibody is eculizumab.

Samsung Bioepis Exhibit No. 1002
Page 1735

In certain embodiments, the compound is administered chronically to said subject. In certain embodiments, the compound is administered systemically to said subject. In certain embodiments, the compound is administered locally to said subject.

In certain embodiments, the method reduces rates of thromboembolism by greater than 25%. In certain embodiments, the method reduces rates of thromboembolism by greater than 50%. In certain embodiments, the method reduces rates of thromboembolism by greater than 75%. In certain embodiments, the method reduces rates of thromboembolism by greater than 90%.

In certain embodiments, the method results in at least a 25% reduction in LDH levels. In certain embodiments, the method results in at least a 50% reduction in LDH levels. In certain embodiments, the method results in at least a 75% reduction in LDH levels. In certain embodiments, the method in a subject results in at least a 90% reduction in LDH levels.

In certain embodiments, the method further comprising administering a second compound, wherein said second compound increases hematopoiesis. In certain embodiments, the second compound is selected from the group consisting of steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin (EPO), pegylated EPO, EPO mimetics, Aranesp®, erythropoiesis stimulating agents, antithymocyte globulin (ATG) and antilymphocyte globulin (ALG). In certain embodiments, EPO is administered with an anti-C5 antibody. In certain embodiments, the antibody is pexelizumab. In certain embodiments, the antibody is eculizumab.

In certain embodiments, the method further comprising administering an antithrombotic compound. In certain embodiments, the antithrombotic compound is an anticoagulant. In certain embodiments, the anticoagulant is administered with an anti-C5 antibody. In certain embodiments, the anticoagulant is an antiplatelet agent. In certain embodiments, the antibody is pexelizumab. In certain embodiments, the antibody is eculizumab.

In another aspect, the application provides a method of determining whether a subject having a hemolytic disorder is susceptible to thrombosis comprising measuring the PNH granulocyte clone size of said subject, wherein if the clone size is greater than 0.1% then said subject is susceptible to thrombosis. In certain

Samsung Bioepis Exhibit No. 1002
Page 1736

embodiments, the clone size is greater than 1%. In certain embodiments, the clone size is greater than 10%. In certain embodiments, the clone size is greater than 50%.

In still another aspect, the application provides a method of increasing PNH red blood cell mass of a subject, said method comprising inhibiting complement in said subject. In certain embodiments, the method comprises administering a compound to the subject, the compound being selected from the group consisting of: i) compounds which bind to one or more complement components, ii) compounds which block the generation of one or more complement components, and iii) compounds which block the activity of one or more complement components.

In certain embodiments, the subject has a PNH granulocyte clone. In certain embodiments, the PNH granulocyte clone is greater than 0.1% of the total granulocyte count. In certain embodiments, the PNH granulocyte clone is greater than 1% of the total granulocyte count. In certain embodiments, the PNH granulocyte clone is greater than 10% of the total granulocyte count. In certain embodiments, the PNH granulocyte clone is greater than 50% of the total granulocyte count.

In certain embodiments, the subject has an LDH level greater than the upper limit of normal. In certain embodiments, the subject has an LDH level greater than or equal to 1.5 times the upper limit of normal. In certain embodiments, the subject has an LDH level greater than or equal to 2.5 times the upper limit of normal. In certain embodiments, the subject has an LDH level greater than or equal to 5 times the upper limit of normal. In certain embodiments, the subject has an LDH level greater than or equal to 10 times the upper limit of normal.

In yet another aspect, the application provides a method of treating hemolytic anemia in a subject, said method comprising inhibiting complement in said subject. In certain embodiments, the method comprises administering a compound to the subject, wherein the compound is selected from the group consisting of: i) compounds which bind to one or more complement components, ii) compounds which block the generation of one or more complement components, and iii) compounds which block the activity of one or more complement components, wherein said method increases red blood cell (RBC) mass.

In certain embodiments, the RBC mass is measured as the absolute number of RBCs. In certain embodiments, the RBC mass is PNH RBC mass. In certain embodiments, the method increases RBC mass by greater than 10%. In certain

Samsung Bioepis Exhibit No. 1002
Page 1737

embodiments, the method increases RBC mass by greater than 25%. In certain embodiments, the method increases RBC mass by greater than 50%. In certain embodiments, the method increases RBC mass by greater than 100%. In certain embodiments, the method increases RBC mass by greater than 2 fold.

In certain embodiments, the method decreases transfusion requirements.

In certain embodiments, the method stabilizes hemoglobin levels.

In certain embodiments, the method causes an increase in hemoglobin levels.

The application contemplates combinations of any of the foregoing aspects and embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A reports biochemical parameters of hemolysis measured during treatment of PNH patients with an anti-C5 antibody.

FIG. 1B graphically depicts the effect of treatment with an anti-C5 antibody on lactate dehydrogenase (LDH) levels.

FIG. 2 shows a urine color scale devised to monitor the incidence of paroxysm of hemoglobinuria in PNH patients.

FIG. 3 is a graph of the effects of eculizumab treatments on patient paroxysm rates, as compared to pre-treatment rates.

FIG. 4 shows urine samples of PNH patients and measurements of hemoglobinuria, dysphagia, LDH, AST, pharmacokinetics (PK) and pharmacodynamics (PD) reflecting the immediate and positive effects of the present methods on hemolysis, symptoms and pharmacodynamics suitable to completely block complement.

FIG. 5 graphically depicts the effect of anti-C5 antibody dosing schedule on hemoglobinuria over time.

FIGS. 6a and 6b are graphs comparing the number of transfusion units required per

Samsung Bioepis Exhibit No. 1002
Page 1738

patient per month, prior to and during treatment with an anti-C5 antibody: FIG. 6a depicts cytopenic patients; and FIG. 6b depicts non-cytopenic patients.

FIG. 7 shows the management of a thrombocytopenic patient by administering an anti-C5 antibody and erythropoietin (EPO).

FIG. 8 graphically depicts the pharmacodynamics of an anti-C5 antibody.

FIG. 9 is a chart of the results of European Organization for Research and Treatment of Cancer questionnaires ("EORTC QLC-C30") completed during the anti-C5 therapy regimen addressing quality of life issues.

FIG. 10 is a chart depicting the effects of anti-C5 antibody treatments on adverse symptoms associated with PNH.

FIG. 11 shows changes in PNH RBC mass during treatment with eculizumab compared with placebo.

FIG. 12 shows the effect of eculizumab and recombinant human erythropoietin on PNH Type III RBC mass and transfusion requirements. The diamonds represent PNH type III RBC counts and solid bars represent the number of packed red blood cell (PRBC) units transfused. The x-axis indicates date.

FIG. 13 shows changes in FACIT-Fatigue score during treatment with eculizumab and for placebo control.

DETAILED DESCRIPTION

The present disclosure relates to a method of treating paroxysmal nocturnal hemoglobinuria ("PNH") and other hemolytic diseases in mammals. Specifically, the methods of treating hemolytic diseases, which are described herein, involve using compounds which bind to or otherwise block the generation and/or activity of one or

Samsung Bioepis Exhibit No. 1002
Page 1739

more complement components. The present methods have been found to provide surprising results. For instance, hemolysis rapidly ceases upon administration of the compound which binds to or otherwise blocks the generation and/or activity of one or more complement components, with LDH and hemoglobinuria being significantly reduced immediately after treatment. Also, hemolytic patients can be rendered less dependent on transfusions or transfusion-independent for extended periods (twelve months or more), well beyond the 120 day life cycle of red blood cells. In addition, type III red blood cell count can be increased dramatically in the midst of other mechanisms of red blood cell lysis (non-complement mediated and/or earlier complement component mediated e.g., Cb3). Another example of a surprising result is that a variety of symptoms resolved, indicating that NO serum levels were increased enough even in the presence of other mechanisms of red blood cell lysis. These and other results reported herein are unexpected and could not be predicted from prior treatments of hemolytic diseases.

Any compound which binds to or otherwise blocks the generation and/or activity of one or more complement components can be used in the present methods. A specific class of such compounds which is particularly useful includes antibodies specific to a human complement component, especially anti-C5 antibodies. The anti-C5 antibody inhibits the complement cascade and, ultimately, prevents red blood cell ("RBC") lysis by the terminal complement complex C5b-9. By inhibiting and/or reducing the lysis of RBCs, the effects of PNH and other hemolytic diseases (including symptoms such as hemoglobinuria, anemia, dysphagia, fatigue, erectile dysfunction, recurrent abdominal pain and thrombosis) are eliminated or decreased.

In another embodiment, soluble forms of the proteins CD55 and CD59, singularly or in combination with each other, can be administered to a subject to inhibit the complement cascade in its alternative pathway. CD55 inhibits at the level of C3, thereby preventing the further progression of the cascade. CD59 inhibits the C5b-8 complex from combining with C9 to form the membrane attack complex (see discussion below).

The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of bacterial and viral pathogens. There are at least 25 proteins involved in the complement cascade, which are found as a complex collection of plasma proteins and membrane cofactors. Complement

Samsung Bioepis Exhibit No. 1002
Page 1740

components achieve their immune defensive functions by interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions. A concise summary of the biologic activities associated with complement activation is provided, for example, in The Merck Manual, 16th Edition.

The complement cascade progresses via the classical pathway, the alternative pathway or the lectin pathway. These pathways share many components, and while they differ in their initial steps, they converge and share the same "terminal complement" components (C5 through C9) responsible for the activation and destruction of target cells. The classical complement pathway is typically initiated by antibody recognition of and binding to an antigenic site on a target cell. The alternative pathway is usually antibody independent, and can be initiated by certain molecules on pathogen surfaces. Additionally, the lectin pathway is typically initiated with binding of mannose-binding lectin ("MBL") to high mannose substrates. These pathways converge at the point where complement component C3 is cleaved by an active protease to yield C3a and C3b.

C3a is an anaphylatoxin (see discussion below). C3b binds to bacteria and other cells, as well as to certain viruses and immune complexes, and tags them for removal from the circulation. C3b in this role is known as opsonin. The opsonic function of C3b is generally considered to be the most important anti-infective action of the complement system. Patients with genetic lesions that block C3b function are prone to infection by a broad variety of pathogenic organisms, while patients with lesions later in the complement cascade sequence, i.e., patients with lesions that block C5 functions, are found to be more prone only to Neisseria infection, and then only somewhat more prone (Fearon, in Intensive Review of Internal Medicine, 2nd Ed. Fanta and Minaker, eds. Brigham and Women's and Beth Israel Hospitals, 1983).

C3b also forms a complex with other components unique to each pathway to form classical or alternative C5 convertase, which cleaves C5 into C5a and C5b. C3 is thus regarded as the central protein in the complement reaction sequence since it is essential to all three activation pathways (Wurzner, et al., Complement Inflamm. 1991, 8:328-340). This property of C3b is regulated by the serum protease Factor I, which acts on C3b to produce iC3b (inactive C3b). While still functional as an

Samsung Bioepis Exhibit No. 1002
Page 1741

opsonin, iC3b can not form an active C5 convertase.

The pro-C5 precursor is cleaved after amino acid 655 and 659, to yield the beta chain as an amino terminal fragment (amino acid residues +1 to 655 of the sequence) and the alpha chain as a carboxyl terminal fragment (amino acid residues 660 to 1658 of the sequence), with four amino acids (amino acid residues 656-659 of the sequence) deleted between the two. C5 is glycosylated, with about 1.5-3 percent of its mass attributed to carbohydrate. Mature C5 is a heterodimer of a 999 amino acid 115 kDa alpha chain that is disulfide linked to a 655 amino acid 75 kDa beta chain. C5 is found in normal serum at approximately 75 μg/mL (0.4 μM). C5 is synthesized as a single chain precursor protein product of a single copy gene (Haviland et al., J. Immunol. 1991, 146:362-368). The cDNA sequence of the transcript of this gene predicts a secreted pro-C5 precursor of 1658 amino acids along with an 18 amino acid leader sequence (see, U.S. Pat. No. 6,355,245).

Cleavage of C5 releases C5a, a potent anaphylatoxin and chemotactic factor, and leads to the formation of the lytic terminal complement complex, C5b-9. C5a is cleaved from the alpha chain of C5 by either alternative or classical C5 convertase as an amino terminal fragment comprising the first 74 amino acids of the alpha chain (i.e., amino acid residues 660-733 of the sequence). Approximately 20 percent of the 11 kDa mass of C5a is attributed to carbohydrate. The cleavage site for convertase action is at, or immediately adjacent to, amino acid residue 733 of the sequence. A compound that binds at, or adjacent, to this cleavage site would have the potential to block access of the C5 convertase enzymes to the cleavage site and thereby act as a complement inhibitor.

C5b combines with C6, C7, and C8 to form the C5b-8 complex at the surface of the target cell. Upon binding of several C9 molecules, the membrane attack complex ("MAC", C5b-9, terminal complement complex--TCC) is formed. When sufficient numbers of MACs insert into target cell membranes, the openings they create (MAC pores) mediate rapid osmotic lysis of the target cells. Lower, non-lytic concentrations of MACs can produce other proinflammatory effects. In particular, membrane insertion of small numbers of the C5b-9 complexes into endothelial cells and platelets can cause deleterious cell activation. In some cases activation may precede cell lysis.

C5a and C5b-9 also have pleiotropic cell activating properties, by amplifying

Samsung Bioepis Exhibit No. 1002
Page 1742

the release of downstream inflammatory factors, such as hydrolytic enzymes, reactive oxygen species, arachidonic acid metabolites and various cytokines. C5 can also be activated by means other than C5 convertase activity. Limited trypsin digestion (Minta and Man, J. Immunol. 1977, 119:1597-1602; Wetsel and Kolb, J. Immunol. 1982, 128:2209-2216) and acid treatment (Yamamoto and Gewurz, J. Immunol. 1978, 120:2008; Damerau et al., Molec. Immunol. 1989, 26:1133-1142) can also cleave C5 and produce active C5b.

As mentioned above, C3a and C5a are anaphylatoxins. These activated complement components can trigger mast cell degranulation, which releases histamine and other mediators of inflammation, resulting in smooth muscle contraction, increased vascular permeability, leukocyte activation, and other inflammatory phenomena including cellular proliferation resulting in hypercellularity. C5a also functions as a chemotactic peptide that serves to attract pro-inflammatory granulocytes to the site of complement activation.

Any compounds which bind to or otherwise block the generation and/or activity of any of the human complement components may be utilized in accordance with the present disclosure. In some embodiments, antibodies specific to a human complement component are useful herein. Some compounds include antibodies directed against complement components C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, Factor D, Factor B, Factor P, MBL, MASP-1, and MASP-2, thus preventing the generation of the anaphylatoxic activity associated with C5a and/or preventing the assembly of the membrane attack complex associated with C5b.

Also useful in the present methods are naturally occurring or soluble forms of complement inhibitory compounds such as CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH. Other compounds which may be utilized to bind to or otherwise block the generation and/or activity of any of the human complement components include, but are not limited to, proteins, protein fragments, peptides, small molecules, RNA aptamers including ARC187 (which is commercially available from Archemix Corp., Cambridge, Mass.), L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, molecules which may be utilized in RNA interference (RNAi) such as double stranded RNA including small interfering RNA (siRNA), locked nucleic acid (LNA) inhibitors, peptide nucleic acid (PNA) inhibitors, etc.

Samsung Bioepis Exhibit No. 1002
Page 1743

Functionally, one suitable class of compounds inhibits the cleavage of C5, which blocks the generation of potent proinflammatory molecules C5a and C5b-9 (terminal complement complex). Preferably, the compound does not prevent the formation of C3b, which subserves critical immunoprotective functions of opsonization and immune complex clearance.

While preventing the generation of these membrane attack complex molecules, inhibition of the complement cascade at C5 preserves the ability to generate C3b, which is critical for opsonization of many pathogenic microorganisms, as well as for immune complex solubilization and clearance. Retaining the capacity to generate C3b appears to be particularly important as a therapeutic factor in complement inhibition for hemolytic diseases, where increased susceptibility to thrombosis, infection, fatigue, lethargy and impaired clearance of immune complexes are pre-existing clinical features of the disease process.

Particularly useful compounds for use herein are antibodies that reduce, directly or indirectly, the conversion of complement component C5 into complement components C5a and C5b. One class of useful antibodies are those having at least one antigen binding site and exhibiting specific binding to human complement component C5. Particularly useful complement inhibitors are compounds which reduce the generation of C5a and/or C5b-9 by greater than about 30%. Anti-C5 antibodies that have the desirable ability to block the generation of C5a have been known in the art since at least 1982 (Moongkarndi et al., Immunobiol. 1982, 162:397; Moongkarndi et al., Immunobiol. 1983, 165:323). Antibodies known in the art that are immunoreactive against C5 or C5 fragments include antibodies against the C5 beta chain (Moongkarndi et al., Immunobiol. 1982, 162:397; Moongkarndi et al., Immunobiol. 1983, 165:323; Wurzner et al., 1991, supra; Mollnes et al., Scand. J. Immunol. 1988, 28:307-312); C5a (see for example, Ames et al., J. Immunol. 1994, 152:4572-4581, U.S. Pat. No. 4,686,100, and European patent publication No. 0 411 306); and antibodies against non-human C5 (see for example, Giclas et al., J. Immunol. Meth. 1987, 105:201-209). Particularly useful anti-C5 antibodies are h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1. Methods for the preparation of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1 are described in U.S. Pat. No. 6,355,245 and "Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chain Fv", Thomas et al.,

Samsung Bioepis Exhibit No. 1002
Page 1744

Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401, 1996, the disclosures of which are incorporated herein in their entirety by this reference. The antibody h5G1.1-mAb is currently undergoing clinical trials under the tradename eculizumab.

Hybridomas producing monoclonal antibodies reactive with complement component C5 can be obtained according to the teachings of Sims, et al., U.S. Pat. No. 5,135,916. Antibodies are prepared using purified components of the complement C5 component as immunogens according to known methods. In accordance with this disclosure, complement component C5, C5a or C5b is preferably used as the immunogen. In accordance with particularly preferred useful embodiments, the immunogen is the alpha chain of C5.

Particularly useful antibodies share the required functional properties discussed in the preceding paragraph and have any of the following characteristics: (1) they compete for binding to portions of C5 that are specifically immunoreactive with 5G1.1; (2) they specifically bind to the C5 alpha chain--such specific binding, and competition for binding can be determined by various methods well known in the art, including the plasmon surface resonance method (Johne et al., J. Immunol. Meth. 1993, 160:191-198); and (3) they block the binding of C5 to either C3 or C4 (which are components of the C5 convertases).

The compound that inhibits the production and/or activity of at least one complement component can be administered in a variety of unit dosage forms. The dose will vary according to the particular compound employed. For example, different antibodies may have different masses and/or affinities, and thus require different dosage levels. Antibodies prepared as fragments (e.g., Fab, $F(ab')_2$, scFv) will also require differing dosages than the equivalent intact immunoglobulins, as they are of considerably smaller mass than intact immunoglobulins, and thus require lower dosages to reach the same molar levels in the patient's blood.

The dose will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician.

Administration of the compound that inhibits the production and/or activity of at least one complement component will preferably be via intravenous infusion by injection but may be in an aerosol form with a suitable pharmaceutical carrier, subcutaneous injection, orally, or sublingually. Other routes of administration may be

Samsung Bioepis Exhibit No. 1002
Page 1745

used if desired.

It is further contemplated that a combination therapy can be used wherein a complement-inhibiting compound is administered in combination with a regimen of known therapy for hemolytic disease. Such regimens include administration of 1) one or more compounds known to increase hematopoiesis (for example, either by boosting production, eliminating stem cell destruction or eliminating stem cell inhibition) in combination with 2) a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. Suitable compounds known to increase hematopoiesis include, for example, steroids, immunosuppressants (such as, cyclosporin), anti-coagulants (such as, warfarin), folic acid, iron and the like, erythropoietin (EPO), antithymocyte globulin (ATG), antilymphocyte globulin (ALG), EPO derivatives, EPO mimetics, and darbepoetin alfa (commercially available as Aranesp® from Amgen, Inc., Thousand Oaks, Calif. (Aranesp® is a man-made form of EPO produced in Chinese hamster ovary (CHO) cells by recombinant DNA technology)). In particularly useful embodiments, erythropoietin (EPO) (a compound known to increase hematopoiesis), EPO derivatives, or darbepoetin alfa may be administered in combination with an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1.

Formulations suitable for injection are found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, Pa., 17th ed. (1985). Such formulations must be sterile and non-pyrogenic, and generally will include a pharmaceutically effective carrier, such as saline, buffered (e.g., phosphate buffered) saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions, and the like. The formulations may contain pharmaceutically acceptable auxiliary substances as required, such as, tonicity adjusting agents, wetting agents, bactericidal agents, preservatives, stabilizers, and the like.

The present disclosure contemplates methods of reducing hemolysis in a patient afflicted with a hemolytic disease by administering one or more compounds which bind to or otherwise block the generation and/or activity of one or more complement components. Reducing hemolysis means that the duration of time a person suffers from hemolysis is reduced by about 25% or more. The effectiveness of

Samsung Bioepis Exhibit No. 1002
Page 1746

the treatment can be evaluated in any of the various manners known to those skilled in the art for determining the level of hemolysis in a patient. One qualitative method for detecting hemolysis is to observe the occurrences of hemoglobinuria. Quite surprisingly, treatment in accordance with the present methods reduces hemolysis as determined by a rapid reduction in hemoglobinuria.

A more qualitative manner of measuring hemolysis is to measure lactate dehydrogenase (LDH) levels in the patient's bloodstream. LDH catalyzes the interconversion of pyruvate and lactate. Red blood cells metabolize glucose to lactate, which is released into the blood and is taken up by the liver. LDH levels are used as an objective indicator of hemolysis. As those skilled in the art will appreciate, measurements of "upper limit of normal" levels of LDH will vary from lab to lab depending on a number of factors including the particular assay employed and the precise manner in which the assay is conducted. Generally speaking, however, the present methods can reduce hemolysis in a patient afflicted with a hemolytic disease as reflected by a reduction of LDH levels in the patients to within 20% of the upper limit of normal LDH levels. Alternatively, the present methods can reduce hemolysis in a patient afflicted with a hemolytic disease as reflected by a reduction of LDH levels in the patients of greater than 50% of the patient's pre-treatment LDH level, preferably greater than 65% of the patient's pre-treatment LDH level, most preferably greater than 80% of the patient's pre-treatment LDH level.

Another quantitative measurement of a reduction in hemolysis is the presence of GPI-deficient red blood cells (PNH red blood cells). As those skilled in the art will appreciate, PNH red blood cells have no GPI-anchor protein expression on the cell surface. The proportion of GPI-deficient cells (PNH cells) can be determined by flow cytometry using, for example, the technique described in Richards, et al., Clin. Appl. Immunol. Rev., vol. 1, pages 315-330, 2001. The absolute number of the PNH cells can then be determined. The present methods can reduce hemolysis in a patient afflicted with a hemolytic disease as reflected by an increase in PNH red blood cells. Preferably an increase in PNH red blood cell levels in the patient of greater than 25% of the total red blood cell count is achieved, more preferably an increase in PNH red blood cell levels in the patients greater than 50% of the total red blood cell count is achieved, most preferably an increase in PNH red blood cell levels in the patients greater than 75% of the total red blood cell count is achieved.

Samsung Bioepis Exhibit No. 1002
Page 1747

Methods of reducing one or more symptoms associated with PNH or other hemolytic diseases are also within the scope of the present disclosure. Such symptoms include, for example, abdominal pain, fatigue, and dyspnea. Symptoms can be the direct result of lysis of red blood cells (e.g., hemoglobinuria, anemia, fatigue, low red blood cell count, etc.) or the symptoms can result from low nitric oxide (NO) levels in the patient's bloodstream (e.g., abdominal pain, erectile dysfunction, dysphagia, thrombosis, etc.). It has recently been reported that patients with greater than 40% PNH granulocyte clone have an increased incidence of thrombosis, abdominal pain, erectile dysfunction and dysphagia, indicating a high hemolytic rate (see Moyo et al., British J. Haematol. 126:133-138 (2004)).

In particularly useful embodiments, the present methods provide a reduction in one or more symptoms associated with PNH or other hemolytic diseases in a patient having a platelet count in excess of 30,000 per microliter (a hypoplastic patient), preferably in excess of 75,000 per microliter, most preferably in excess of 150,000 per microliter. In other embodiments, the present methods provide a reduction in one or more symptoms associated with PNH or other hemolytic diseases in a patient where the proportion of PNH type III red blood cells of the subject's total red blood cell content is greater than 10%, preferably greater than 25%, most preferably in excess of 50%. In yet other embodiments, the present methods provide a reduction in one or more symptoms associated with PNH or other hemolytic diseases in a patient having a reticulocyte count in excess of 80×10^9 per liter, more preferably in excess of 120×10^9 per liter, most preferably in excess of 150×10^9 per liter. Patients in the most preferable ranges recited above have active bone marrow and will produce adequate numbers of red blood cells. While in a patient afflicted with PNH or other hemolytic disease the red blood cells may be defective in one or more ways (e.g., GPI deficient), the present methods are particularly useful in protecting such cells from lysis resulting from complement activation. Thus, patients within the preferred ranges benefit most from the present methods.

In one aspect, a method of reducing fatigue is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing fatigue means the duration of time a person suffers from fatigue is reduced by about 25% or more. Fatigue is a symptom

Samsung Bioepis Exhibit No. 1002
Page 1748

believed to be associated with intravascular hemolysis as the fatigue relents when hemoglobinuria resolves even when the anemia persists. By reducing the lysis of red blood cells, the present methods reduce fatigue. Patients within the above-mentioned preferred ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

In another aspect, a method of reducing abdominal pain is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing abdominal pain means the duration of time a person suffers from abdominal pain is reduced by about 25% or more. Abdominal pain is a symptom resulting from the inability of a patient's natural levels of haptoglobin to process all the free hemoglobin released into the bloodstream as a result of intravascular hemolysis, resulting in the scavenging of NO and intestinal dystonia and spasms. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby reducing abdominal pain. Patients within the above-mentioned preferred ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

In another aspect, a method of reducing dysphagia is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing dysphagia means the duration of time a person has dysphagia attacks is reduced by about 25% or more. Dysphagia is a symptom resulting from the inability of a patient's natural levels of haptoglobin to process all the free hemoglobin released into the bloodstream as a result of intravascular hemolysis, resulting in the scavenging of NO and esophageal spasms. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby reducing dysphagia. Patients within the above-mentioned preferred ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

In yet another aspect, a method of reducing erectile dysfunction is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the

Samsung Bioepis Exhibit No. 1002
Page 1749

generation and/or activity of one or more complement components. Reducing erectile dysfunction means the duration of time a person suffers from erectile dysfunction is reduced by about 25% or more. Erectile dysfunction is a symptom believed to be associated with scavenging of NO by free hemoglobin released into the bloodstream as a result of intravascular hemolysis. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of NO and reducing erectile dysfunction. Patients within the above-mentioned preferred ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

In yet another aspect, a method of reducing hemoglobinuria is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing hemoglobinuria means a reduction in the number of times a person has red, brown, or darker urine, wherein the reduction is typically about 25% or more. Hemoglobinuria is a symptom resulting from the inability of a patient's natural levels of haptoglobin to process all the free hemoglobin released into the bloodstream as a result of intravascular hemolysis. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream and urine thereby reducing hemoglobinuria. Quite surprisingly, the reduction in hemoglobinuria occurs rapidly. Patients within the above-mentioned preferred ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

In still another aspect, a method of reducing thrombosis is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing thrombosis means the duration of time a person has thrombosis attacks is reduced by about 25% or more or that the frequency of thrombosis attacks is reduced by about 25% or more over a period of one or more years. Thrombosis is a symptom believed to be associated with scavenging of NO by free hemoglobin released into the bloodstream as a result of intravascular hemolysis. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of NO and reducing thrombosis.

Samsung Bioepis Exhibit No. 1002
Page 1750

Thrombosis is thought to be multi-factorial in etiology including NO scavenging by free hemoglobin, exposure of prothrombotic surfaces from lysed red blood cell membranes, and changes in the endothelium surface by cell free heme. The intravascular release of free hemoglobin may directly contribute to small vessel thrombosis. NO has been shown to inhibit platelet aggregation, induce disaggregation of aggregated platelets and inhibit platelet adhesion. Conversely, NO scavenging by hemoglobin or the reduction of NO generation by the inhibition of arginine metabolism results in an increase in platelet aggregation. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of NO and reducing thrombosis.

In particularly useful embodiments, the present methods reduce thrombosis, especially in patients having a platelet count in excess of 30,000 per microliter, preferably in excess of 75,000 per microliter, most preferably in excess of 150,000 per microliter. In other embodiments, the present methods reduce thrombosis in patients where the proportion of PNH type III red blood cells of the subject's total red blood cell content is greater than 1%, preferably greater than 10%, more preferably greater than 25%, even more preferably in excess of 50%, and most preferably in excess of 75% (see, e.g., Hall et al., Blood 102:3587-3591 (2003); Audebert et al., J. Neurol. 252:1379-1386 (2005)). In yet other embodiments, the present methods reduce transfusion thrombosis in patients having a reticulocyte count in excess of 80×10^9 per liter, more preferably in excess of 120×10^9 per liter, most preferably in excess of 150×10^9 per liter.

In still another aspect, a method of reducing anemia is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing anemia means the duration of time a person has anemia is reduced by about 25% or more. Anemia in hemolytic diseases results from the blood's reduced capacity to carry oxygen due to the loss of red blood cell mass. By reducing the lysis of red blood cells, the present methods assist red blood cell levels to increase thereby reducing anemia.

In another aspect, a method of increasing the total endogenous red blood cell count in a patient afflicted with a hemolytic disease is contemplated. By increasing the patient's RBC count, fatigue, anemia and the patient's need for blood transfusions

Samsung Bioepis Exhibit No. 1002
Page 1751

is reduced. The reduction in transfusions can be in frequency of transfusions, amount of blood units transfused, or both.

The method of increasing red blood cell count in a patient afflicted with a hemolytic disease includes the step of administering a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components to a patient afflicted with a hemolytic disease. In particularly useful embodiments, the present methods increase red blood cell count in a patient afflicted with a hemolytic disease, especially patients having a platelet count in excess of 30,000 per microliter, preferably in excess of 75,000 per microliter, most preferably in excess of 150,000 per microliter. In other embodiments, the present methods increase red blood cell count in a patient afflicted with a hemolytic disease where the proportion of PNH type III red blood cells of the subject's total red blood cell content is greater than 1%, preferably greater than 10%, more preferably greater than 25%, even more preferably in excess of 50%, and most preferably in excess of 75%. In yet other embodiments, the present methods increase red blood cell count in a patient afflicted with a hemolytic disease having a reticulocyte count in excess of 80×10^9 per liter, more preferably in excess of 120×10^9 per liter, most preferably in excess of 150×10^9 per liter. In some embodiments, the methods of the present disclosure may result in a decrease in the frequency of transfusions by about 50%, typically a decrease in the frequency of transfusions by about 70%, more typically a decrease in the frequency of transfusions by about 90%.

In yet another aspect, the present disclosure contemplates a method of rendering a subject afflicted with a hemolytic disease less dependent on transfusions or transfusion-independent by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. As those skilled in the art will appreciate, the normal life cycle for a red blood cell is about 120 days. Treatment for six months or more is required for the evaluation of transfusion independence given the long half life of red blood cells. It has unexpectedly been found that in some patients transfusion-independence can be maintained for twelve months or more, in some cases more than four years, long beyond the 120 day life cycle of red blood cells. In particularly useful

Samsung Bioepis Exhibit No. 1002
Page 1752

embodiments, the present methods provide decreased dependence on transfusions or transfusion-independence in a patient afflicted with a hemolytic disease, especially patients having a platelet count in excess of 30,000 per microliter, preferably in excess of 75,000 per microliter, most preferably in excess of 150,000 per microliter. In other embodiments, the present methods provide decreased dependence on transfusions or transfusion-independence in a patient afflicted with a hemolytic disease where the proportion of PNH type III red blood cells of the subject's total red blood cell content is greater than 1%, preferably greater than 10%, more preferably greater than 25%, even more preferably in excess of 50%, and most preferably in excess of 75%. In yet other embodiments, the present methods provide decreased dependence on transfusions or transfusion-independence in a patient afflicted with a hemolytic disease having a reticulocyte count in excess of 80×10^9 per liter, more preferably in excess of 120×10^9 per liter, most preferably in excess of 150×10^9 per liter.

Methods of increasing the nitric oxide (NO) levels in a patient having PNH or some other hemolytic disease are also within the scope of the present disclosure. These methods of increasing NO levels include the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Low NO levels arise in patients afflicted with PNH or other hemolytic diseases as a result of scavenging of NO by free hemoglobin released into the bloodstream as a result of intravascular hemolysis. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of NO. In particularly useful embodiments, NO homeostasis is restored as evidenced by a resolution of symptoms attributable to NO deficiencies.

Without intending to limit it in any manner, the present application will be more fully described by the following examples.

Samsung Bioepis Exhibit No. 1002
Page 1753

EXAMPLES

EXAMPLE 1

Eleven patients participated in therapy trials to evaluate the effects of anti-C5 antibody on PNH and symptoms associated therewith. PNH patients were transfusion-dependent and hemolytic. Patients were defined as transfusion dependent with a history of four or more transfusions within twelve months. The median number of transfusions within the patient pool was nine in the previous twelve months. The median number of transfusion units used in the previous twelve months was twenty-two for the patient pool.

Over the course of four weeks, each of 11 patients received a weekly 600 mg intravenous infusion of anti-C5 antibody for approximately thirty minutes. The specific anti-C5 antibody used in the study was eculizumab. Patients received 900 mg of eculizumab 1 week later and then 900 mg on a bi-weekly basis. The first twelve weeks of the study constituted the pilot study. Following completion of the initial acute phase twelve week study, all patients participated in an extension study conducted to a total of 64 weeks. Ten of the eleven patients participated in an extension study conducted to a total of two years.

The effect of anti-C5 antibody treatments on PNH type III red blood cells ("RBCs") was tested. "PNH Type" refers to the density of GPI-anchored proteins expressed on the cell surface. Type I is normal expression, Type II is intermediate expression, and Type III has no GPI-anchor protein expression on the cell surface. The proportion of GPI-deficient cells is determined by flow cytometry in the manner described in Richards, et al., Clin. Appl. Immunol. Rev., vol.1, pages 315-330, 2001. As compared to pre-therapy conditions, PNH Type III red blood cells increased more than 50% during the extension study. The increase from a pre-study mean value of 36.7% of all red blood cells to a 64 week mean value of 58.4% of Type III red blood cells indicated that hemolysis had decreased sharply. See Table 1, below. Eculizumab therapy protected PNH type III RBCs from complement-mediated lysis, prolonging the cells' survival. This protection of the PNH-affected cells reduced the need for transfusions, paroxysms and overall hemolysis in all patients in the trial.

Samsung Bioepis Exhibit No. 1002
Page 1754

Table 1: PNH Cell Populations Pre- and Post-Eculizumab Treatment in All Patients

	Proportion of PNH Cells (%)			
PNH Cell Type	baseline	12 weeks	64 weeks	p-value[a]
Type III RBCs	36.7 +/- 5.9	59.2 +/- 8.0	58.4 +/- 8.5	0.005
Type II RBCs	5.3 +/- 1.4	7.5 +/- 2.1	13.2 +/- 2.4	0.013
Type III WBCs	92.1 +/- 4.6	89.9 +/- 6.6	91.1 +/- 5.8	N.S.
Type III Platelets	92.4 +/- 2.4	93.3 +/- 2.8	92.8 +/- 2.6	N.S.

[a]comparison of mean change from baseline to 64 weeks

During the course of the two year extension study, it was found that PNH red cells with a complete deficiency of GPI-linked proteins (Type III red cells) progressively increased during the treatment period from a mean of 36.7% to 58.9% (p=0.001) while partially deficient PNH red cells (Type II) increased from 5.3% to 8.7% (p=0.01). There was no concomitant change in the proportion of PNH neutrophils in any of the patients during eculizumab therapy, indicating that the increase in the proportion of PNH red cells was due to a reduction in hemolysis and transfusions rather than a change in the PNH clone(s) themselves.

The effect of anti-C5 antibody treatments on lactate dehydrogenase levels ("LDH") was measured on all eleven patients. LDH catalyzes the interconversion of pyruvate and lactate. Red blood cells metabolize glucose to lactate, which is released into the blood and is taken up by the liver. LDH levels are used as an objective indicator of hemolysis. The LDH levels were decreased by greater than 80% as compared to pre-treatment levels. The LDH levels were lowered from a pre-study mean value of 3111 U/L to a mean value of 594 U/L during the pilot study and a mean value of 622 U/L after 64 weeks (p=0.002 for 64 week comparison; see FIGS. 1A and 1B).

Similarly, aspartate aminotransferase (AST) levels, another marker of red blood cell hemolysis, decreased from a mean baseline value of 76 IU/L to 26 IU/L

Samsung Bioepis Exhibit No. 1002
Page 1755

and 30 IU/L during the 12 and 64 weeks of treatment, respectively (p=0.02 for 64 week comparison). Levels of haptoglobin, hemoglobin and bilirubin, and numbers of reticulocytes, did not change significantly from prestudy values during the 64 week treatment period.

Paroxysm rates were measured and compared to pre-treatment levels. Paroxysm as used in this disclosure is defined as incidences of dark-colored urine with a colorimetric level of 6 of more on a scale of 1-10. FIG. 2 shows the urine color scale devised to monitor the incidence of paroxysm of hemoglobinuria in patients with PNH before and during treatment. As compared to pre-treatment levels, the paroxysm percentage rate was reduced by 93% (see, FIG. 3) from 3.0 paroxysms per patient per month before eculizumab treatment to 0.1 paroxysm per patient per month during the initial 12 weeks and 0.2 paroxysm per patient per month during the 64 week treatment (FIG. 3 ($p<0.001$)).

Serum hemolytic activity in nine of the eleven patients was completely blocked throughout the 64 week treatment period with trough levels of eculizumab at equilibrium ranging from approximately 35 μg/mL to 350 μg/mL. During the extension study, 2 patients did not sustain levels of eculizumab necessary to consistently block complement. This breakthrough in serum hemolytic activity occurred in the last 2 days of the 14 day dosing interval, a pattern that was repeated between multiple doses. In one of the patients, as seen in FIG. 4, break-through of complement blockade resulted in hemoglobinuria, dysphagia, and increased LDH and AST, which correlated with the return of serum hemolytic activity. At the next dose, symptoms resolved (FIG. 5) and reduction in the dosing interval from 900 mg every 14 days to 900 mg every 12 days resulted in a regain of complement control which was maintained over the extension study to 64 weeks in both patients. This patient showed a 24 hour resolution of dysphagia and hemoglobinuria. A reduction in the dosing interval from 14 to 12 days was sufficient to maintain levels of eculizumab above 35 μg/mL and effectively and consistently blocked serum hemolytic activity for the remainder of the extension study for both patients.

The patients' need for transfusions was also reduced by the treatment with eculizumab. FIG. 6a compares the number of transfusion units required per patient per month, prior to and during treatment with an anti-C5 antibody for cytopenic patients, while FIG. 6b compares the number of transfusion units required per patient per

Samsung Bioepis Exhibit No. 1002
Page 1756

month, prior to and during treatment with an anti-C5 antibody for non-cytopenic patients. A significant reduction in the need for transfusion was also noted in the entire group (mean transfusion rates decreased from 2.1 units per patient per month during a 1 year period prior to treatment to 0.6 units per patient per month during the initial 12 weeks and 0.5 units per patient per month during the combined 64 week treatment period), with non-cytopenic patients benefiting the most. In fact, four of the non-thrombocytopenic patients with normal platelet counts (≥150,000 per microliter) became transfusion-independent during the 64 week treatment.

The effect of eculizumab administered in combination with erythropoietin (EPO) was also evaluated in a thrombocytopenic patient. EPO (NeoRecormon™, Roche Pharmaceuticals, Basel, Switzerland) was administered in an amount of 18,000 I.U. three times per week beginning in week 23 of the study. As shown in FIG. 7, the frequency of transfusions required for this patient was significantly reduced, and soon halted.

For the two year extension study, 10 of the 11 patients from the initial 3 month study continued to receive 900 mg of eculizumab every other week. (One patient discontinued eculizumab therapy after 23 months.) Six of the 11 patients had normal platelet counts (no clinical evidence of marrow failure) whereas 5 of the 11 had low platelet counts. For the patient who discontinued eculizumab therapy after 23 months, intravascular hemolysis was successfully controlled by eculizumab, but the patient continued to be transfused even after erythropoietin therapy. This patient had the most severe hypoplasia at the start of eculizumab therapy with a platelet count below 30 x 10^9/L, suggesting that the ongoing transfusions were likely a result of the underlying bone marrow failure.

Results of the two year extension study also demonstrated that there was a statistically significant decrease in transfusion requirements for the patients. Three patients remained transfusion independent during the entire two year treatment period, and four cytopenic patients became transfusion independent, three following treatment with EPO (NeoRecormon™). The reduction in transfusion requirements was found to be most pronounced in patients with a good marrow reserve.

Pharmacodynamic levels were measured and recorded according to eculizumab doses. The pharmacodynamic analysis of eculizumab was determined by measuring the capacity of patient serum samples to lyse chicken erythrocytes in a

Samsung Bioepis Exhibit No. 1002
Page 1757

standard total human serum complement hemolytic assay. Briefly, patient samples or human control serum (Quidel, San Diego, Calif.) was diluted to 40% vol/vol with gelatin veronal-buffered saline (GVB2+, Advanced Research technologies, San Diego, Calif.) and added in triplicate to a 96-well plate such that the final concentration of serum in each well was 20%. The plate was then incubated at room temperature while chicken erythrocytes (Lampire Biologics, Malvern, Pa.) were washed. The chicken erythrocytes were sensitized by the addition of anti-chicken red blood cell polyclonal antibody (0.1% vol/vol). The cells were then washed and resuspended in GVB2+ buffer. Chicken erythrocytes (2.5 x 10^6cells/30 μL) were added to the plate containing human control serum or patient samples and incubated at 37° C for 30 min. Each plate contained six additional wells of identically prepared chicken erythrocytes of which four wells were incubated with 20% serum containing 2 mM EDTA as the blank and two wells were incubated with GVB2+ buffer alone as a negative control for spontaneous hemolysis. The plate was then centrifuged and the supernatant transferred to a new flat bottom 96-well plate. Hemoglobin release was determined at OD 415 nm using a microplate reader. The percent hemolysis was determined using the following formula: Percent Hemolysis = 100 x ((OD patient sample - OD blank)/(OD human serum control - OD blank)).

The graph of the pharmacodynamics (FIG. 8), the study of the physiological effects, shows the percentage of serum hemolytic activity (i.e. the percentage of cell lysis) over time. Cell lysis was dramatically reduced in the majority of the patients to below 20% of normal serum hemolytic activity while under eculizumab treatment. Two patients exhibited a breakthrough in hemolytic activity, but complement blockade was permanently restored by reducing the dosing interval to 12 days (See, FIG. 4).

Improvement of quality of life issues was also evaluated using the European Organization for Research and Treatment of Cancer Core (http://www.eortc.be) questionnaires ("EORTC QLC-C30"). Each of the participating patients completed the QLC-30 questionnaire before and during the eculizumab therapy. Overall improvements were observed in global health status, physical functioning, role functioning, emotional functioning, cognitive functioning, fatigue, pain, dyspnea and insomnia. (See FIG. 9).

Patients in the two year study experienced a reduction in adverse symptoms

Samsung Bioepis Exhibit No. 1002
Page 1758

associated with PNH. For example, as set forth in FIG. 10, there was a demonstrated decrease of abdominal pain, dysphagia, and erectile dysfunction after administration of eculizumab in those patients reporting those symptoms before administration of eculizumab.

EXAMPLE 2

Description of Clinical Studies

The safety and efficacy of eculizumab was assessed in three separate studies including an 87 patient randomized, double-blind, placebo-controlled 26 week phase 3 study (Study C04-001), an ongoing 97 patient open-label 52 week phase 3 study (Study C04-002), and an 11 patient open-label 12 week phase 2 study (Study C02-001; this study had two study-specific extension studies [E02-001 and X03-001] totaling an additional 156 weeks). All patients successfully completing Studies C04-001, C04-002, or C02-001/E02-001/X03-001 were eligible to enroll in an ongoing open-label 104 week phase 3 extension study (Study E05-001) which is anticipated to enroll approximately 190 patients. The E05-001 study provides additional long-term safety and efficacy data of eculizumab in the overall population of PNH patients and includes the collection of thromboembolic event rates with eculizumab treatment across the pooled eculizumab treatment groups from the parent studies described above. The pre-specified secondary endpoint of thromboembolic event rate in Study E05-001 was designed to assess the thromboembolic event rate in each of the Study E05-001 patients before eculizumab treatment, during eculizumab treatment in each of the Study C04-001, C04-002, and C02-001/E02-001/X03-001 patients, and during eculizumab treatment in the overall patient population for all studies. Thrombotic event rates were captured as major adverse vascular events (MAVE, See Table 2).

In all studies, eculizumab-treated patients were administered 600 mg study drug every week for 4 weeks, 900 mg in week 5, and then a 900 mg dose every 14 ± 2 days for the study duration.

In Study C04-001, C04-002, and C02-001/E02-001/X03-001, eculizumab treatment was associated with highly statistically and clinically significant improvements in all pre-specified primary and secondary endpoints.

Samsung Bioepis Exhibit No. 1002
Page 1759

Table 2: LIST of MAJOR ADVERSE VASCULAR EVENTS (MAVE)

Thrombophlebitis/Deep Vein Thrombosis	Renal Vein Thrombosis
Pulmonary Embolus	Mesenteric Vein Thrombosis
Cerebrovascular Accident	Portal Vein Thrombosis (Budd-Chiari)
Amputation	Gangrene
Myocardial Infarction	Acute Peripheral Vascular Occlusion
Transient Ischemic Attack	Sudden Death
Unstable Angina	

Effect of Eculizumab on the Pathophysiological Pathways Leading to Clinically Symptomatic Thrombosis

Thromboembolic (TE) events are frequently tied directly to intravascular hemolysis in PNH. Intravascular hemolysis leads to accumulation of free hemoglobin in the plasma which has been demonstrated to deplete nitric oxide and subsequently lead to thrombus formation.

Treatment with eculizumab markedly reduces intravascular hemolysis, as measured by a decrease in median LDH, from 2,042 U/L pre-treatment to 261 U/L at 26 weeks with eculizumab treatment in the combined C04-001 and C04-002 studies ($P<0.001$).

Treatment with eculizumab markedly reduces circulating levels of cell-free hemoglobin, as measured by median free hemoglobin levels, from 36.7 mg/dL pre-treatment to 5.6 mg/dL at 26 weeks with eculizumab treatment in the combined C04-001 and C04-002 studies ($P<0.001$).

Treatment with eculizumab effectively reduces the consumption of nitric oxide, as measured by the median change in nitric oxide consumption, with median pre-treatment nitric oxide of 9.3 μM decreasing by 67.1% at week 26 with eculizumab treatment and with pre-treatment nitric oxide of 9.9 μM increasing by 14.9% at week 26 with placebo in the C04-001 study ($P<0.001$).

Effect of Eculizumab on Thrombotic Rate in Overall Study Population

Eculizumab-treatment TE events were determined for all patients that entered into and received eculizumab in the C04-001, C04-002, C02-001, E02-001, X03-001 and E05-001 PNH clinical studies on an intention-to-treat basis. TE events were defined by the MAVE criteria (see Table 2 above) in the C04-001, C04-002, and E05-001 studies (primary adverse event and medical history listings were used for the

Samsung Bioepis Exhibit No. 1002
Page 1760

C02-001, E02-001 and X03-001 studies). Patient years of eculizumab exposure were calculated for the completed C04-001, C02-001, E02-001 and X03-001 studies. For the C04-002 study, patient years of eculizumab exposure were determined for each patient after 26 weeks of treatment (the 6 month interim analysis). In the E05-001 study, patient years of exposure was determined for all patients through April 2006.

The pre-treatment patient years was determined from the earlier of diagnosis of PNH or first thrombotic event prior to enrollment into the parent PNH clinical studies (C04-001, C04-002, C02-001) and also included patient years from placebo-treated patients in the C04-001 study. The total pre-eculizumab treatment TE events included all TE events in all patients prior to enrollment in C04-001, C04-002, and C02-001 plus the TE events during placebo treatment in the C04-001 study (i.e., total pre-eculizumab treatment period TE events equals the sum of pre-eculizumab treatment TE events in C04-001, C04-002, and C02-001 in Table 3 plus the pre-C04-001 TE events in Table 4 plus the placebo-treatment TE events in Table 4). The total eculizumab period TE events included all TE events during the period commencing from the first eculizumab dose. The primary TE analysis (i.e., E05-001 secondary endpoint) was performed with a signed rank test.

Compared to the rate of thromboembolic events before treatment, eculizumab treatment resulted in a reduction in the TE event rate in the same patients in each of the individual clinical studies and a significant reduction in the TE event rate overall. The overall TE event rate was reduced from 7.49 TE events per 100 patient years pre-eculizumab treatment to 1.22 TE events per 100 patient years in the same patients with eculizumab treatment ($P<0.001$). This represented a relative reduction of 84% and an absolute reduction of 6.27 TE events per 100 patient years. Thromboembolic event rates are shown in Table 3.

Samsung Bioepis Exhibit No. 1002
Page 1761

Table 3: Overall Thromboembolic Events in Patients Prior to Start of Eculizumab Treatment and During Eculizumab Treatment in C04-001, C04-002, C02-001/E02-001/X03-001 and E05-001

	C04-001	C04-002	C02-001/ E02-001/ X03-001	E05-001 (All studies combined)
Pre-Treatment				
Patients (n)	43	97	11	195
MAVE Events (n)	16	93	5	126
Patient Years (n)	309.0	718.3	161.7	1683.4
MAVE Event Rate (n per 100 patient years)	5.18	12.95	3.09	7.49
Eculizumab Treatment				
Patients (n)	43	97	11	195
MAVE Events (n)	0	2	0	2
Patient Years (n)	21.8	48.2	35.8	164.1
MAVE Event Rate (n per100 patient years)	0.00	4.15	0.00	1.22[1]

[1]P<0.001 Eculizumab Treatment vs. Pre-Treatment

The apparent heterogeneity in the different pre-study TE event rates may have been related in part to the different inclusion criteria of the three individual studies and/or the different sites involved in the individual studies. However, as opposed to earlier reports, current TE event ascertainment was systematic, prospective, and performed on a multicenter, international, and controlled basis in the C04-001, C04-002, and E05-001 studies. For these reasons, it is likely that the current pre-study TE event rates more likely represent the TE event rate in this PNH patient population prior to enrollment in the eculizumab PNH studies, although even these estimates may underestimate the true TE event rate as discussed below. Further, despite heterogeneity in the individual pre-study TE event rates, eculizumab treatment consistently resulted in a marked reduction in TE event rate in each individual study.

Tests for Robustness of Eculizumab Effect on Thrombosis

Because of the striking reduction in TE event rate observed above, five post-hoc analyses were performed to test the robustness of the observed effect. The major confounding issues that were identified were possible reduction in TE event rate reporting in the randomized clinical trial as compared to the medical history, reduction in TE event rates over time during the pre-eculizumab treatment period, quantitative imbalance between the pre-eculizumab treatment and treatment period

Samsung Bioepis Exhibit No. 1002
Page 1762

quantity of patient years, impact of eculizumab treatment on patients with previous TE, reduction in TE event rates during the pre-eculizumab treatment period due to concomitant anticoagulant therapy.

Evaluation of Potential for Reduction in TE Event Rate Reporting in the Randomized Clinical Trial as Compared to the Medical History

In order to control for the potential confounding impact of an unexpected reduction in TE reporting during the randomized clinical trial as compared to pre-enrollment medical history, TE events were compared pre-enrollment and in placebo-treated C04-001 patients.

The TE event rate in patients treated with placebo was not reduced when compared to the rate in the same patients prior to placebo treatment. The TE event rate was 2.34 events per 100 patient years pre-placebo treatment and 4.38 events per 100 patient years in the same patients with placebo treatment. Thromboembolic event rates are shown in Table 4.

This analysis does not support the view that there was any intrinsic reduction in TE event rate reporting during the eculizumab clinical studies.

Table 4: Thromboembolic Events in the Same Patients Prior to Placebo Treatment and with Placebo Treatment in C04-001

	C04-001
Pre-Treatment	
Patients (n)	44
MAVE Events (n)	11
Patient Years (n)	470.4
MAVE Event Rate (n per 100 patient years)	2.34
Placebo Treatment	
Patients (n)	44
MAVE Events (n)	1
Patient Years (n)	22.9
MAVE Event Rate (n per100 patient years)	4.38

Twelve Months before Treatment vs. Eculizumab Treatment:

In order to evaluate for the potential of both (i) an unexpected reduction in the TE event rate immediately preceding trial entry, and also (ii) a quantitative imbalance

Samsung Bioepis Exhibit No. 1002
Page 1763

between the quantity of patient years associated with the pre-eculizumab treatment and eculizumab treatment periods, a single analysis was performed; pre-eculizumab treatment TE event rates were truncated and only examined during the 12 months immediately preceding eculizumab treatment and compared to the available eculizumab treatment period. This single analysis served to both remove more distant years from the analysis and therefore focus more so on the most recent medical condition of the patients and to also equalize the quantity of patient years considered in the analysis prior to treatment with the quantity of patient years currently available with eculizumab treatment.

Compared to the TE event rate during only the 12 month period immediately preceding commencement of eculizumab treatment, eculizumab treatment resulted in a reduction in TE event rate in the same patients in each of the individual clinical studies and a significant reduction in the TE event rate overall. The TE event rate was reduced from 17.21 events per 100 patient years pre-eculizumab treatment to 1.22 events per 100 patient years in the same patients with eculizumab treatment (P=0.013). This represented a relative reduction of 93%, and an absolute reduction of 15.99 TE events per 100 patient years. Thromboembolic event rates are shown in Table 5.

It is noteworthy that the TE event rate in the 12 month period immediately preceding eculizumab treatment is markedly increased at 17.21 TE events per 100 patient years, as compared to the aggregate event rate of 7.49 TE events per 100 patient years for the entire period of time extending from the earlier of first TE/PNH diagnosis to enrollment into one of the eculizumab PNH trials. Thus, the data demonstrate that the TE event rates during the 12 month period immediately preceding eculizumab treatment were not reduced as compared to the overall pre-eculizumab treatment event rate. This crescendo TE event rate immediately preceding trial enrollment may be indicative of a substantial survivor bias in the pre-eculizumab treatment dataset. Additionally, this crescendo pattern of the TE event rate in the period immediately preceding commencement of eculizumab treatment was followed by a comparative arrest of the TE event rate with eculizumab treatment.

Truncating the analysis to equalize the quantity of patient years in the two comparison groups, as shown in Table 5 below, did not mitigate the observed beneficial impact of eculizumab on the TE event rate. With an approximately equal

Samsung Bioepis Exhibit No. 1002
Page 1764

quantity of patient years distributed before and during eculizumab treatment, the observed relative and absolute reductions in TE event rates with eculizumab treatment were, if anything, greater than those observed with the primary analysis.

Table 5: Thromboembolic Events in Patients during the 12 Months Prior to Start of Eculizumab Treatment and During Eculizumab Treatment in C04-001, C04-002, C02-001/E02-001/X03-001 and E05-001

	C04-001	C04-002	C02-001/ E02-001/ X03-001	E05-001 (All studies combined)
Pre-Treatment				
Patients (n)	43	97	11	195
MAVE Events (n)	6	23	3	33
Patient Years (n)	42.9	93.8	11.0	191.8
MAVE Event Rate (n per 100 patient years)	13.98	24.51	27.27	17.21
SOLIRIS™ Treatment				
Patients (n)	43	97	11	195
MAVE Events (n)	0	2	0	2
Patient Years (n)	21.8	48.2	35.8	164.1
MAVE Event Rate (n per 100 patient years)	0.00	4.15	0.00	1.22[1]

[1]P=0.013 Eculizumab vs. Pre-Treatment

Evaluation of Impact of Eculizumab Treatment on Patients with Previous TE

In order to control for and identify the impact of previous thrombosis on the analysis, the effect of eculizumab on the TE event rate in patients with previous TE was examined. Patients who did not have a TE event pre-eculizumab treatment were excluded from the analysis.

Compared to the rate of thromboembolic events in patients with previous TE before eculizumab treatment, eculizumab treatment resulted in a reduction in TE event rate in the same patients in each of the individual clinical studies and a significant reduction in TE event rate overall. The TE event rate was reduced from 21.95 TE events per 100 patient years pre-eculizumab treatment to 3.42 TE events per 100 patient years in the same patients with eculizumab treatment ($P<0.001$). This represented a reduction of 84%, and an absolute reduction of 18.53 TE events per 100 patient years. Thromboembolic event rates are shown in Table 6.

Samsung Bioepis Exhibit No. 1002
Page 1765

Thus, in patients with the highest TE event rate pre-eculizumab treatment, eculizumab treatment caused a commensurate and highly significant reduction in TE event rates.

Table 6: Thromboembolic Events in Patients with Previous Thrombotic Events Prior to Start of Eculizumab Treatment and During Eculizumab Treatment in C04-001, C04-002, C02-001/E02-001/X03-001 and E05-001

	C04-001	C04-002	C02-001/ E02-001/ X03-001	E05-001 (All studies combined)
Pre-Treatment				
Patients (n)	9	42	3	63
MAVE Events (n)	16	93	5	126
Patient Years (n)	78.7	329.2	17.6	574.2
MAVE Event Rate (n per 100 patient years)	20.34	28.25	28.43	21.95
Eculizumab Treatment				
Patients (n)	9	42	3	63
MAVE Events (n)	0	2	0	2
Patient Years (n)	4.6	20.7	9.1	58.5
MAVE Event Rate (n per 100 patient years)	0.00	9.68	0.00	3.42[1]

[1]P<0.001 Eculizumab vs. Pre-Treatment

Evaluation of Impact of Eculizumab Treatment on Patients Treated Concomitantly with Anticoagulant Therapy

In order to evaluate the potential impact of other anti-thrombotic therapies (which can comprise both anticoagulant and anti-platelet therapy) to reduce TE event rates over time prior to eculizumab treatment, the potentially confounding effect of anticoagulant therapy was controlled by specifically examining the effect of eculizumab treatment on the TE event rate in patients with previous anticoagulation therapy. TE event rates in patients who were never anticoagulated were also examined; in these analyses, TE events prior to initiation of anticoagulant therapy were excluded.

Compared to the TE event rate in patients treated with anticoagulant therapy before commencement of eculizumab treatment, eculizumab treatment resulted in a reduction in the TE event rate in the same patients in each of the individual clinical studies and a significant reduction in the TE event rate overall. The TE event rate was reduced from 14.00 TE events per 100 patient years with anticoagulant therapy but

Samsung Bioepis Exhibit No. 1002
Page 1766

prior to commencement of eculizumab treatment to 0.00 TE events per 100 patient years with eculizumab treatment in the same patients (P<0.001). This represented a relative reduction of 100%, and an absolute reduction of 14.00 TE events per 100 patient years. Thromboembolic event rates are shown in Table 7.

Compared to the negligible rate of thromboembolic events in patients without anticoagulant therapy before eculizumab treatment, eculizumab treatment resulted in no meaningful change in the thrombotic event rate. The TE event rate was 1.31 TE events per 100 patient years pre-eculizumab treatment and 2.90 TE events per 100 patient years in the same patients with eculizumab treatment (P=1.000). Thromboembolic event rates are shown in Table 8.

Table 7: Thromboembolic Events in Patients with Previous Anticoagulant Treatment Prior to Start of Eculizumab Treatment and During Eculizumab Treatment in C04-001, C04-002, C02-001/E02-001/X03-001 and E05-001

	C04-001	**C04-002**	**C02-001/ E02-001/ X03-001**	**E05-001 (All studies combined)**
Pre-Treatment				
Patients (n)	23	51	9	103
MAVE Events (n)	11	35	4	54
Patient Years (n)	72.7	168.6	45.9	385.7
MAVE Event Rate (n per 100 patient years)	15.13	20.76	8.71	14.00
Eculizumab Treatment				
Patients (n)	23	51	9	103
MAVE Events (n)	0	0	0	0
Patient Years (n)	11.9	24.8	28.8	100.1
MAVE Event Rate (n per 100 patient years)	0.00	0.00	0.00	0.00[1]

[1]P<0.001 Eculizumab vs. Pre-Treatment

Samsung Bioepis Exhibit No. 1002
Page 1767

Table 8: Thromboembolic Events in Patients without Previous Anticoagulant Treatment Prior to Start of Eculizumab Treatment and During Eculizumab Treatment in C04-001, C04-002, C02-001/E02-001/X03-001 and E05-001

	C04-001	C04-002	C02-001/ E02-001/ X03-001	E05-001 (All studies combined)
Pre-Treatment				
Patients (n)	20	46	2	92
MAVE Events (n)	0	7	0	10
Patient Years (n)	122.4	319.4	69.0	764.3
MAVE Event Rate (n per 100 patient years)	0.00	2.19	0.00	1.31
Eculizumab Treatment				
Patients (n)	20	46	2	92
MAVE Events (n)	0	2	0	2
Patient Years (n)	9.9	22.2	7.0	69.0
MAVE Event Rate (n per 100 patient years)	0.00	8.99	0.00	2.90[1]

[1]P=1.000 Eculizumab vs. Pre-Treatment

Eculizumab has been demonstrated to be safe and well tolerated for the treatment of PNH. In studies of patients diagnosed with PNH, there were no apparent significant safety concerns associated with eculizumab therapy. Adverse event frequency was similar in eculizumab and placebo-treated patients and the overall frequency of serious adverse events was less with eculizumab than with placebo. There was one reported infection with *Neisseria* species in a vaccinated PNH patient that was treated effectively and resolved without clinical sequelae. The overall frequency of infections was similar with eculizumab and placebo. Serious hemolysis following discontinuation of eculizumab in PNH patients was not observed and patients that discontinued eculizumab were effectively managed by standard of care. The incidence of bone marrow failure disorders was unchanged with eculizumab treatment. In addition, no dose-related toxicities were observed in these studies.

EXAMPLE 3

Eculizumab, a complement inhibitor, was shown to reduce intravascular hemolysis and transfusion requirements in patients with PNH. Eculizumab-treated patients, as compared to placebo, showed an 85.8% decrease in intravascular hemolysis (as measured by LDH area under a curve, $p<0.001$). This reduction in hemolysis with eculizumab resulted in a 2.5-fold increase in PNH RBC mass from a

Samsung Bioepis Exhibit No. 1002
Page 1768

median of $0.81x10^{12}$ cells/L at baseline to $2.05x10^{12}$ cells/L at 26 weeks ($p<0.001$), while the PNH RBC mass in placebo-treated patients remained relatively unchanged (from a median of $1.09x10^{12}$ cells/L to $1.16x10^{12}$ cells/L) (FIG. 11). The increase in PNH RBC mass was associated with an overall increase in hemoglobin levels in eculizumab-treated patients relative to placebo ($p<0.001$, mixed model analysis). The number of PRBC units transfused decreased from a median of 10.0/patient with placebo to 0.0/patient with eculizumab ($p<0.001$), and 51.2% of eculizumab-treated patients became transfusion independent (versus 0.0% of placebo patients, $p<0.001$). Even patients who required some transfusions while on eculizumab showed a marked reduction in transfusion requirements from a median of 10.0 units per patient with placebo to 6.0 units/patient with eculizumab ($p<0.001$). The reduction in PRBC units transfused with eculizumab was observed regardless of transfusion requirements prior to treatment, with statistical significance reached in 3 of 3 pre-treatment transfusion strata (4 to 14 units/year; 15-25 units/year; and >25 units/year, $p<0.001$ for each stratum) (see Table 9). Significant reductions were observed in intravascular hemolysis (LDH) in eculizumab-treated patients that achieved transfusion independence ($p<0.001$) as well as those that did not ($p<0.001$) (see Table 10). Taken together, these data demonstrate that effective control of intravascular hemolysis in PNH with eculizumab results in a substantial improvement in anemia, as evidenced by an increase in endogenous RBC mass, an improvement in hemoglobin levels, and a reduction in transfusion requirements. Substantial and significant reductions in intravascular hemolysis and improvements in anemia with eculizumab are demonstrated regardless of historical transfusion requirements or whether patients achieve transfusion independence during treatment. See Hillmen et al., N. Engl. J. Med. 355:1233-1243 (2006).

Samsung Bioepis Exhibit No. 1002
Page 1769

Table 9 - Transfusion Requirement during Treatment by Pretreatment Transfusion Strata

		Median Packed Red Cells Transfused (units/patient)		
Transfusion Stratum (Units)	No. Patients	Placebo	Eculizumab	P value*
Overall	87	10.0	0.0	<0.001
4-14	30	6.0	0.0	<0.001
15-25	35	10.0	2.0	<0.001
>25	22	18.0	3.0	<0.001

Table 10 - Hemolysis (LDH AUC) during Treatment by Pretreatment Transfusion Strata

		Median Lactate Dehydrogenase Area under the Curve (Units/L x Day)		
Transfusion Stratum (Units)	No. Patients	Placebo	Eculizumab	P value*
Overall	87	411,822	58,587	<0.001
4-14	30	398,573	53,610	<0.001
15-25	35	420,338	56,127	<0.001
>25	22	441,880	67,181	<0.001

Samsung Bioepis Exhibit No. 1002
Page 1770

Randomization strata were based on transfusion data over a period of 12-months prior to screening.

*P value was calculated using Wilcoxon's rank sum test.

EXAMPLE 4

A 48-year old transfusion-dependent male was diagnosed with aplastic anemia in May 1988 and with PNH in September 1993. He has been transfusion dependent due to PNH starting in September 1993, requiring transfusions of packed red blood cells (PRBCs) every 4 to 6 weeks. He received eculizumab infusions starting May 22, 2002 and is currently dosed at 900 mg every other week. On November 6, 2002, after 6 months of receiving eculizumab, rHuEpo (NeoRecormon®) therapy was initiated at the following doses: 450 IU/kg/week in 3 divided doses during the first 2 months; 900 IU/kg/week in 3 divided doses during the next 15 months; and 750 IU/kg/week in 3 divided doses until May 3, 2006. At that time he was switched to Aranesp® at a dose of 300 mcg every 2 weeks. The dose was increased to 500 mcg every 2 weeks on 28th June 2006.

Intravascular hemolysis was assessed by measuring levels of the enzyme lactate dehydrogenase (LDH). Levels of erythropoiesis were determined by measuring reticulocyte counts. PNH RBC mass was calculated by multiplying the absolute number of RBCs by the proportion of PNH type III RBCs as assessed by flow cytometry. Hemoglobin levels and PRBC transfusion requirements were also monitored. All assessments have been collected to the present date and results are reported through August 2006.

During the year prior to eculizumab therapy, the mean LDH level was 2,075 IU/L (more than 4 times that of the upper limit of the normal range), the mean hemoglobin level was 10.5 g/dL, and the mean reticulocyte count was 77.5 x 10^9/L (Table 11). The absolute number of PNH type III RBCs was 1.1 x 10^{12}/L, and the proportion of these cells constituted less than 50% of the total RBC mass. The patient required 1.8 units of PRBCs per month during the pre-treatment period (Table 11), receiving a total of 9 transfusions and 22 units (Fig. 12).

Samsung Bioepis Exhibit No. 1002
Page 1771

Table 11: Hematological Parameters Before and After Eculizumab and rHuEpo Therapies.

	Mean ± SD		
Parameter	**Pre-treatment (1 year)**	**Eculizumab alone (0.5 year)**	**Eculizumab RHuEPO (3.7 years)**
LDH, IU/L (normal range 150-480)	2075 ± 1590	456 ± 76	679 ± 146
Hemoglobin, g/dL (normal range 13.5-18.0)	10.5 ± 1.5	10.2 ± 0.9	11.4 ± 1.1
Reticulocytes, $x10^9$/L (normal range 20-80)	77.5 ± 10.6	96.4 ± 29.5	205.3 ± 43.6
PNH type III RBCs, $x10^{12}$/L*	1.1 ± 0.3	1.9 ± 0.1	2.5 ± 0.3
Units transfused per month	1.8	1.0	0.1

*Calculated as (proportion of PNH type III RBCs) x (total number of RBCs) ÷ 100

After starting eculizumab treatment, hemolysis was rapidly and consistently reduced as indicated by a 78% decrease in the mean LDH level (Table 11). A concomitant increase (73%) in the PNH type III RBC mass was also demonstrated, supporting enhanced survival of these cells. Further, the average number of transfusions required each month was reduced by 44%. RBC hemoglobin was stable even though transfusion requirement decreased, indicating a net increase in endogenous hemoglobin levels (Table 11).

After 6 months of eculizumab treatment, the patient received concomitant rHuEpo therapy resulting in a mean reticulocyte count increase of 113% (Table 11). This increase in erythropoiesis was associated with an additional 32% increase in the PNH type III RBC mass over that achieved with eculizumab treatment alone. In addition, RBC hemoglobin levels showed an increase from 10.2 g/dL to 11.4 g/dL during the same period. This improvement in anemia resulted in a further decrease in transfusion requirements, eventually leading to transfusion-independence for more than two years (Fig. 12). One transfusion was given after the two-years of transfusion independence and this coincided with a transient decrease in erythropoiesis, as evidenced by a drop in the reticulocyte count (data not shown). There was no evidence of an increase in intravascular hemolysis and LDH levels have remained

Samsung Bioepis Exhibit No. 1002
Page 1772

within the normal range or just above the upper limit of the normal range during the entire treatment period. This patient continues to receive eculizumab and rHuEpo and has received only 1 transfusion in more than 3 years.

EXAMPLE 5

Pulmonary hypertension (PHT) is an emerging common complication of hereditary hemolytic anemias. It has been mechanistically and epidemiologically linked to intravascular hemolysis and decreased nitric oxide (NO) bioavailability. While this complication has been described in approximately 30% of adult patients with sickle cell disease and thalassemia, the prevalence of PHT in patients with paroxysmal nocturnal hemoglobinuria (PNH), an acquired disease with the highest levels of intravascular hemolysis observed, has never been determined. PNH patients frequently have symptoms consistent with both hemolysis and PHT including severe fatigue and dyspnea on exertion. Therefore, we examined for the presence of PHT in PNH and explored potential mechanisms associated with its development by measuring the ability of plasma to instantaneously consume NO using ozone-based chemiluminescence.

Doppler echocardiography was performed in 28 hemolytic PNH patients to estimate pulmonary artery systolic pressures. Systolic PHT was defined by a tricuspid regurgitant jet velocity (TRV) $\geq$2.5m/s at rest. Fourteen (50%) patients had elevated pulmonary artery systolic pressures. Twelve (43%) had mild to moderate PHT (mean TRV 2.6m/s $\pm$ 0.01) while two (7%) had moderate to severe pressures (mean TRV 3.7m/s $\pm$ 0.02). Plasma from PNH patients (n=32) consumed 34.6 $\pm$ 8.3μM NO while normal subjects (n=9) consumed 2.2 $\pm$ 0.6μM NO (p=0.0001). LDH levels correlated with NO consumption (r=0.6342, p<0.0002). In a separate cohort of 7 patients treated with eculizumab for a median of 3 years to reduce hemolysis, the ability to consume NO appeared lower (13.2 $\pm$ 4.8μM NO).

EXAMPLE 6

PNH patients suffer from diverse and serious hemolysis-induced morbidities leading to a poor quality of life (QoL). Fatigue in PNH patients may be disabling and levels are similar to anemic cancer patients. Fatigue is multifactoral, related to both the underlying anemia and hemolysis. Patients suffer from reduced global health

Samsung Bioepis Exhibit No. 1002
Page 1773

status, patient functioning, pain and dyspnea. Treatment with the complement inhibitor eculizumab reduces intravascular hemolysis and improves anemia. The impact of eculizumab treatment on levels of fatigue and other patient reported outcomes was prospectively examined in a double-blind placebo-controlled study (TRIUMPH) using two distinct instruments, the FACIT-Fatigue and the EORTC QLQ-C30. Improvements in QoL were quantified using standardized effect sizes (SES), a measure of the magnitude of the clinical benefit in various instruments. Eculizumab treatment, as compared to placebo, was associated with a very large and significant improvement in fatigue as measured by the FACIT-fatigue scale (SES=1.13, P<0.001) as well as the EORTC-QLQ-C30 fatigue subscale ((SES=1.12, P<0.001). Similarly, the percentage of patients achieving a pre-specified minimally important difference (MID) was 53.7% versus 20.5% of eculizumab- and placebo-treated patients, respectively (P=0.003) using the FACIT-Fatigue; 67.6% versus 24.4%, respectively (P<0.001) with the EORTC QLQ-C30. Treatment independent univariate analyses showed that reduction in intravascular hemolysis (decreased LDH levels) and improvement in anemia (increased hemoglobin levels) were both significantly associated with an improvement in fatigue. Further multivariate analyses indicated that reduction in hemolysis was more predictive than improvement in anemia of an improvement in fatigue. Eculizumab treatment was also associated with significant improvements with moderate to large SES in the following EORTC-QLQ-C30 subscales: global health status (0.87, P<0.001); role functioning (0.93, P<0.001); social functioning (0.57, P=0.003); cognitive functioning (0.78, P=0.002); physical functioning (1.01, P<0.001); emotional functioning (0.51, P=0.008); pain (0.65, P=0.002); dyspnea (0.69, P<0.001); and appetite loss (0.50, P<0.001). These data demonstrate that resolution of intravascular hemolysis with eculizumab treatment results in large and clinically meaningful improvements in patient reported outcomes including fatigue, global health status, patient functioning, and disease-related symptoms in PNH.

EXAMPLE 7

In paroxysmal nocturnal hemoglobinuria (PNH), lack of the GPI-anchored terminal complement inhibitor CD59 from blood cells renders erythrocytes susceptible to chronic hemolysis resulting in anemia, fatigue, thrombosis, poor quality

Samsung Bioepis Exhibit No. 1002
Page 1774

of the life (QoL), and a dependency on transfusions. Eculizumab, a complement inhibitor, reduced intravascular hemolysis and transfusion requirements in transfusion dependent patients with normal or near-normal platelet counts in a randomized placebo-controlled trial (TRIUMPH). SHEPHERD, an open-label, non-placebo controlled 52-week phase III clinical study, is underway to evaluate the safety and efficacy of eculizumab in a broader PNH population including patients with significant thrombocytopenia and/or lower transfusion requirements. Eculizumab was dosed as follows: 600 mg IV every 7 days x 4; 900 mg 7 days later; and then 900 mg every 14±2 days. Eculizumab was administered to 97 patients at 33 international sites. In a pre-specified 6-month interim analysis, the most frequent adverse events were headache (50%), nasopharyngitis (23%), and nausea (16%); most were mild to moderate in severity. No infections or serious adverse events were reported as "probably" or "definitely" related to drug. Intravascular hemolysis, the central clinical manifestation in PNH and the primary surrogate efficacy endpoint of the trial, was significantly reduced in eculizumab patients as assessed by change in lactate dehydrogenase (LDH) area under the curve ($p<0.001$). LDH levels decreased from a median of 2,051 U/L at baseline to 270 U/L at 26 weeks ($p<0.001$; normal range 103-223 U/L). Control of intravascular hemolysis resulted in an improvement in anemia as transfusion requirements decreased from a median of 4.0 PRBC units/patient pre-treatment to 0.0 during treatment ($p<0.001$), approximately 50% of the patients were rendered transfusion independent ($P<0.001$), and hemoglobin levels increased ($p<0.001$). Fatigue, as measured by both the FACIT-Fatigue and EORTC QLQ-C30 instruments, was significantly improved with eculizumab treatment as compared to baseline ($p<0.001$ for each) (FIG. 13 and Table 12). Other EORTC-QLQ-C30 patient reported outcomes demonstrating improvement included global health status ($p<0.001$), all 5 patient functioning subscales ($p<0.001$) and 7 of 9 symptom/single item subscales ($p\leq0.03$). These results demonstrate that the beneficial effects of eculizumab in PNH are applicable to a much broader patient population than previously studied and further underscore that eculizumab treatment markedly reduces intravascular hemolysis, thereby providing clinical benefit to treated patients.

Table 12: Fatigue and other outcomes, as measured by both the FACIT-Fatigue and EORTC QLQ-C30 instruments.

Samsung Bioepis Exhibit No. 1002
Page 1775

	MID (%)*	SES†	Change from Baseline P-Value‡
FACIT-Fatigue	74.5	1.01	<0.001
EORTC QLQ-C30			
Fatigue	80.9	1.08	<0.001
Global Health Status	59.6	0.73	<0.001
Functioning scales			
Physical	50.0	0.86	<0.001
Role	55.3	0.70	<0.001
Cognitive	39.4	0.40	<0.001
Social	54.3	0.61	<0.001
Emotional	44.7	0.58	<0.001
Dyspnea	55.3	0.75	<0.001
Pain	30.9	0.30	0.004
Appetite loss	20.2	0.31	<0.001
Insomnia	35.1	0.48	<0.001
Financial difficulties	15.1	0.08	0.804
Constipation	10.8	0.08	0.758
Nausea/ vomiting	18.1	0.05	0.034
Diarrhea	17.0	0.27	<0.001

EXAMPLE 8

Paroxysmal nocturnal hemoglobinuria (PNH) is characterized by clonal expansion of PNH red cells that are highly sensitive to lysis by terminal complement. The primary lesion in PNH is bone marrow failure in the form of immune-mediated aplastic anemia and peripheral blood cytopenias of varying severity. In Example 1, the successful control of hemolysis and transfusion in 11 patients with the complement inhibitor eculizumab is described. Ten of these 11 patients remained on eculizumab therapy after approximately 3 years with maintained reductions in intravascular hemolysis and transfusion. The effectiveness of eculizumab therapy in

Samsung Bioepis Exhibit No. 1002
Page 1776

these patients is through the protection of the PNH red cell from complement-mediated lysis and the expansion of this cell population. Flow cytometry studies have shown that the percentage of PNH red cells increased significantly from a mean of 36.7% before treatment to 58.4% at week 64 of therapy. Importantly, granulocyte, monocyte and platelet PNH clone sizes were >90% before treatment and remained stable for all patients throughout the trial suggesting that the majority of hematopoiesis is derived from PNH stem cells. It is hypothesized that the PNH red cell clone should approach the clone size of other myeloid hematopoietic cells in a given patient when hemolysis is prevented by eculizumab therapy as this more accurately depicts PNH stem cell activity.

In all patients hemolysis was substantially reduced by 21 days. In 9 of 11 patients, there was a rapid rise in PNH red cell count with the mean absolute number of PNH red cells increasing from 1.37 x 1012/L before treatment to 1.50 x 10^{12}/L at 2 weeks (P=0.21), 1.74 x 10^{12}/L at 4 weeks (P=0.002), and 2.11 x 10^{12}/L at 12 weeks (P=0.001) of eculizumab treatment. The maximum theoretical red cell response was achieved in a mean of 178 days (range 49 – 419 days). The mean absolute number of PNH red cells increased to 2.37 x 10^{12}/L at maximum response (P=0.001), an increase of 73% (range 36% – 207%). All patients achieved a maximum response prior to 18 months of treatment and clone size was subsequently stable. In 2 patients, despite the effectiveness of eculizumab in resolving hemolysis, there was no change in absolute numbers of PNH red cells pre and post-treatment. This is likely due to a combination of a lower degree of hemolysis and more profound bone marrow insufficiency in these patients. The determination of absolute PNH red cell counts during the first 12 months of eculizumab therapy may identify which patients will become transfusion independent and which patients may benefit from additional growth factor support to boost erythropoiesis. Furthermore, long-term eculizumab therapy appeared to be associated with a stable PNH red cell clone size in this initial clinical study.

Samsung Bioepis Exhibit No. 1002
Page 1777

EXAMPLE 9

Surprisingly, in the C04-002 study, eculizumab treatment, as compared to baseline, was associated with an apparent increase in parameters of platelet activation (mixed model analysis, overall). Statistically significant increases were observed in monocyte-platelet aggregates (mean increase of 7.9%, P=0.002), neutrophil-platelet aggregates (mean increase of 5.3%, P<0.001) and the percentage of P-Selectin positive platelets (mean increase of 3.7%, P<0.001). Similarly, in eculizumab-treated patients in the C04-001 study increases were observed in monocyte-platelet aggregates (mean increase of 15.0%, P=0.056), neutrophil-platelet aggregates (mean increase of 11.2%, P=0.077) and the percentage of P-Selectin positive platelets (mean increase of 5.1%, P=0.044).

In the combined C04-001 and C04-002 studies, significant increases were also observed in monocyte-platelet aggregates (mean increase of 10.1%, P<0.001), neutrophil-platelet aggregates (mean increase of 7.0%, P<0.001) and the percentage of P-Selectin positive platelets (mean increase of 4.1%, P<0.001). In this placebo-controlled C04-001 study, the eculizumab cohort as well as the placebo cohort showed similar increases in parameters of platelet activation from baseline (Table 13A-F). In these placebo-treated patients, increases from baseline were observed in monocyte-platelet aggregates (mean increase of 2.2%, P=0.771), neutrophil-platelet aggregates (mean increase of 6.9%, P=0.135) and the percentage of P-Selectin positive platelets (mean increase of 5.9%, P-0.001) (Table 14A-C).

Samsung Bioepis Exhibit No. 1002
Page 1778

Attorney Docket : ALXN-PW2-114

TABLE 13A: C04-002; C04-001 and C04-002 Compared and Combined: Mixed model analysis of change in Platelet Activation Markers; Change from Baseline
MONOCYTE PLATELET AGGREGATION

Study		Study Week		Statistic	DF	P Value (a)
C04-001 (N = 43)	Mean Baseline Value		44.282			
	Least Square Means	1	9.294	0.98	34	0.333249423
		2	14.009	1.48	34	0.148210796
		4	27.935	2.95	34	0.005713194
		14	16.012	1.64	34	0.110194214
		26	10.827	1.11	34	0.275177698
	Overall (b)	Overall	15.005	1.98	34	0.055927296
C-04-002 (N = 97)	Mean Baseline Value		26.310			
	Least Square Means	1	2.967	0.65	95	0.518317428
		2	2.691	0.62	95	0.534704269
		4	9.596	2.22	95	0.028644828
		14	11.193	2.54	95	0.012540534
		26	11.838	2.69	95	0.008417504
	Overall (b)	Overall	7.940	3.17	95	0.002037650

NOTE: (a) P value was based on T test.
(b) Overall was based on the average of least square means from Week 1 to Week 26.

Samsung Bioepis Exhibit No. 1002
Page 1779

Attorney Docket : ALXN-PW2-114

TABLE 13B: C04-002; C04-001 and C04-002 Compared and Combined: Mixed model analysis of change in Platelet Activation Markers; Change from Baseline
MONOCYTE PLATELET AGGREGATION

Study		Study Week		Statistic	DF	P Value (a)
Combined (N = 140)	Mean Baseline Value		31.302			
	Least Square Means	1	4.971	1.15	133	0.250281818
		2	5.794	1.39	133	0.166169537
		4	14.650	3.52	133	0.000591120
		14	12.842	3.02	133	0.003059012
		26	11.824	2.78	133	0.006256488
	Overall (b)	Overall	10.092	3.47	133	0.000691639
Placebo (N = 44)	Mean Baseline Value		39.444			
	Least Square Means	1	4.768	0.55	44	0.585815062
		2	-3.814	-0.45	44	0.652851604
		4	5.484	0.63	44	0.531000233
		14	-4.964	-0.55	44	0.583493606
		26	13.523	1.61	44	0.115481853
	Overall (b)	Overall	2.181	0.37	44	0.711338659

NOTE: (a) P value was based on T test.
(b) Overall was based on the average of least square means from Week 1 to Week 26.

Samsung Bioepis Exhibit No. 1002
Page 1780

Attorney Docket : ALXN-PW2-114

TABLE 13C: C04-002; C04-001 and C04-002 Compared and Combined: Mixed model analysis of change in Platelet Activation Markers; Change from Baseline
NEUTROPHIL-PLATELET AGGREGATION

Study		Study Week		Statistic	DF	P Value (a)
C04-001 (N = 43)	Mean Baseline Value		23.555			
	Least Square Means	1	6.057	0.75	34	0.457636650
		2	6.877	0.85	34	0.399618945
		4	21.467	2.66	34	0.011756090
		14	16.246	1.95	34	0.059947232
		26	6.857	0.82	34	0.417142286
	Overall (b)	Overall	11.222	1.83	34	0.076671903
C-04-002 (N = 97)	Mean Baseline Value		10.586			
	Least Square Means	1	3.558	1.27	94	0.208460346
		2	0.353	0.13	94	0.894343104
		4	4.011	1.51	94	0.133423112
		14	9.505	3.52	94	0.000663614
		26	8.476	3.14	94	0.002261817
	Overall (b)	Overall	5.275	3.51	94	0.000679427

NOTE: (a) P value was based on T test.
(b) Overall was based on the average of least square means from Week 1 to Week 26.

Samsung Bioepis Exhibit No. 1002
Page 1781

Attorney Docket : ALXN-PW2-114

TABLE 13D: C04-002; C04-001 and C04-002 Compared and Combined: Mixed model analysis of change in Platelet Activation Markers; Change from Baseline
NEUTROPHIL-PLATELET AGGREGATION

Study		Study Week		Statistic	DF	P Value (a)
Combined (N = 140)	Mean Baseline Value		14.188			
	Least Square Means	1	4.232	1.38	133	0.170014676
		2	2.156	0.73	133	0.467887940
		4	8.851	2.99	133	0.003344370
		14	11.514	3.80	133	0.000220613
		26	8.246	2.72	133	0.007382065
	Overall (b)	Overall	6.999	3.44	133	0.000781995
Placebo (N = 44)	Mean Baseline Value		16.172			
	Least Square Means	1	5.275	0.77	44	0.448005308
		2	-0.160	-0.02	44	0.980942735
		4	9.719	1.41	44	0.165084720
		14	4.415	0.62	44	0.539239191
		26	17.220	2.58	44	0.013226012
	Overall (b)	Overall	6.919	1.52	44	0.135363716

NOTE: (a) P value was based on T test.
(b) Overall was based on the average of least square means from Week 1 to Week 26.

Samsung Bioepis Exhibit No. 1002
Page 1782

Attorney Docket : ALXN-PW2-114

TABLE 13E: C04-002; C04-001 and C04-002 Compared and Combined: Mixed model analysis of change in Platelet Activation Markers; Change from Baseline
P-SELECTIN EXPRESSION

Study		Study Week		Statistic	DF	P Value (a)
C04-001 (N = 43)	Mean Baseline Value		7.941			
	Least Square Means	1	2.157	0.50	34	0.621911155
		2	5.479	1.26	34	0.214825093
		4	7.478	1.73	34	0.093582884
		14	6.591	1.45	34	0.157269235
		26	6.011	1.32	34	0.195994953
	Overall (b)	Overall	5.149	2.09	34	0.044482961
C-04-002 (N = 97)	Mean Baseline Value		7.517			
	Least Square Means	1	4.462	2.12	95	0.036933290
		2	4.009	2.02	95	0.046211034
		4	3.817	1.92	95	0.057451135
		14	2.535	1.25	95	0.213238625
		26	3.035	1.50	95	0.137140051
	Overall (b)	Overall	3.671	3.45	95	0.000837199

NOTE: (a) P value was based on T test.
(b) Overall was based on the average of least square means from Week 1 to Week 26.

Samsung Bioepis Exhibit No. 1002
Page 1783

Attorney Docket : ALXN-PW2-114

TABLE 13F: C04-002; C04-001 and C04-002 Compared and Combined: Mixed model analysis of change in Platelet Activation Markers; Change from Baseline
P-SELECTIN EXPRESSION

Study		Study Week		Statistic	DF	P Value (a)
Combined (N = 140)	Mean Baseline Value		7.635			
	Least Square Means	1	3.763	1.92	133	0.056451906
		2	4.333	2.31	133	0.022390009
		4	4.750	2.53	133	0.012475659
		14	3.665	1.90	133	0.059358752
		26	3.788	1.96	133	0.051519764
	Overall (b)	Overall	4.106	3.86	133	0.000178203
Placebo (N = 44)	Mean Baseline Value		6.585			
	Least Square Means	1	8.053	2.14	43	0.038297488
		2	5.063	1.40	43	0.168857949
		4	6.484	1.72	43	0.092313078
		14	6.383	1.62	43	0.112358382
		26	3.403	0.94	43	0.352152288
	Overall (b)	Overall	5.851	3.49	43	0.001115025

NOTE: (a) P value was based on T test.
(b) Overall was based on the average of least square means from Week 1 to Week 26.

Samsung Bioepis Exhibit No. 1002
Page 1784

Attorney Docket : ALXN-PW2-114

TABLE 14A: C04-001: Mixed Model Analyses of Platelet Activation Markers; Change from Baseline
MONOCYTE-PLATELET AGGREGATION
Population: ITT

Effect	Degree of Freedom		F Statistic	P Value
	Numerator	Denominator		
Baseline Platelet Assay	1	104	11.26	0.001105679
Week	5	104	1.33	0.255322788
Treatment	1	104	3.01	0.085849037

NOTE: Analysis based on North American ITT patients only as described in the protocol.

TABLE 14B: C04-001: Mixed Model Analyses of Platelet Activation Markers; Change from Baseline
NEUTROPHIL-PLATELET AGGREGATION
Population: ITT

Effect	Degree of Freedom		F Statistic	P Value
	Numerator	Denominator		
Baseline Platelet Assay	1	104	4.28	0.040938932
Week	5	104	2.20	0.060319683
Treatment	1	104	1.21	0.274747130

NOTE: Analysis based on North American ITT patients only as described in the protocol.

Samsung Bioepis Exhibit No. 1002
Page 1785

TABLE 14C: C04-001: Mixed Model Analyses of Platelet Activation Markers; Change from Baseline
P-SELECTIN EXPRESSION
Population: ITT

Effect	Degree of Freedom		F Statistic	P Value
	Numerator	Denominator		
Baseline Platelet Assay	1	103	9.01	0.003374798
Week	5	103	0.91	0.480577712
Treatment	1	103	0.26	0.613587524

NOTE: Analysis based on North American ITT patients only as described in the protocol

INCORPORATION BY REFERENCE

All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

EQUIVALENTS

While specific embodiments of the subject inventions are explicitly disclosed herein, the above specification is illustrative and not restrictive. Many variations of the inventions will become apparent to those skilled in the art upon review of this specification and the claims below. The full scope of the inventions should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

Samsung Bioepis Exhibit No. 1002
Page 1786

Attorney Docket : ALXN-PW2-114

What is claimed is:

1. A method of reducing the occurrence of thrombosis in a subject, said method comprising inhibiting complement in said subject.

2. The method of claim 1, wherein said method comprises administering a compound to said subject, wherein the compound is selected from the group consisting of: a) compounds which bind to one or more complement components, b) compounds which block the generation of one or more complement components, and c) compounds which block the activity of one or more complement components.

3. The method of claim 1, wherein said subject has a paroxysmal nocturnal hemoglobinuria (PNH) granulocyte clone greater than 0.1% of the total granulocyte count.

4. The method of claim 1, wherein said subject has a PNH granulocyte clone greater than 1% of the total granulocyte count.

5. The method of claim 1, wherein said subject has a PNH granulocyte clone greater than 10% of the total granulocyte count.

6. The method of claim 1, wherein said subject has a PNH granulocyte clone greater than 50% of the total granulocyte count.

7. The method of claim 2, wherein the compound is selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic

Samsung Bioepis Exhibit No. 1002
Page 1787

acid inhibitors.

8. The method of claim 2, wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

9. The method of claim 2, wherein said compound inhibits C5b activity.

10. The method of claim 2, wherein said compound inhibits cleavage of C5.

11. The method of claim 2, wherein said compound inhibits terminal complement.

12. The method of claim 2, wherein said compound inhibits C5a activity or inhibits binding of C5a to its receptor.

13. The method of claim 1, wherein said subject is a human.

14. The method of claim 1, wherein said subject has a history of one or more thrombotic events.

15. The method of claim 7, wherein said compound is an antibody or antibody fragment.

16. The method of claim 15, wherein said antibody or antibody fragment is selected from the group consisting of a polyclonal antibody, a monoclonal antibody or antibody fragment, a diabody, a chimerized or chimeric antibody or antibody fragment, a humanized antibody or antibody fragment, a deimmunized human antibody or antibody fragment, a fully human antibody or antibody fragment, a single chain antibody, an Fv, an Fab, an Fab', an Fd, and an $F(ab')_2$.

17. The method of claim 15, wherein said antibody is pexelizumab.

Samsung Bioepis Exhibit No. 1002
Page 1788

18. The method of claim 15, wherein said antibody is eculizumab.

19. The method of claim 2, wherein said compound is administered chronically to said subject.

20. The method of claim 2, wherein said compound is administered systemically to said subject.

21. The method of claim 2, wherein said compound is administered locally to said subject.

22. The method of claim 1, wherein said method reduces rates of thromboembolism by greater than 25%.

23. The method of claim 1, wherein said method reduces rates of thromboembolism by greater than 50%.

24. The method of claim 1, wherein said method reduces rates of thromboembolism by greater than 75%.

25. The method of claim 1, wherein said method reduces rates of thromboembolism by greater than 90%.

26. The method of claim 1, wherein said method results in at least a 25% reduction in LDH levels.

27. The method of claim 1, wherein said method results in at least a 50% reduction in LDH levels.

Samsung Bioepis Exhibit No. 1002
Page 1789

28. The method of claim 1, wherein said method results in at least a 75% reduction in LDH levels.

29. The method of claim 1, wherein said method in a subject results in at least a 90% reduction in LDH levels.

30. The method of claim 2, further comprising administering a second compound, wherein said second compound increases hematopoiesis.

31. The method of claim 30, wherein the second compound is selected from the group consisting of steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin (EPO), pegylated EPO, EPO mimetics, Aranesp®, erythropoiesis stimulating agents, antithymocyte globulin (ATG) and antilymphocyte globulin (ALG).

32. The method of claim 31, wherein EPO is administered with an anti-C5 antibody.

33. The method of claim 32, wherein said antibody is pexelizumab.

34. The method of claim 32, wherein said antibody is eculizumab.

35. The method of claim 2, further comprising administering an antithrombotic compound.

36. The method of claim 35, wherein said antithrombotic compound is an anticoagulant.

37. The method of claim 36, wherein said anticoagulant is administered with an anti-C5 antibody.

38. The method of claim 36, wherein said anticoagulant is an antiplatelet agent.

Samsung Bioepis Exhibit No. 1002
Page 1790

39. The method of claim 37, wherein said antibody is pexelizumab.

40. The method of claim 37, wherein said antibody is eculizumab.

41. A method of reducing the occurrence of thrombosis in a subject who has a higher than normal lactate dehydrogenase (LDH) level, said method comprising inhibiting complement in said subject.

42. The method of claim 41 comprising administering a compound to said subject, wherein the compound is selected from the group consisting of: a) compounds which bind to one or more complement components, b) compounds which block the generation of one or more complement components, and c) compounds which block the activity of one or more complement components.

43. The method of claim 41, wherein said subject has an LDH level greater than the upper limit of normal.

44. The method of claim 41, wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of normal.

45. The method of claim 41, wherein said subject has an LDH level greater than or equal to 2.5 times the upper limit of normal.

46. The method of claim 41, wherein said subject has an LDH level greater than or equal to 5 times the upper limit of normal.

47. The method of claim 41, wherein said subject has an LDH level greater than or equal to 10 times the upper limit of normal.

Samsung Bioepis Exhibit No. 1002
Page 1791

48. The method of claim 42, wherein the compound is selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

49. The method of claim 42, wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

50. The method of claim 42, wherein said compound inhibits C5b activity.

51. The method of claim 42, wherein said compound inhibits cleavage of C5.

52. The method of claim 42, wherein said compound inhibits terminal complement.

53. The method of claim 42, wherein said compound inhibits C5a activity or inhibits binding of C5a to its receptor.

54. The method of claim 41, wherein said subject is a human.

55. The method of claim 41, wherein said subject has a history of one or more thrombotic events.

56. The method of claim 42, wherein said compound is an antibody or antibody fragment.

57. The method of claim 56, wherein said antibody or antibody fragment is selected from the group consisting of a polyclonal antibody, a monoclonal antibody or antibody fragment, a diabody, a chimerized or chimeric antibody or antibody

Samsung Bioepis Exhibit No. 1002
Page 1792

fragment, a humanized antibody or antibody fragment, a deimmunized human antibody or antibody fragment, a fully human antibody or antibody fragment, a single chain antibody, an Fv, an Fab, an Fab', an Fd, and an $F(ab')_2$.

58. The method of claim 56, wherein said antibody is pexelizumab.

59. The method of claim 56, wherein said antibody is eculizumab.

60. The method of claim 42, wherein said compound is administered chronically to said subject.

61. The method of claim 42, wherein said compound is administered systemically to said subject.

62. The method of claim 42, wherein said compound is administered locally to said subject.

63. The method of claim 41, wherein said method reduces rates of thromboembolism by greater than 25%.

64. The method of claim 41, wherein said method reduces rates of thromboembolism by greater than 50%.

65. The method of claim 41, wherein said method reduces rates of thromboembolism by greater than 75%.

66. The method of claim 41, wherein said method reduces rates of thromboembolism by greater than 90%.

67. The method of claim 41, wherein said method results in at least a 25% reduction in LDH levels.

Samsung Bioepis Exhibit No. 1002
Page 1793

68. The method of claim 41, wherein said method results in at least a 50% reduction in LDH levels.

69. The method of claim 41, wherein said method results in at least a 75% reduction in LDH levels.

70. The method of claim 41, wherein said method results in at least a 90% reduction in LDH levels.

71. The method of claim 42, further comprising administering a second compound, wherein said second compound increases hematopoiesis.

72. The method of claim 71, wherein the second compound is selected from the group consisting of steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin (EPO), pegylated EPO, EPO mimetics, Aranesp®, erythropoiesis stimulating agents, antithymocyte globulin (ATG) and antilymphocyte globulin (ALG).

73. The method of claim 72, wherein EPO is administered with an anti-C5 antibody.

74. The method of claim 73, wherein said antibody is pexelizumab.

75. The method of claim 73, wherein said antibody is eculizumab.

76. The method of claim 42, further comprising administering an antithrombotic compound.

77. The method of claim 76, wherein said antithrombotic compound is an anticoagulant.

Samsung Bioepis Exhibit No. 1002
Page 1794

78. The method of claim 77, wherein said anticoagulant is administered with an anti-C5 antibody.

79. The method of claim 77, wherein said anticoagulant is an antiplatelet agent.

80. The method of claim 78, wherein said antibody is pexelizumab.

81. The method of claim 78, wherein said antibody is eculizumab.

82. A method of reducing the occurrence of thrombosis in a subject who has a PNH granulocyte clone and an LDH level greater than the upper limit of normal, said method comprising inhibiting complement in said subject.

83. The method of claim 82 comprising administering a compound to said subject, wherein the compound is selected from the group consisting of: a) compounds which bind to one or more complement components, b) compounds which block the generation of one or more complement components, and c) compounds which block the activity of one or more complement components.

84. The method of claim 82, wherein said subject has a PNH granulocyte clone greater than 0.1% of the total granulocyte count.

85. The method of claim 82, wherein said subject has a PNH granulocyte clone greater than 0.1% of the total granulocyte count.

86. The method of claim 82, wherein said subject has a PNH granulocyte clone greater than 1% of the total granulocyte count.

87. The method of claim 82, wherein said subject has a PNH granulocyte clone greater than 10% of the total granulocyte count.

Samsung Bioepis Exhibit No. 1002
Page 1795

88. The method of claim 82, wherein said subject has a PNH granulocyte clone greater than 50% of the total granulocyte count.

89. The method of claim 83, wherein the compound is selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

90. The method of claim 83, wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

91. The method of claim 83, wherein said compound inhibits C5b activity.

92. The method of claim 83, wherein said compound inhibits cleavage of C5.

93. The method of claim 83, wherein said compound inhibits terminal complement.

94. The method of claim 83, wherein said compound inhibits C5a activity or inhibits binding of C5a to its receptor.

95. The method of claim 82, wherein said subject is a human.

96. The method of claim 82, wherein said subject has a history of one or more thrombotic events.

97. The method of claim 89, wherein said compound is an antibody or antibody fragment.

Samsung Bioepis Exhibit No. 1002
Page 1796

98. The method of claim 97, wherein said antibody or antibody fragment is selected from the group consisting of a polyclonal antibody, a monoclonal antibody or antibody fragment, a diabody, a chimerized or chimeric antibody or antibody fragment, a humanized antibody or antibody fragment, a deimmunized human antibody or antibody fragment, a fully human antibody or antibody fragment, a single chain antibody, an Fv, an Fab, an Fab', an Fd, and an $F(ab')_2$.

99. The method of claim 97, wherein said antibody is pexelizumab.

100. The method of claim 97, wherein said antibody is eculizumab.

101. The method of claim 83, wherein said compound is administered chronically to said subject.

102. The method of claim 83, wherein said compound is administered systemically to said subject.

103. The method of claim 83, wherein said compound is administered locally to said subject.

104. The method of claim 82, wherein said method reduces rates of thromboembolism by greater than 25%.

105. The method of claim 82, wherein said method reduces rates of thromboembolism by greater than 50%.

106. The method of claim 82, wherein said method reduces rates of thromboembolism by greater than 75%.

107. The method of claim 82, wherein said method reduces rates of thromboembolism by greater than 90%.

Samsung Bioepis Exhibit No. 1002
Page 1797

108. The method of claim 82, wherein said method results in at least a 25% reduction in LDH levels.

109. The method of claim 82, wherein said method results in at least a 50% reduction in LDH levels.

110. The method of claim 82, wherein said method results in at least a 75% reduction in LDH levels.

111. The method of claim 82, wherein said method results in at least a 90% reduction in LDH levels.

112. The method of claim 83, further comprising administering a second compound, wherein said second compound increases hematopoiesis.

113. The method of claim 112, wherein the second compound is selected from the group consisting of steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin (EPO), pegylated EPO, EPO mimetics, Aranesp®, erythropoiesis stimulating agents, antithymocyte globulin (ATG) and antilymphocyte globulin (ALG).

114. The method of claim 113, wherein EPO is administered with an anti-C5 antibody.

115. The method of claim 114, wherein said antibody is pexelizumab.

116. The method of claim 114, wherein said antibody is eculizumab.

117. The method of claim 83, further comprising administering an antithrombotic compound.

Samsung Bioepis Exhibit No. 1002
Page 1798

118. The method of claim 117, wherein said antithrombotic compound is an anticoagulant.

119. The method of claim 118, wherein said anticoagulant is administered with an anti-C5 antibody.

120. The method of claim 118, wherein said anticoagulant is an antiplatelet agent.

121. The method of claim 119, wherein said antibody is pexelizumab.

122. The method of claim 119, wherein said antibody is eculizumab.

123. A method of reducing the occurrence of thrombosis in a subject suffering from a lower than normal nitric oxide (NO) level, said method comprising inhibiting complement in said subject.

124. The method of claim 123 comprising administering a compound to said subject, wherein the compound is selected from the group consisting of: i) compounds which bind to one or more complement components, ii) compounds which block the generation of one or more complement components, and iii) compounds which block the activity of one or more complement components, wherein said method increases serum nitric oxide (NO) levels.

125. The method of claim 123, wherein said method increases NO levels by greater than 25%.

126. The method of claim 123, wherein said method increases NO levels by greater than 50%.

127. The method of claim 123, wherein said method increases NO levels by greater than 100%.

Samsung Bioepis Exhibit No. 1002
Page 1799

128. The method of claim 123, wherein said method increases NO levels by greater than 3 fold.

129. The method of claim 123, wherein the subject has PNH.

130. The method of claim 124, wherein the compound is selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

131. The method of claim 124, wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

132. The method of claim 124, wherein said compound inhibits C5b activity.

133. The method of claim 124, wherein said compound inhibits cleavage of C5.

134. The method of claim 124, wherein said compound inhibits terminal complement.

135. The method of claim 124, wherein said compound inhibits C5a activity or inhibits binding of C5a to its receptor.

136. The method of claim 123, wherein said subject is a human.

137. The method of claim 123, wherein said subject has a history of one or more thrombotic events.

Samsung Bioepis Exhibit No. 1002
Page 1800

138. The method of claim 130, wherein said compound is an antibody or antibody fragment.

139. The method of claim 138, wherein said antibody or antibody fragment is selected from the group consisting of a polyclonal antibody, a monoclonal antibody or antibody fragment, a diabody, a chimerized or chimeric antibody or antibody fragment, a humanized antibody or antibody fragment, a deimmunized human antibody or antibody fragment, a fully human antibody or antibody fragment, a single chain antibody, an Fv, an Fab, an Fab', an Fd, and an $F(ab')_2$.

140. The method of claim 138, wherein said antibody is pexelizumab.

141. The method of claim 138, wherein said antibody is eculizumab.

142. The method of claim 124, wherein said compound is administered chronically to said subject.

143. The method of claim 124, wherein said compound is administered systemically to said subject.

144. The method of claim 124, wherein said compound is administered locally to said subject.

145. The method of claim 123, wherein said method reduces rates of thromboembolism by greater than 25%.

146. The method of claim 123, wherein said method reduces rates of thromboembolism by greater than 50%.

147. The method of claim 123, wherein said method reduces rates of thromboembolism by greater than 75%.

Samsung Bioepis Exhibit No. 1002
Page 1801

148. The method of claim 123, wherein said method reduces rates of thromboembolism by greater than 90%.

149. The method of claim 123, wherein said method results in at least a 25% reduction in LDH levels.

150. The method of claim 123, wherein said method results in at least a 50% reduction in LDH levels.

151. The method of claim 123, wherein said method results in at least a 75% reduction in LDH levels.

152. The method of claim 123, wherein said method results in at least a 90% reduction in LDH levels.

153. The method of claim 124, further comprising administering a second compound, wherein said second compound increases hematopoiesis.

154. The method of claim 153, wherein the second compound is selected from the group consisting of steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin (EPO), pegylated EPO, EPO mimetics, Aranesp®, erythropoiesis stimulating agents, antithymocyte globulin (ATG) and antilymphocyte globulin (ALG).

155. The method of claim 154, wherein EPO is administered with an anti-C5 antibody.

156. The method of claim 155, wherein said antibody is pexelizumab.

157. The method of claim 155, wherein said antibody is eculizumab.

Samsung Bioepis Exhibit No. 1002
Page 1802

158. The method of claim 124, further comprising administering an antithrombotic compound.

159. The method of claim 158, wherein said antithrombotic compound is an anticoagulant.

160. The method of claim 159, wherein said anticoagulant is administered with an anti-C5 antibody.

161. The method of claim 159, wherein said anticoagulant is an antiplatelet agent.

162. The method of claim 160, wherein said antibody is pexelizumab.

163. The method of claim 160, wherein said antibody is eculizumab.

164. A method of determining whether a subject having a hemolytic disorder is susceptible to thrombosis comprising measuring the PNH granulocyte clone size of said subject, wherein if the clone size is greater than 0.1% then said subject is susceptible to thrombosis.

165. The method of claim 164, wherein said clone size is greater than 1%.

166. The method of claim 164, wherein said clone size is greater than 10%.

167. The method of claim 164, wherein said clone size is greater than 50%.

168. A method of increasing PNH red blood cell mass of a subject, said method comprising inhibiting complement in said subject.

169. The method of claim 168 comprising administering a compound to the subject, the compound being selected from the group consisting of: i) compounds which

Samsung Bioepis Exhibit No. 1002
Page 1803

bind to one or more complement components, ii) compounds which block the generation of one or more complement components, and iii) compounds which block the activity of one or more complement components.

170. The method of claim 168, wherein said subject has a PNH granulocyte clone.

171. The method of claim 170, wherein said PNH granulocyte clone is greater than 0.1% of the total granulocyte count.

172. The method of claim 170, wherein said PNH granulocyte clone is greater than 1% of the total granulocyte count.

173. The method of claim 170, wherein said PNH granulocyte clone is greater than 10% of the total granulocyte count.

174. The method of claim 170, wherein said PNH granulocyte clone is greater than 50% of the total granulocyte count.

175. The method of claim 168, wherein said subject has an LDH level greater than the upper limit of normal.

176. The method of claim 175, wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of normal.

177. The method of claim 175, wherein said subject has an LDH level greater than or equal to 2.5 times the upper limit of normal.

178. The method of claim 175, wherein said subject has an LDH level greater than or equal to 5 times the upper limit of normal.

Samsung Bioepis Exhibit No. 1002
Page 1804

179. The method of claim 175, wherein said subject has an LDH level greater than or equal to 10 times the upper limit of normal.

180. A method of treating hemolytic anemia in a subject, said method comprising inhibiting complement in said subject.

181. The method of claim 180, wherein said method comprises administering a compound to the subject, wherein the compound is selected from the group consisting of: i) compounds which bind to one or more complement components, ii) compounds which block the generation of one or more complement components, and iii) compounds which block the activity of one or more complement components, wherein said method increases red blood cell (RBC) mass.

182. The method of claim 181, wherein RBC mass is measured as the absolute number of RBCs.

183. The method of claim 181, wherein RBC mass is PNH RBC mass.

184. The method of claim 183, wherein said method increases RBC mass by greater than 10%.

185. The method of claim 183, wherein said method increases RBC mass by greater than 25%.

186. The method of claim 183, wherein said method increases RBC mass by greater than 50%.

187. The method of claim 183, wherein said method increases RBC mass by greater than 100%.

Samsung Bioepis Exhibit No. 1002
Page 1805

188. The method of claim 183, wherein said method increases RBC mass by greater than 2 fold.

189. The method of claim 180, wherein said method decreases transfusion requirements.

190. The method of claim 180, wherein said method stabilizes hemoglobin levels.

191. The method of claim 180, wherein said method causes an increase in hemoglobin levels.

Samsung Bioepis Exhibit No. 1002
Page 1806

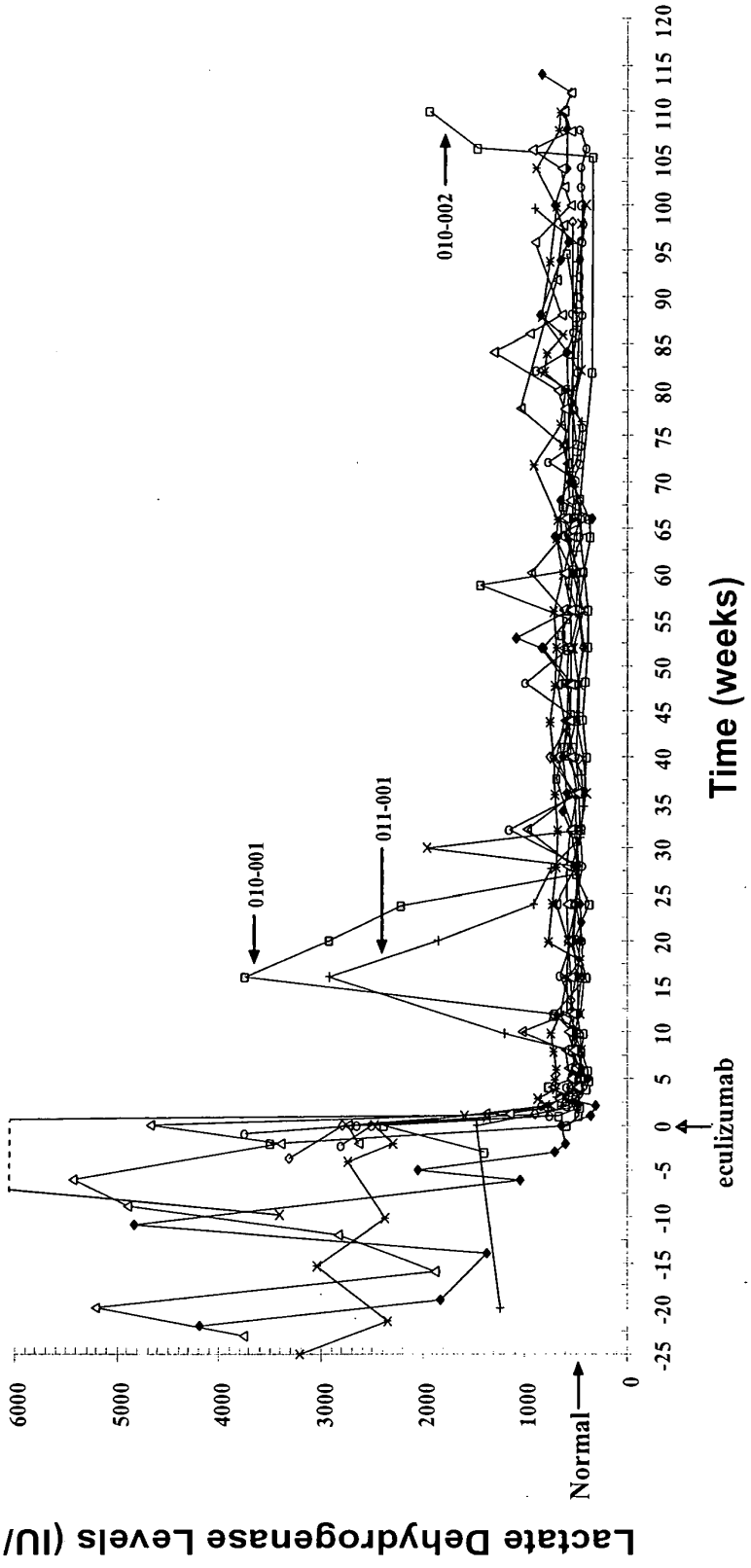
Figure 1A.
Biochemical Parameters of Hemolysis During Eculizumab Treatment

Biochemical Marker	Normal range	Time of Analysis			p-value[a]
		Pre-study[b]	12 weeks	64 weeks	
LDH (IU/L)	150 - 480	3110.7 +/- 598.4	594.0 +/- 31.7	622.4 +/-41.1	0.002
AST (IU/L)	10 - 40	76.2 +/- 16.0	26.2 +/- 2.3	30.1 +/- 3.2	0.02
Haptoglobin (g/L)	0.5 - 2	<0.06	<0.06	0.14 +/- 0.07[c]	N.S.[d]
Hemoglobin (g/dL)	11.5 - 18	10.0 +/- 0.4	10.3 +/- 0.4	10.4 +/- 0.4	N.S.
Bilirubin	3 - 15	25.9 +/- 4.3	28.2 +/- 4.4	28.7 +/- 4.0	N.S.
Reticulocytes (x10^3/mm^3)	20 - 80	161.4 +/- 25.9	191.2 +/- 23.6	189.6 +/- 21.8	N.S.

[a]from comparisons of mean change from pre-study to 64 weeks
[b]values represent means during 52 week period prior to treatment except for AST which represents the baseline mean
[c]10 of 11 patients were below the detectable limit of haptoglobin (<0.06 g/L); 1 patient had a value of 0.69 g/L.
[d]not significant

Samsung Bioepis Exhibit No. 1002
Page 1807

Figure 1B



Samsung Bioepis Exhibit No. 1002
Page 1808

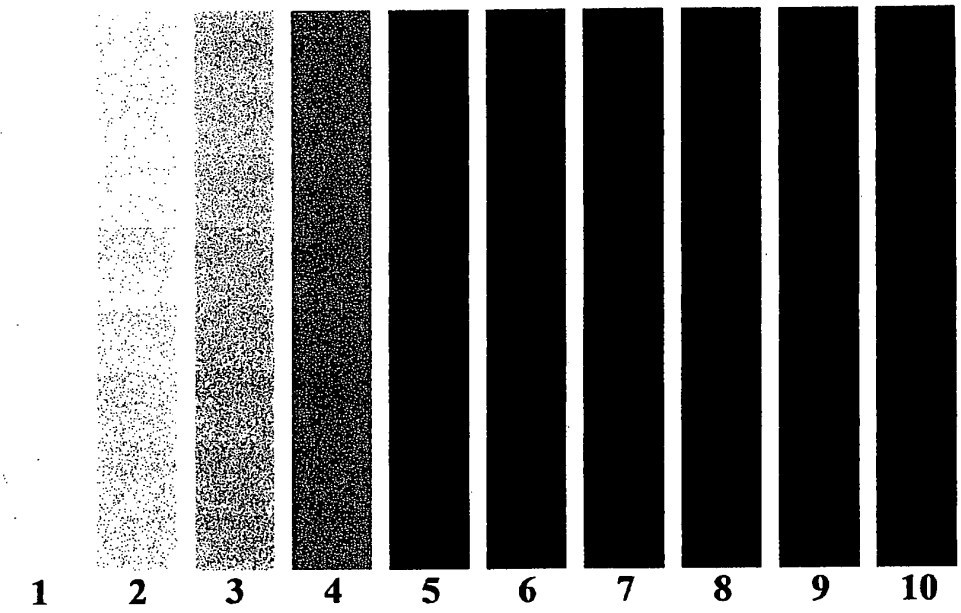
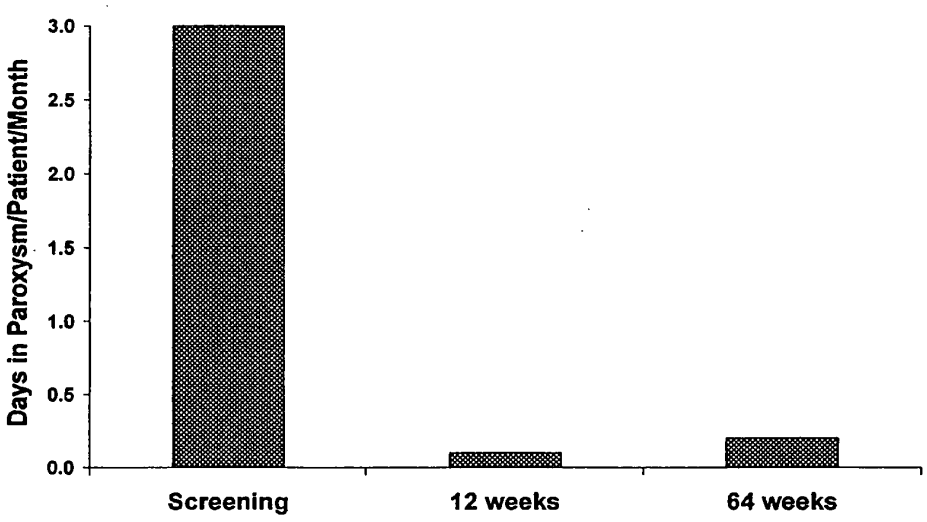


Figure 2

Samsung Bioepis Exhibit No. 1002
Page 1809

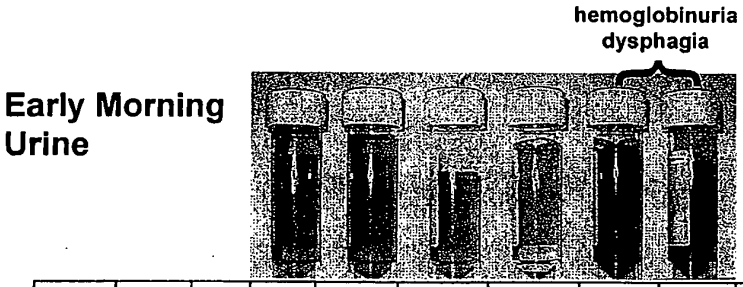
Figure 3.
Effect of Eculizumab on Paroxysm Rate (n=8)



Bars represent the paroxysm rates (number of paroxysms per patient per month) during the screening period (pre-eculizumab treatment), and during the first 12 weeks and the entire 64 weeks of eculizumab treatment. Three patients were not included in the analysis as either pretreatment urine scores were inadvertently not collected (2 patients) or an iron chelating agent that resulted in artificially colored urine was administered during the extension study (1 patient).

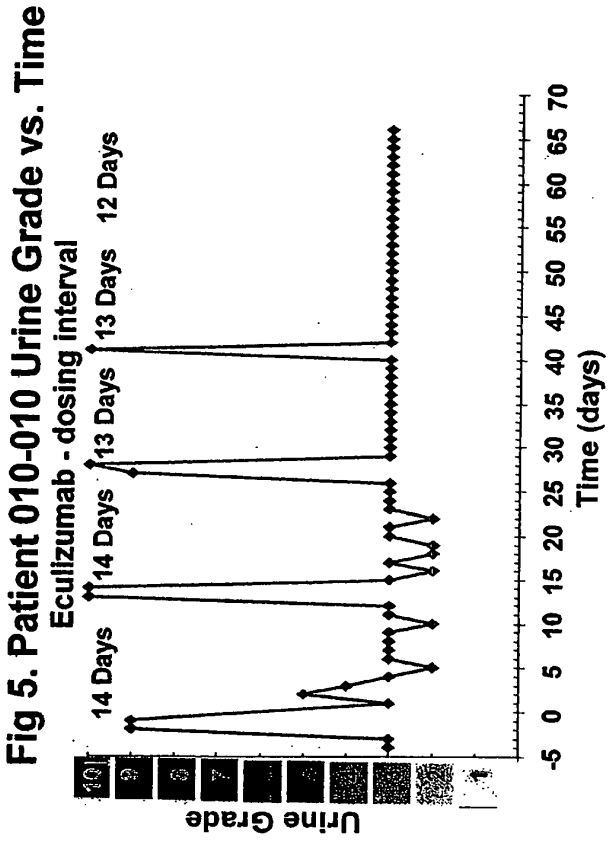
Samsung Bioepis Exhibit No. 1002
Page 1810

Figure 4.
Analysis of Complement Breakthrough



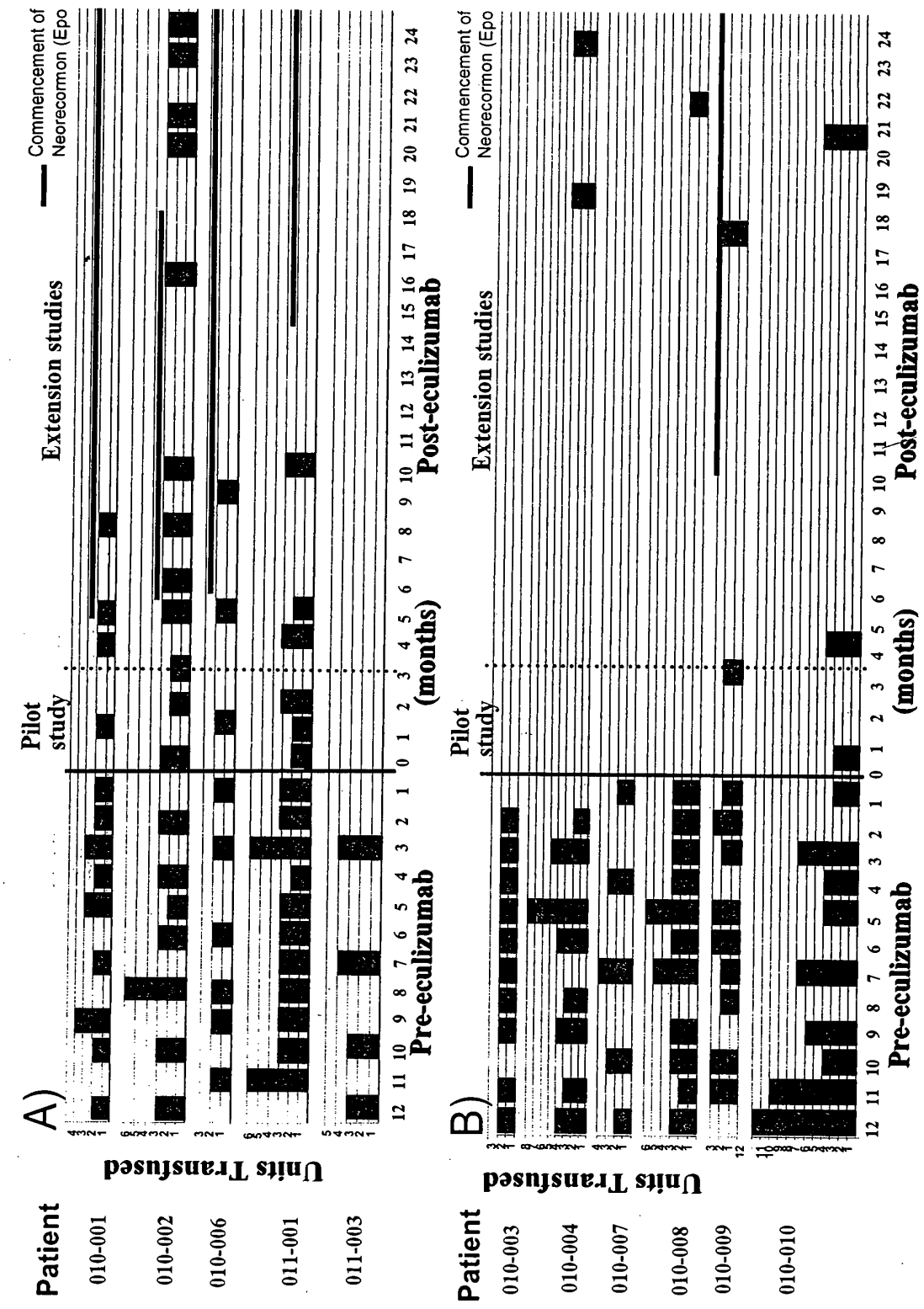
Visit	4							5			9
Day	0*	1-8	9	10	11	12	13	0*	1-2		0*
Urine	10	2-3	3	3	3	3	9	10	3		2
LDH	2624	–	784	–	–	697	1687	2917	–		495
AST	119	–	38	–	–	31	87	–	–		–
PK	28	–	51	–	–	35	31	23	–		51
PD	72	–	1	–	–	2	56	56	–		2

*Dose of eculizumab



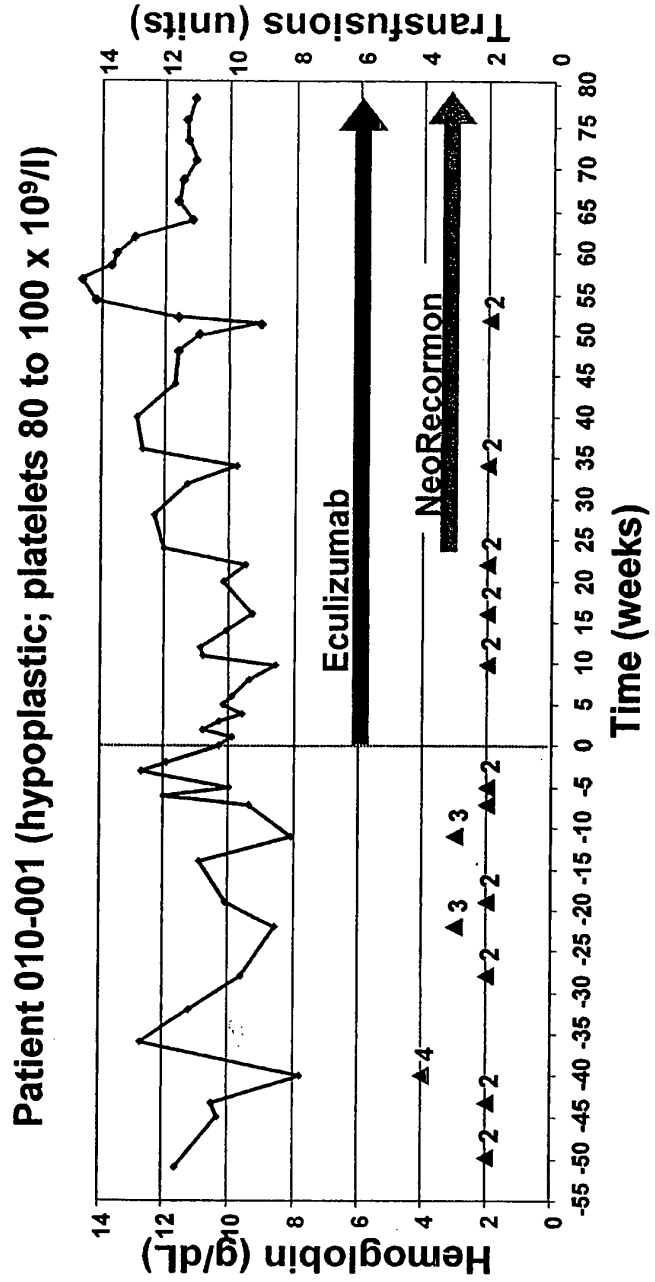
Samsung Bioepis Exhibit No. 1002
Page 1812

Figure 6A-6B



Samsung Bioepis Exhibit No. 1002
Page 1813

Figure 7 Management of thrombocytopenic patient with erythropoietin



Transfusions reduced with eculizumab and transfusion independence with combined eculizumab and erythropoietin (NeoRecormon 18,000U 3x/week)

Samsung Bioepis Exhibit No. 1002
Page 1814

Figure 8.
Eculizumab Pharmacodynamics Pre- and Post-dose

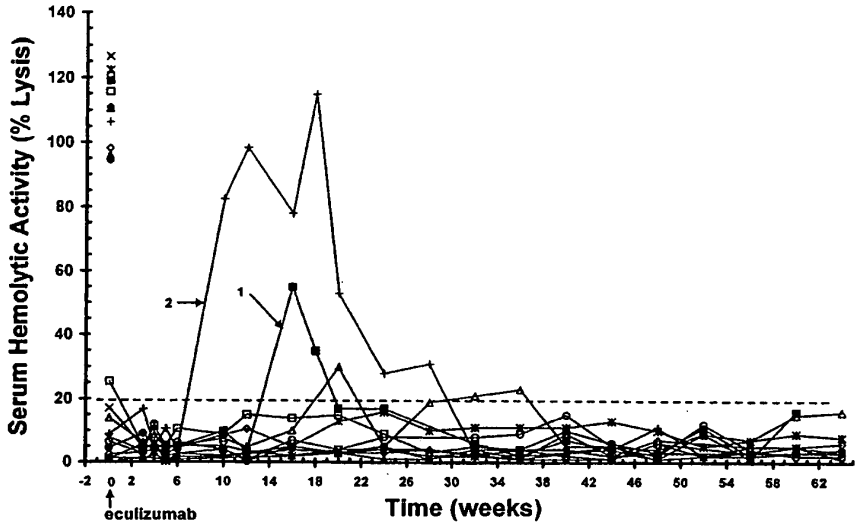


Figure 8 shows serum hemolytic activity (PD) during the 64 week treatment period as determined by the ability of serum to lyse antibody-presensitized chicken erythrocytes. The percentage of hemolytic activity at which complement is considered to be effectively inhibited (≤ 20 %) is indicated by dashed line. Two patients that demonstrated trough serum hemolytic activity values less than 20% are identified (patients 1 and 2).

Samsung Bioepis Exhibit No. 1002
Page 1815

Figure 9.
Eculizumab Pharmacodynamics Pre- and Post-dose

Domain (a)	Mean Base-Line Score (b)	64 Week Change from Base-line (c)	p-value (d)
Global Health Status	56.1	13.8	0.009
Physical Functioning	70.9	14.3	<0.001
Emotional Functioning	70.5	12.5	<0.001
Role Functioning	66.7	14.5	0.003
Cognitive Functioning	77.3	10.3	0.001
Fatigue	47.5	-17.8	<0.001
Dyspnea	39.4	-16.6	0.002
Insomnia	30.3	-8.2	0.031
Pain	21.2	-8.2	0.023
Constipation	3.0	4.1	<0.001

a) the quality of life was assessed using the European Organization for Research and Treatment of Cancer QLQ-C30 instrument
b) numbers represent mean values of linearly transformed scores
c) values represent least-square means; positive change indicates improvement for Global Health Status and Functional Scales while a negative change indicates improvement for Symptom Scales
d) values are from a mixed analysis-of-covariance model with visit as a fixed effect, patient as a random effect, and baseline as a covariate

Samsung Bioepis Exhibit No. 1002
Page 1816

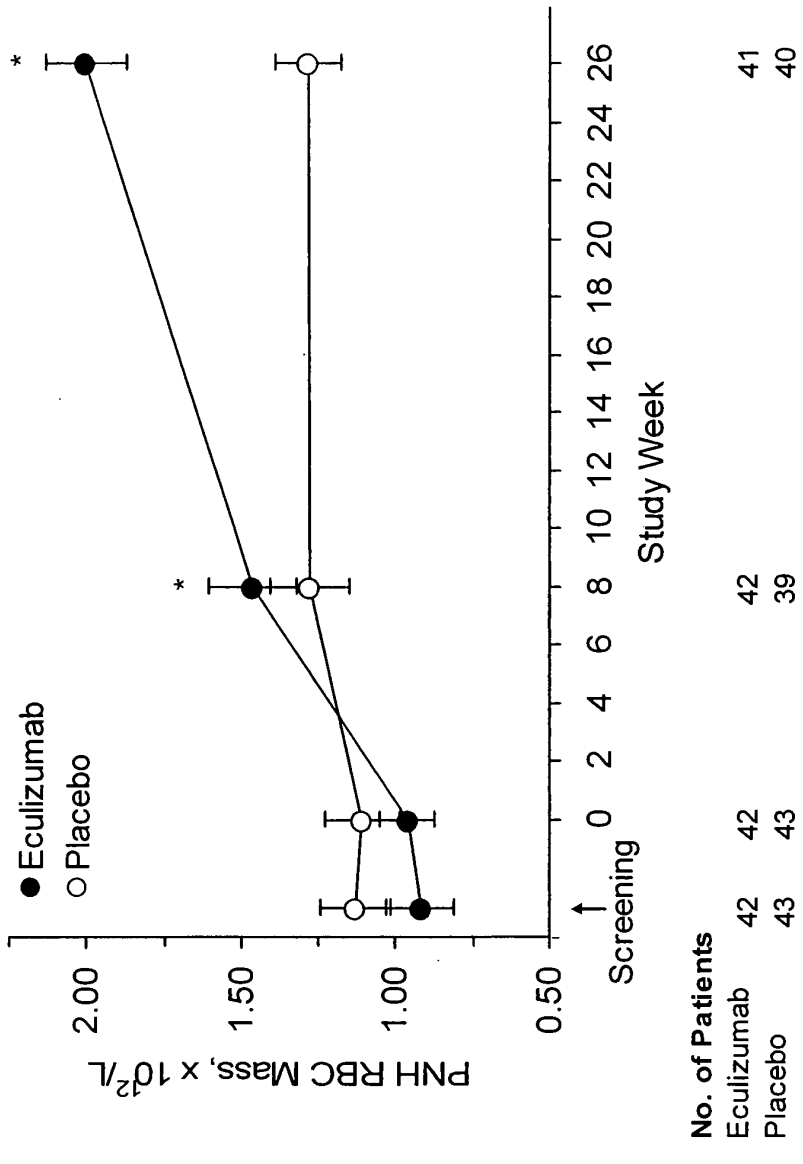
Figure 10.
Symptoms Pre- and 2 Years Post-Eculizumab

Patient	Pre-Eculizumab			Post-Eculizumab		
	Abdominal Pain	Dysphagia	Erectile Dysfunction	Abdominal Pain	Dysphagia	Erectile Dysfunction
010-001	Every 4 – 8 weeks	Every 4 – 8 weeks	Every 4 – 8 weeks	None	None	None
010-002	None	None	--	None	None	--
010-003	Every week	Every week	Every week	None	None	None
010-004	Every 4 – 6 weeks	None	--	None	None	--
010-006	None	None	None	None	None	None
010-007	None	At least every 4 weeks	--	None	None	--
010-008	None	Every 10 weeks	Every 10 weeks	None	None	Intermittent
010-009	None	None	None	None	None	None
010-010	Every 4 weeks	Every 4 weeks	None	None	2 episodes in 2 years*	None
011-001	None	None	--	None	None	--
011-003	None	None	--	None	None	--

*This patient experienced a transient breakthrough in complement blockade with a return of hemolysis and symptoms. Increasing the dosing frequency to 12 days re-established complete complement blockade and prevented further symptoms.

Samsung Bioepis Exhibit No. 1002
Page 1817

Figure 11



PNH RBC mass = (proportion of PNH RBCs) x (total number of RBCs) ÷ 100.
*P < 0.001, based on a Wilcoxon's rank sum test.

Samsung Bioepis Exhibit No. 1002
Page 1818

Figure 12

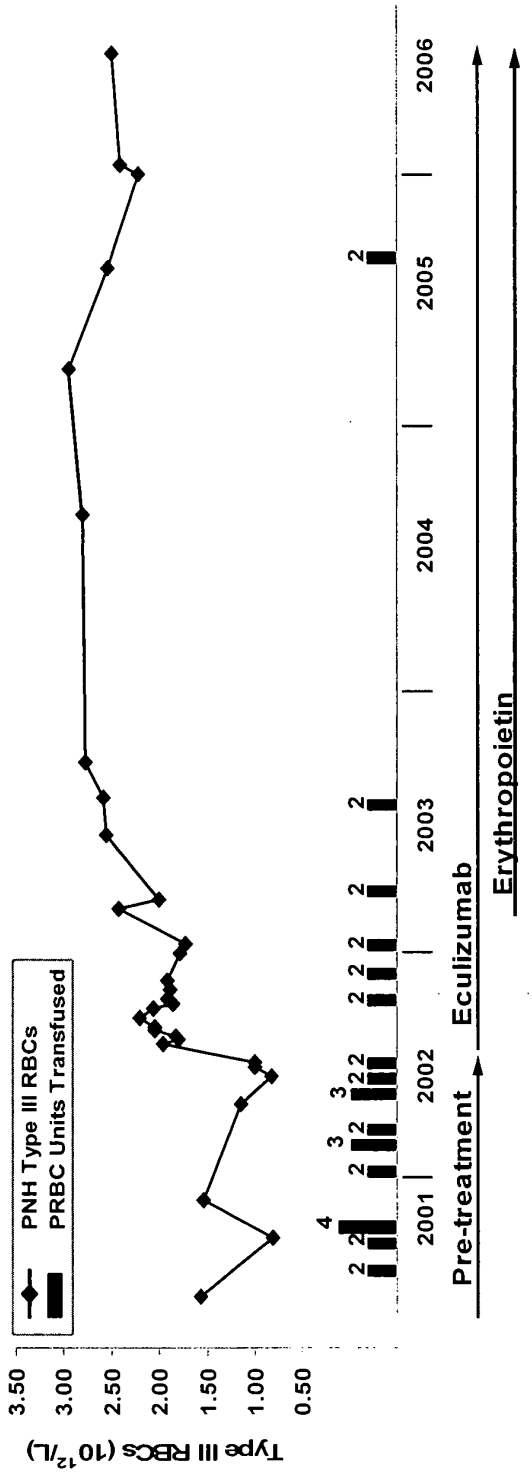
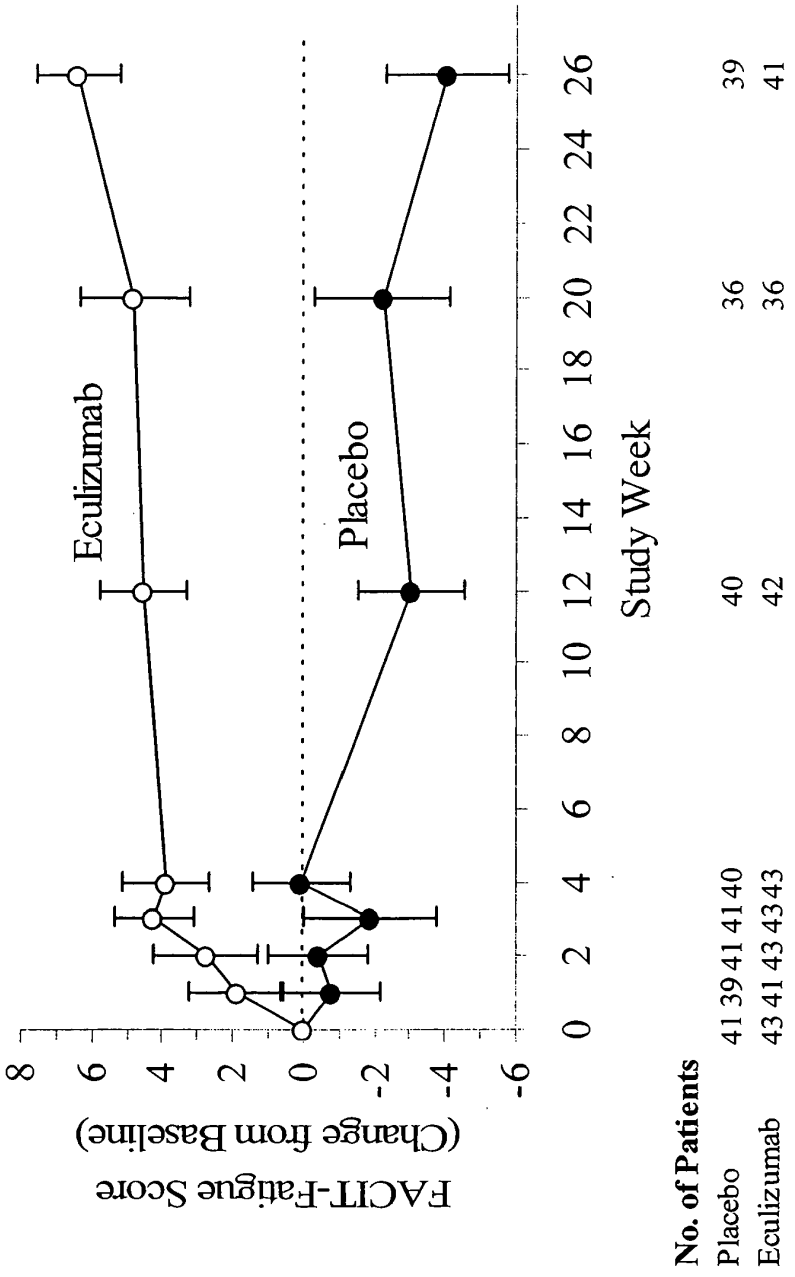

PNH Type III RBCs
PRBC Units Transfused
Type III RBCs (10^{12}/L)
3.50
3.00
2.50
2.00
1.50
1.00
0.50
2001
2002
2003
2004
2005
2006
Pre-treatment
Eculizumab
Erythropoietin

Figure 13



Samsung Bioepis Exhibit No. 1002
Page 1820

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(71) Applicant: **Alexion Pharmaceuticals, Inc.**
Cheshire, CT 06410 (US)

(72) Inventors:
- **Rother, Russell P.**
Oklahoma City, OK 73151 (US)
- **Wang, Hao**
London, Ontario N5X 4H2 (CA)
- **Zhong, Zhen**
deceased (CA)

(74) Representative: **Vossius & Partner**
Siebertstrasse 4
81675 München (DE)

Remarks:
This application was filed on 18-07-2014 as a divisional application to the application mentioned under INID code 62.

(54) Prolongation of survival of an allograft by inhibiting complement activity

(57) Methods of prolonging survival of allotransplanted cells, tissues or organs are presented. These methods are directed to administering to the allotransplanted recipient an inhibitor of complement activity together with one or more immunosuppressants. The inhibitor of complement activity is administered chronically. These methods have been determined to aid in preventing chronic rejection of allografts. These methods can additionally be used in cases in which the recipient has been presensitized to the allograft or in which there is an ABO mismatch between the allograft and the recipient.

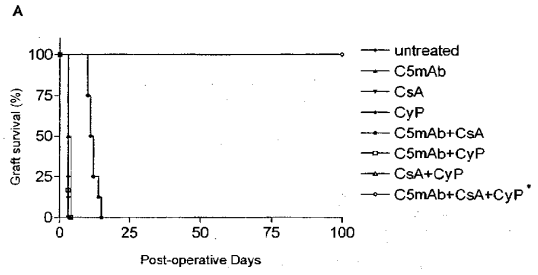


Figure 2A

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EP 2 815 767 A1

Description

RELATED APPLICATIONS

[0001] This application claims the benefit of and priority to U.S. Provisional Patent Application No. 60/571,444, filed May 14, 2004, the disclosure of which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

[0002] The present disclosure relates to methods for prolonging survival of an allograft in a mammal. In particular, the present disclosure relates to prolonging survival of an allograft by administering an inhibitor of complement or terminal complement formation, especially an inhibitor of complement C5 cleavage, in addition to one or more drugs that are immunosuppressant.

BACKGROUND

[0003] Organ transplantation is the preferred treatment for most patients with chronic organ failure. Although transplantation of kidney, liver, lung, and heart offers an excellent opportunity for rehabilitation as recipients return to a more normal lifestyle, it is limited by the medical/surgical suitability of potential recipients, an increasing shortage of donors, and premature failure of transplanted organ function.

[0004] Transplantation of cells, tissues and organs has become very common and is often a life-saving procedure. Organ transplantation is the preferred treatment for most patients with chronic organ failure. Despite great improvement in treatments to inhibit rejection, rejection continues to be the single largest impediment to successful organ transplantation. Rejection includes not only acute rejection but also chronic rejection. One-year survival rates for transplanted kidneys average 88.3% with kidneys from deceased donors and 94.4% with kidneys received from living donors. The corresponding five year survival rates for the transplanted kidneys are 63.3% and 76.5% (OPTN/SRTR Annual Report, 2002). For livers the one year survival rates are 80.2% and 76.5% for livers from deceased and living donors, respectively. The corresponding five year liver graft survival rates are 63.5% and 73.0% (OPTN/SRTR Annual Report, 2002). The use of immunosuppressant drugs, especially cyclosporin A and more recently tacrolimus, has dramatically improved the success rate of organ transplantation especially by preventing acute rejection. But as the numbers above show, there is still a need to improve the success rates, both short-term and especially long-term. As seen from the above numbers for kidney and liver transplants, the five year failure rates for these transplanted organs are on the order of 25-35%. In the year 2001 alone there were more than 23,000 patients who received an organ transplant of which approximately 19,000 were kidney or liver (OPTN/SRTR Annual Report, 2002). For this one year of transplants alone, with present techniques it can be expected that approximately 5,000-6,000 of these transplanted kidneys and livers will fail within 5 years. These numbers do not even include other transplanted organs or transplanted tissues or cells such as bone marrow.

[0005] There are multiple types of transplants. These are described in Abbas et al., 2000. A graft transplanted from one individual to the same individual is called an autologous graft or autograft. A graft transplanted between two genetically identical or syngeneic individual is called a syngeneic graft. A graft transplanted between two genetically different individuals of the same species is called an allogeneic graft or allograft. A graft transplanted between individuals of different species is called a xenogeneic graft or xenograft. The molecules that are recognized as foreign on allografts are called alloantigens and those on xenografts are called xenoantigens. The lymphocytes or antibodies that react with alloantigens or xenoantigens are described as being alloreactive or xenoreactive, respectively.

[0006] Currently more than 40,000 kidney, heart, lung, liver and pancreas transplants are performed in the United States each year (Abbas et al., 2000). Other possible transplants include, but are not limited to, vascular tissue, eye, cornea, lens, skin, bone marrow, muscle, connective tissue, gastrointestinal tissue, nervous tissue, bone, stem cells, islets, cartilage, hepatocytes, and hematopoietic cells. Unfortunately, there are many more candidates for a transplant than there are donors. To overcome this shortage, a major effort is being made to learn how to use xenografts. While progress is being made in this field, the fact is that at present most transplants are allografts. An allogeneic transplant, while presently being more likely to be successful than a xenogeneic transplant, must surmount numerous obstacles to be successful. There are several types of immunological attacks made by the recipient against the donor organ which can lead to rejection of the allograft. These include hyperacute rejection, acute vascular rejection (including accelerated humoral rejection and de novo acute humoral rejection), and chronic rejection. Rejection is normally a result of T-cell mediated or humoral antibody attack, but may include additional secondary factors such as the effects of complement and cytokines.

[0007] An ever growing gap between the number of patients requiring organ transplantation and the number of donor organs available has become a major problem throughout the world. Park et al., 2003. Individuals who have developed

Samsung Bioepis Exhibit No. 1002
Page 1822

anti-HLA antibodies are said to be immunized or sensitized. Gloor, 2005. HLA sensitization is the major barrier to optimal utilization of organs from living donors in clinical transplantation (Warren et al., 2004) due to the development of severe antibody-mediated rejection (ABMR). For example, more than 50% of all individuals awaiting kidney transplantation are presensitized patients (Glotz et al., 2002) who have elevated levels of broadly reactive alloantibodies, resulting from multiple transfusions, prior failed allografts, or pregnancy (Kupiec-Weglinski, 1996). The role of ABMR is currently one of the most dynamic areas of study in transplantation, due to recognition that this type of rejection can lead to either acute or chronic loss of allograft function. Mehra et al., 2003. Numerous cases of ABMR, including hyperacute rejection (HAR) or accelerated humoral rejection (ACHR), have been reported that are characterized by acute allograft injury that is resistant to potent anti-T cell therapy, the detection of circulating donor specific antibodies, and the deposition of complement components in the graft. ABMR with elevated circulating alloantibodies and complement activation that occurs in 20-30% of acute rejection cases has a poorer prognosis than cellular rejection. Mauiyyedi et al., 2002.

[0008] Highly presensitized patients, who exhibit high levels of alloantibodies, usually suffer an immediate and aggressive HAR. In clinical practice, with great efforts and significant advances in technology, HAR is avoided by obtaining a pretransplant lymphocytotoxic crossmatch to identify sensitized patients with antibodies specific for donor HLA antigens. However, circulating antibodies against donor HLA or other non-MHC endothelial antigens may also be responsible for a delayed form of acute humoral rejection, which is associated with an increased incidence of graft loss. Collins et al., 1999. Therefore, development of a novel presensitized animal model to mimic ABMR in clinical settings would be beneficial to studies on the mechanism, and to the much needed progress in the management of allograft rejection in presensitized hosts.

[0009] Some highly presensitized patients can benefit from intervention programs such as immunoadsorption (Palmer et al.,1989; Ross et al., 1993; Kriaa et al., 1995), plasmapheresis and intravenous immunoglobulin (Sonnenday et al., 2002; Rocha et al., 2003), that have been designed and implemented to temporarily eliminate anti-donor antibodies. However, in addition to their benefits, the aforementioned therapies carry with them numerous drawbacks as some individuals are less susceptible to their effects (Kriaa et al., 1995; Hakim et al., 1990; Glotz et al., 1993; Tyan et al., 1994); they are extremely expensive, time-consuming, and risky (Salama et al., 2001). Moreover, the transient and variable effect of these protocols has limited their impact. Glotz et al., 2002; Kupin et al., 1991; Schweitzer et al., 2000. Therefore, developing novel strategies to reduce the risk and cost in prevention of ABMR would be beneficial to presensitized recipients receiving an allograft.

SUMMARY

[0010] Accordingly, methods of prolonging survival of transplanted cells, tissues or organs are provided. In particular, methods of prolonging survival of allotransplanted cells, tissues or organs are provided. These methods are directed to using one or more immunosuppressants in addition to an inhibitor of complement activity. Use of one or more immunosuppressants and an inhibitor of complement activity in the manufacture of one or more medicaments or medicament packages is also provided. Such medicaments or medicament packages are useful in prolonging survival of an allograft in a subject mammal.

[0011] In certain embodiments, the inhibition of complement activity is effected by chronic administration of a drug directed against complement C5. A preferred drug that inhibits complement activity is an antibody specific to one or more components of complement, for example, C5. In certain preferred embodiments, the antibody inhibits the cleavage of C5 and thereby inhibits the formation of both C5a and C5b-9. The antibody may be, e.g., a monoclonal antibody, a chimeric antibody (e.g., a humanized antibody), an antibody fragment (e.g., Fab), a single chain antibody, an Fv, or a domain antibody. The recipient is also treated with one or more immunosuppressive drugs, for example, cyclosporin A.

[0012] In certain embodiments, either an MHC mismatched recipient (i.e., a mammalian recipient of an MHC mismatched allograft), a presensitized recipient or an ABO mismatched recipient (i.e., a mammalian recipient of an AMB mismatched allograft) is treated. In this model, the recipient is again chronically treated with a complement inhibitor, preferably an anti-C5 monoclonal antibody, together with immunosuppressive drugs, preferably a chronic administration of cyclosporin A and a short-term administration of cyclophosphamide. This triple therapy results in extended graft survival in the presensitized allotransplant recipient.

[0013] The present disclosure also provides methods of prolonging survival of an allograft in a mammalian recipient by administering to the recipient agents that modulate the level and/or ratio of subclasses and/or isotypes of anti-donor immunoglobulins (Ig) in the recipient. In certain embodiments, an agent that reduces the level of anti-donor IgG1 in the recipient is preferred. In certain embodiments, an agent that increases the level of anti-donor IgG2a and/or IgG2b in the recipient is preferred. In certain embodiments, an agent that reduces the ratio of anti-donor IgG1/anti-donor IgG2a or IgG2b in the recipient is preferred.

[0014] The present disclosure also provides a method of prolonging survival of an allograft in a second mammalian recipient using an allograft that has been accommodated in a first mammalian recipient (i.e., the allograft has prolonged survival in the first recipient). The present disclosure further provides an allograft that is resistant to anti-donor antibodies

Samsung Bioepis Exhibit No. 1002
Page 1823

in a mammalian recipient, and the allograft is prepared from a first recipient that has accommodated the allograft. In preferred embodiments, the first recipient has accommodated the allograft by receiving a treatment as described herein, such a triple therapy treatment involving administering to the first recipient a drug that inhibits complement activity and two immunosuppressive agents.

[0015] Further provided are pharmaceutical packages. A pharmaceutical package of the present disclosure may comprise a drug that inhibits complement activity and at least one immunosuppressive agent. The pharmaceutical package may further comprise a label for chronic administration. The pharmaceutical package may also comprise a label for self-administration by a patient, for example, a recipient of a transplant graft, or instructions for a caretaker of a recipient of a transplant graft. In certain embodiments, the drug and the agent in the pharmaceutical package are in a formulation or separate formulations that are suitable for chronic administration and/or self-administration.

[0016] The present disclosure also provides lyophilized formulations and formulations suitable for injection. Certain embodiments provide a lyophilized antibody formulation comprising an antibody that inhibits complement activity and a lyoprotectant. In preferred embodiments, the antibody formulation is suitable for chronic administration, for example, the antibody formulation is stable. Alternative embodiments provide an injection system comprising a syringe; the syringe comprises a cartridge containing an antibody that inhibits complement activity and is in a formulation suitable for injection.

[0017] An antibody employed in various embodiments of the present disclosure preferably inhibits the formation of terminal complement or C5a. In certain embodiments, antibody inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment. The whole antibody or antibody fragment may be a human, humanized, chimerized or deimmunized antibody or antibody fragment. In certain embodiments, the whole antibody or antibody fragment may inhibit cleavage of complement C5. In certain embodiments, the antibody fragment is a Fab, an F(ab')2, an Fv, a domain antibody, or a single-chain antibody. In preferred embodiments, the antibody fragment is pexelizumab. In alternative preferred embodiments, the whole antibody is eculizumab.

[0018] In certain embodiments, a drug, such as an antibody, that inhibits complement activity is present in unit dosage form, which can be particularly suitable for self-administration. Similarly, an immunosuppressive agent of the present disclosure may also be present in unit dosage form.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019]

Figures 1A-1D show anti-donor antibody levels in presensitized versus unsensitized recipients under different treatments.

Figures 2A and 2B show comparison between triple therapy using anti-C5 antibody, CsA and CyP in presensitized allograft recipients and combination therapy using only anti-C5 antibody and CsA in presensitized allograft recipients. Figure 2A compares heart-allograft survival in various recipients under different treatments as indicated. Figure 2B shows histology and immunohistology, for example, for lymphocyte infiltration in heart allografts of recipients in different groups.

Figure 3 shows blocked terminal complement activity by anti-C5 antibody as compared to immunosuppressive agents.

Figures 4A-4D compare levels of anti-donor antibodies in presensitized recipients of allografts under monotherapy with anti-C5 antibody alone, double combination therapy with anti-C5 antibody and CsA, and triple combination therapy with anti-C5 antibody, CsA and CyP.

Figures 5A and 5B show change of ratios of IgG isotypes in allograft recipients that were untreated or under different treatments.

Figure 6 shows high-level expression of Bcl-2 and Bcl-xl proteins in long-term surviving heart grafts as compared to heart grafts of untreated animals.

Figure 7 shows improved second transplantation (re-transplantation) of an accommodated graft from a first transplantation recipient.

Figure 8 shows results from re-transplantation experiments.

Figure 9 shows results from re-transplantation experiments.

Samsung Bioepis Exhibit No. 1002
Page 1824

DETAILED DESCRIPTION

Overview: Rejection of Transplants or Grafts

[0020] Hyperacute rejection occurs within minutes to hours after transplant and is due to preformed antibodies to the transplanted tissue antigens. It is characterized by hemorrhage and thrombotic occlusion of the graft vasculature. The binding of antibody to endothelium activates complement, and antibody and complement induce a number of changes in the graft endothelium that promote intravascular thrombosis and lead to vascular occlusion, the result being that the grafted organ suffers irreversible ischemic damage (Abbas et al., 2000). Hyperacute rejection is often mediated by preexisting IgM alloantibodies, e.g., those directed against the ABO blood group antigens expressed on red blood cells. This type of rejection, mediated by natural antibodies, is the main reason for rejection of xenotransplants. Hyperacute rejection due to natural IgM antibodies is no longer a major problem with allografts because allografts are usually selected to match the donor and recipient ABO type. Hyperacute rejection of an ABO matched allograft may still occur, usually mediated by IgG antibodies directed against protein alloantigens, such as foreign MHC molecules, or against less well defined alloantigens expressed on vascular endothelial cells. Such antibodies may arise as a result of prior exposure to alloantigens through blood transfusion, prior transplantation, or multiple pregnancies (this prior exposure being referred to as "presensitization"). Abbas et al., 2000.

[0021] Acute rejection is a process of vascular and parenchymal injury mediated by T cells, macrophages, and antibodies that usually begins after the first week of transplantation. Abbas et al., 2001. T lymphocytes play a central role in acute rejection by responding to alloantigens, including MHC molecules, present on vascular endothelial and parenchymal cells. The activated T cells cause direct lysis of graft cells or produce cytokines that recruit and activate inflammatory cells, which cause necrosis. Both $CD4^+$ and $CD8^+$ cells may contribute to acute rejection. The destruction of allogeneic cells in a graft is highly specific and a hallmark of $CD8^+$ cytotoxic T lymphocyte killing. Abbas et al., 2000. $CD4^+$ T cells may be important in mediating acute graft rejection by secreting cytokines and inducing delayed-type hypersensitivity-like reactions in grafts, with some evidence available that indicates that $CD4^+$ T cells are sufficient to mediate acute rejection. Abbas et al., 2000. Antibodies can also mediate acute rejection after a graft recipient mounts a humoral immune response to vessel wall antigens and the antibodies that are produced bind to the vessel wall and activate complement. Abbas et al., 2000.

[0022] Chronic rejection is characterized by fibrosis with loss of normal organ structures occurring over a prolonged period. The pathogenesis of chronic rejection is less well understood than that of acute rejection. Graft arterial occlusion may occur as a result of the proliferation of intimal smooth muscle cells (Abbas et al., 2000). This process is called accelerated or graft arteriosclerosis and can develop in any vascularized organ transplant within 6 months to a year after transplantation.

[0023] For a transplant to be successful, the several modes of rejection must be overcome. Multiple approaches are utilized in preventing rejection. This may require administration of immunosuppressants, often several types to prevent the various modes of attack, e.g., inhibition of T-cell attack, antibodies, and cytokine and complement effects. Prescreening of donors to match them with recipients is also a major factor in preventing rejection, especially in preventing hyperacute rejection. Immunoadsorption of anti-HLA antibodies prior to grafting may reduce hyperacute rejection. Prior to transplantation the recipient or host may be administered anti-T cell reagents, e.g., the monoclonal antibody OKT3, Anti-Thymocyte Globulin (ATG), cyclosporin A, or tacrolimus (FK 506). Additionally, glucocorticoids and/or azathioprine may be administered to the host prior to transplant. Drugs used to aid in preventing transplant rejection include, but are not limited to, ATG or ALG, OKT3, daclizumab, basiliximab, corticosteroids, 15-deoxyspergualin, cyclosporins, tacrolimus, azathioprine, methotrexate, mycophenolate mofetil, 6-mercaptopurine, bredinin, brequinar, leflunamide, cyclophosphamide, sirolimus, anti-CD4 monoclonal antibodies, CTLA4-Ig, anti-CD 154 monoclonal antibodies, anti-LFA1 monoclonal antibodies, anti-LFA-3 monoclonal antibodies, anti-CD2 monoclonal antibodies, and anti-CD45.

[0024] Allografts are rejected in part by the activation of T cells. The transplant recipient mounts a rejection response following $CD4^+$ T cell recognition of foreign antigens in the allograft. These antigens are encoded by the major histocompatibility complex (MHC). There are both Class I and Class II MHC molecules. In humans the class I MHC molecules are HLA-A, HLA-B, and HLA-C. The class II MHC molecules in humans are called HLA-DR, HLA-DQ and HLA-DP. In mice the class I MHC molecules are H-2K, H-2D and H-2L and the class II MHC molecules are I-A and I-E. When $CD4^+$ T cells bind the foreign MHC antigens they are activated and undergo clonal proliferation. The activated T cells secrete cytokines which aid in activating monocytes/macrophages, B cells and cytotoxic $CD8^+$ T cells. The activated monocytes/macrophages release agents which result in tissue damage, the B cells produce alloantibodies which lead to complement mediated tissue destruction, and the $CD8^+$ T cells kill graft cells in an antigen-specific manner through induction of apoptosis and cell lysis.

Samsung Bioepis Exhibit No. 1002
Page 1825

Immunosuppressive Agents

[0025] The numerous drugs utilized to delay graft rejection (i.e., to prolong their survival) work in a variety of ways. Immunosuppressive agents are widely used. See Stepkowski, 2000, for a review of the mechanism of action of several immunosuppressive drugs. Cyclosporin A is one of the most widely used immunosuppressive drugs for inhibiting graft rejection. It is an inhibitor of interleukin-2 or IL-2 (it prevents mRNA transcription of interleukin-2). More directly, cyclosporin inhibits calcineurin activation that normally occurs upon T cell receptor stimulation. Calcineurin dephosphorylates NFAT (nuclear factor of activated T cells) enabling it to enter the nucleus and bind to interleukin-2 promoter. By blocking this process, cyclosporin A inhibits the activation of the $CD4^+$ T cells and the resulting cascade of events which would otherwise occur. Tacrolimus is another immunosuppressant that acts by inhibiting the production of interleukin-2.

[0026] Rapamycin (Sirolimus), SDZ RAD, and interleukin-2 receptor blockers are drugs that inhibit the action of interleukin-2 and therefore prevent the cascade of events described above.

[0027] Inhibitors of purine or pyrimidine biosynthesis are also used to inhibit graft rejection. These prevent DNA synthesis and thereby inhibit cell division including the ability of T cells to divide. The result is the inhibition of T cell activity by preventing the formation of new T cells. Inhibitors of purine synthesis include azathioprine, methotrexate, mycophenolate mofetil (MMF) and mizoribine (bredinin). Inhibitors of pyrimidine synthesis include brequinar sodium and leflunomide. Cyclophosphamide is an inhibitor of both purine and pyrimidine synthesis.

[0028] Yet another method for inhibiting T cell activation is to treat the recipient with antibodies to T cells. OKT3 is a murine monoclonal antibody against CD3 which is part of the T cell receptor. This antibody inhibits the T cell receptor and suppresses T cell activation.

[0029] Numerous other drugs and methods for delaying allotransplant rejection are known to and used by those of skill in the art. One approach has been to deplete T cells, e.g., by irradiation. This has often been used in bone marrow transplants, especially if there is a partial mismatch of major HLA. Administration to the recipient of an inhibitor (blocker) of the CD40 ligand-CD40 interaction and/or a blocker of the CD28-B7 interaction has been used (U.S. Patent 6,280,957). Published PCT patent application WO 01/37860 teaches the administration of an anti-CD3 monoclonal antibody and IL-5 to inhibit the Thl immune response. Published PCT patent application WO 00/27421 teaches a method for prophylaxis or treatment of corneal transplant rejection by administering a tumor necrosis factor-α antagonist. Glotz et al. (2002) show that administration of intravenous immunoglobulins (IVIg) can induce a profound and sustained decrease in the titers of anti-HLA antibodies thereby allowing a transplant of an HLA-mismatched organ. Similar protocols have included plasma exchanges (Taube et al., 1984) or immunoadsorption techniques coupled to immunosuppressive agents (Hiesse et al., 1992) or a combination of these (Montgomery et al., 2000). Changelian et al. (2003) teach a model in which immunosuppression is caused by an oral inhibitor of Janus kinase 3 (JAK3) which is an enzyme necessary for the proper signaling of cytokine receptors which use the common gamma chain (yc) (Interleukins-2, -4, -7, -9, -15, -21), the result being an inhibition of T cell activation. Antisense nucleic acids against ICAM-1 have been used alone or in combination with a monoclonal antibody specific for leukocyte-function associated antigen 1 (LFA-1) in a study of heart allograft transplantation (Stepkowski, 2000). Similarly, an anti-ICAM-1 antibody has been used in combination with anti-LFA-1 antibody to treat heart allografts (Stepkowski, 2000). Antisense oligonucleotides have additionally been used in conjunction with cyclosporin in rat heart or kidney allograft models, resulting in a synergistic effect to prolong the survival of the grafts (Stepkowski, 2000). Chronic transplant rejection has been treated by administering an antagonist of TGF-β which is a cytokine involved in differentiation, proliferation and apoptosis (U.S. Patent Application Publication US 2003/0180301).

Complement and Transplant/Graft Rejection

[0030] The role of complement in transplant rejection is well known. This is especially true in the case of xenotransplantation, but complement also plays a role in allotransplant rejection. For review, see Platt and Saadi, 1999. One aspect of complement's role is that ischemia-reperfusion injury may occur at the time that an organ graft is reperfused with the blood of the recipient. Complement may also cause some manifestations of allograft rejection.

[0031] The complement system is described in detail in U.S. Patent 6,355,245. The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of cellular and viral pathogens. There are at least 25 complement proteins, which are found as a complex collection of plasma proteins and membrane cofactors. The plasma proteins make up about 10% of the globulins in vertebrate serum. Complement components achieve their immune defensive functions by interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions.

[0032] The complement cascade progresses via the classical pathway or the alternative pathway. These pathways share many components and, while they differ in their initial steps, they converge and share the same "terminal complement" components (C5 through C9) responsible for the activation and destruction of target cells.

Samsung Bioepis Exhibit No. 1002
Page 1826

[0033] The classical complement pathway is typically initiated by antibody recognition of and binding to an antigenic site on a target cell. The alternative pathway is usually antibody independent and can be initiated by certain molecules on pathogen surfaces. Both pathways converge at the point where complement component C3 is cleaved by an active protease (which is different in each pathway) to yield C3a and C3b. Other pathways activating complement attack can act later in the sequence of events leading to various aspects of complement function.

[0034] C3a is an anaphylatoxin. C3b binds to bacterial and other cells, as well as to certain viruses and immune complexes, and tags them for removal from the circulation. C3b in this role is known as opsonin. The opsonic function of C3b is considered to be the most important anti-infective action of the complement system. Patients with genetic lesions that block C3b function are prone to infection by a broad variety of pathogenic organisms, while patients with lesions later in the complement cascade sequence, i.e., patients with lesions that block C5 functions, are found to be more prone only to Neisseria infection, and then only somewhat more prone (Fearon, 1983).

[0035] C3b also forms a complex with other components unique to each pathway to form classical or alternative C5 convertase, which cleaves C5 into C5a and C5b. C3 is thus regarded as the central protein in the complement reaction sequence since it is essential to both the alternative and classical pathways (Wurzner et al., 1991). This property of C3b is regulated by the serum protease Factor I, which acts on C3b to produce iC3b. While still functional as opsonin, iC3b cannot form an active C5 convertase.

[0036] C5 is a 190 kDa beta globulin found in normal serum at approximately 75 μg/mL (0.4 μM). C5 is glycosylated, with about 1.5-3 percent of its mass attributed to carbohydrate. Mature C5 is a heterodimer of a 999 amino acid 115 kDa alpha chain that is disulfide linked to a 656 amino acid 75 kDa beta chain. C5 is synthesized as a single chain precursor protein product of a single copy gene (Haviland et al., 1991). The cDNA sequence of the transcript of this gene predicts a secreted pro-C5 precursor of 1659 amino acids along with an 18 amino acid leader sequence.

[0037] The pro-C5 precursor is cleaved after amino acid 655 and 659, to yield the beta chain as an amino terminal fragment (amino acid residues +1 to 655) and the alpha chain as a carboxyl terminal fragment (amino acid residues 660 to 1658), with four amino acids deleted between the two.

[0038] C5a is cleaved from the alpha chain of C5 by either alternative or classical C5 convertase as an amino terminal fragment comprising the first 74 amino acids of the alpha chain (i.e., amino acid residues 660-733). Approximately 20 percent of the 11 kDa mass of C5a is attributed to carbohydrate. The cleavage site for convertase action is at or immediately adjacent to amino acid residue 733. A compound that would bind at or adjacent to this cleavage site would have the potential to block access of the C5 convertase enzymes to the cleavage site and thereby act as a complement inhibitor.

[0039] C5 can also be activated by means other than C5 convertase activity. Limited trypsin digestion (Minta and Man, 1977; Wetsel and Kolb, 1982) and acid treatment (Yamamoto and Gewurz, 1978; Vogt et al., 1989) can also cleave C5 and produce active C5b.

[0040] C5a is another anaphylatoxin. C5b combines with C6, C7, and C8 to form the C5b-8 complex at the surface of the target cell. Upon binding of several C9 molecules, the membrane attack complex (MAC, C5b-9, terminal complement complex-TCC) is formed. When sufficient numbers of MACs insert into target cell membranes the openings they create (MAC pores) mediate rapid osmotic lysis of the target cells. Lower, non-lytic concentrations of MACs can produce other effects. In particular, membrane insertion of small numbers of the C5b-9 complexes into endothelial cells and platelets can cause deleterious cell activation. In some cases activation may precede cell lysis.

[0041] As mentioned above, C3a and C5a are anaphylatoxins. These activated complement components can trigger mast cell degranulation, which releases histamine and other mediators of inflammation, resulting in smooth muscle contraction, increased vascular permeability, leukocyte activation, and other inflammatory phenomena including cellular proliferation resulting in hypercellularity. C5a also functions as a chemotactic peptide that serves to attract pro-inflammatory granulocytes to the site of complement activation.

[0042] Complement-binding recipient antibodies to donor alloantigens are considered to be the main cause of hyperacute graft rejection. Owing to pretransplant crossmatch testing, this prototype of humoral rejection is now rarely observed (Regele et al., 2001). Data are now showing that humoral immune mechanisms might contribute to other types of allograft rejection (Regele et al., 2001). High levels of panel reactive antibodies indicating humoral presensitization were found to be associated with inferior kidney graft survival (Opelz, 1992), the appearance of alloantibodies during the post-transplant period has been reported to predict poor graft outcome (Jeannet et al., 1970; Halloran et al., 1992), and selective removal of recipient IgG by immunoadsorption reversed some rejection episodes indicating the contribution of humoral immune mechanisms to rejection (Persson et al., 1995; Böhmig et al., 2000). Complement activation within a graft might indicate antibody-mediated graft injury. The complement cleavage product C4d is a marker for activation of the antibody-dependent classical pathway. Capillary C4d deposits in kidney allograft biopsies were associated with poor graft outcome.

[0043] Recently increasing evidence indicates that complement activation significantly contributes to the sensitization of allograft recipients and the development of tissue injury in allografts (Platt et al., 1999). Antibodies are the most thoroughly investigated mediators of activating the classical complement pathway. Clinically, alloantibodies are known

Samsung Bioepis Exhibit No. 1002
Page 1827

to activate complement (Baldwin et al., 2001). Halloran and Collins indicate that C4d deposition in peritubular capillaries of renal allografts is a sensitive and diagnostic marker of acute humoral rejection that correlates strongly with the presence of circulating donor-specific antibodies (Collins et al., 1999; Halloran, 2003). Further supporting evidence is seen in animals with complement inhibition (Pratt et al., 1996; Pruitt et al., 1991; Forbes et al., 1978) or deficiency (Pratt et al., 2000; Baurer et al., 1995) which exhibit significantly reduced inflammatory injury and lowered anti-donor immune responses. In ABMR, complement is suggested to be activated by the classical pathway and to play a key role in the pathogenesis (Collard et al., 1997). Although the role of complement in HAR or acute vascular rejection (AVR) following xenotransplantation has been well documented (Platt et al., 1999), precise mechanisms of complement in the pathogenesis of ABMR following allotransplantation has not yet been elucidated.

[0044] The C5 component of complement is cleaved to form products with multiple proinflammatory effects and thus represents an attractive target for complement inhibition within the immune-mediated inflammatory response. As described above, C5a is a powerful anaphylatoxin and chemotactic factor. Cellular activation by C5a induces the release of multiple additional inflammatory mediators (Jose et al., 1983). The complement activation pathways (classical, alternative, or mannan-binding lectin pathway) ultimately lead to the formation of the cytolytic membrane attack complex C5b-9 (Kirschfunk, 2001), which can mediate both direct tissue injury by cell lysis, and proinflammatory cell activation at sublytic doses (Saadi et al., 1995; Papadimitriou et al., 1991). Therefore, blocking both C5a and C5b-9 generation may be required for the optimal inhibition of complement-mediated inflammatory response following transplantation. At the same time, inhibition of the complement cascade at C5 does not impair the generation of C3b, preserving C3b-mediated opsonization of pathogenic microorganisms as well as solubilization and clearance of immune complexes (Liszewski, 1993).

[0045] The beneficial effect of anti-C5 mAb has previously been reported in several experimental models including myocardial reperfusion (Vakeva et al., 1998), systemic lupus erythematosus (Wang et al., 1996) and rheumatoid arthritis (Wang et al., 1995); as well as in human clinical trials (Kirschfink, 2001) of autoimmune disease, cardiopulmonary bypass and acute myocardial infarction. In addition, complement inactivation by a functionally blocking anti-C5 monoclonal Ab (mAb) prevented HAR in xenotransplantation models (Kroshus et al., 1995; Wang et al., 1999).

[0046] Methods of delaying allotransplant rejection by administration of complement inhibitors have been tested. Published PCT patent application WO 92/10205 discloses the use of a combination of cyclosporin and a soluble complement receptor (sCR1) to inhibit rejection of a cardiac allotransplant in a presensitized rat model. Complement receptor 1 binds complements C3b and C4b. Soluble forms of complement receptor 1 occur naturally or can be generated via recombinant DNA procedures. These soluble complement receptors have inhibited in vitro the consequences of complement activation (U.S. Patent 6,057,131). In WO 92/10205, rats, which had been presensitized to the cardiac allograft they were receiving, were administered cyclosporin A intramuscularly at 10 mg/kg/day beginning two days prior to transplant and continued until the time of graft rejection. Additionally, soluble complement receptor 1 (sCR1) was administered as a single intravenous bolus at 15 mg/kg immediately prior to reperfusion of the graft. Control animals with no drug treatment had the graft rejected at an average of 3.8 days. Those administered cyclosporin A alone rejected the grafts at an average of 57 days (this was quite variable with two rats rejecting quickly at 2 and 4 days and a third rat rejecting at 166 days). Rats administered sCR1 alone rejected the grafts at an average of 44 days. Those rats administered the combination of cyclosporin A and sCR1 rejected the grafts at an average of 147 days. The combination of chronic cyclosporin A and single bolus sCR1 was seen to result in a synergistic effect greatly prolonging the time until graft rejection. Earlier studies by Pruitt and Bollinger (1991) used a similar model of a presensitized rat allograft to show that administration of sCR1 alone to inactivate complement resulted in increased time before graft rejection.

[0047] Sims et al. (U.S. Patent 5,135,916) suggest using inhibitors of complement, e.g., CD59 or antibodies against C7 or C9 to block the formation of the C5b-9 complex, to treat the vascular endothelium of organs and tissues to be transplanted. This would prevent the C5b-9 initiated cell necrosis. The C5b-9 inactivators would be added to the perfusate or storage medium to protect the vascular lining cells from ongoing complement activation during in vitro storage. Additionally the organ or tissue would be protected from the cytolytic and thrombotic effects arising from complement activation initiated upon transplantation, thereby circumventing complement mediated acute rejection. Sims et al. (U.S. Patent 5,573,940 and U.S. Patent 6,100,443) also teach a method of expressing CD59 in the transplanted tissue or organ to protect the transplanted organ from rejection. This can be accomplished by transfecting the cells being transplanted.

[0048] Although the several drugs developed to date in combination with methods of prescreening donors and recipients to match the donor allograft to the recipient have over time increased the average length of time of survival of allografts, many allografts are nonetheless rejected during the life-time of the recipient. In general, the prior art advances have mainly been directed to overcoming acute graft rejection. Further, the role of activated terminal complement components in antibody-mediated allograft rejection has not been examined using inhibitors that specifically target the complement cascade at the C5 protein level. The methods described herein and as exemplified in the Examples advance the allotransplant art by inhibiting chronic rejection of allografts, in particular, allografts in a presensitized recipient. New methods are presented for further prolonging allograft survival by using a proper combination of immunosuppressive

Samsung Bioepis Exhibit No. 1002
Page 1828

drugs in combination with a chronic administration of a complement inhibitor.

Methods and Uses

[0049] The methods disclosed herein are used to prolong allograft survival. The methods generally include administering an inhibitor of complement activity in combination with one or more immunosuppressants.

[0050] Suitable complement inhibitors are known to those of skill in the art. Antibodies can be made to individual components of activated complement, e.g., antibodies to C5a, C7, C9, etc. (see, e.g., U.S. Patent 6,534,058; published U.S. patent application US 2003/0129187; and U.S. Patent 5,660,825). Proteins are known which inhibit complement-mediated lysis, including CD59, CD55, CD46 and other inhibitors of C8 and C9 (see, e.g., U.S. Patent 6,100,443). U.S. Patent 6,355,245 teaches an antibody which binds to C5 and prevents it from being cleaved into C5a and C5b thereby preventing the formation not only of C5a but also the C5b-9 complex. Proteins known as complement receptors and which bind complement are also known (see, Published PCT Patent Application WO 92/10205 and U.S. Patent 6,057,131). Use of soluble forms of complement receptors, e.g., soluble CR1, can inhibit the consequences of complement activation such as neutrophil oxidative burst, complement mediated hemolysis, and C3a and C5a production. Those of skill in the art recognize the above as some, but not all, of the known methods of inhibiting complement and its activation.

[0051] Suitable immunosuppressants include, but are not limited to, ATG or ALG, OKT3, daclizumab, basiliximab, corticosteroids, 15-deoxyspergualin, cyclosporins, tacrolimus, azathioprine, methotrexate, mycophenolate mofetil, 6-mercaptopurine, bredinin, brequinar, leflunamide, cyclophosphamide, sirolimus, anti-CD4 monoclonal antibodies, CTLA4-Ig, anti-CD154 monoclonal antibodies, anti-LFA1 monoclonal antibodies, anti-LFA-3 monoclonal antibodies, anti-CD2 monoclonal antibodies, and anti-CD45.

[0052] An allograft can include a transplanted organ, part of an organ, tissue or cell. These include, but are not limited to, heart, kidney, lung, pancreas, liver, vascular tissue, eye, cornea, lens, skin, bone marrow, muscle, connective tissue, gastrointestinal tissue, nervous tissue, bone, stem cells, islets, cartilage, hepatocytes, and hematopoietic cells.

[0053] At least part of the reason for the failure of allografts is that one response by the recipient of an allograft is the activation of complement. This results in the formation of C5a and C5b-9 which are potent proinflammatory molecules which aid in causing graft failure. Without wishing to be bound by any proposed theory, Applicants theorized that inhibiting the formation of C5a and C5b-9 or inhibiting C5a and C5b-9 which was present would aid in preventing graft failure. Furthermore, it was theorized that so long as the allograft is present, the recipient will continue to attempt to mount an immune response against the graft, and this response will include attempts to produce C5a and C5b-9. If not prevented, this complement response will lead to acute vascular rejection in the short term and could contribute to chronic graft rejection in the long term. Prior art methods of using inhibitors of complement activity were limited to administering these inhibitors only at the time of transplant. This was helpful in preventing acute rejection, but as the results disclosed herein illustrate, improved results are obtained by administration of such inhibitors for a longer term. This long-term administration aids in preventing a chronic rejection of the allograft as opposed to only aiding in preventing an acute rejection. The result is a longer term survival of the allograft as compared to either not administering an inhibitor of complement activity or administering such an inhibitor only at the time of transplant of the allograft. Although very commonly it is desirable that the allograft will survive for the remaining lifetime of the recipient, there are times when the allograft is needed only for a shorter length of time, e.g., a bridge organ to bridge the time until the recipient's own organ can recover on its own, at which time the allograft will no longer be needed. The length of time such a graft will be needed will vary, but will usually be longer than the time at which acute rejection would occur and may be long enough for chronic rejection to occur. This period of desired survival for a bridge graft may be several months, e.g., six months.

[0054] To prove that long-term inhibition of complement activity will prolong allograft survival, experiments were performed in which complement activation was inhibited in a chronic fashion and not merely at the time of transplant. Chronic treatment means treatment during an extended period up to the lifetime of the allograft. This can be daily treatment but is not limited to daily treatment. Chronic treatment will maintain an effective amount of the drug in the allograft recipient. For example, a preferred method is to include the anti-C5 monoclonal antibody eculizumab in the treatment. In studies of persons suffering from paroxysmal nocturnal hemoglobinuria (PNH), eculizumab has been administered at a dose of 900 mg/patient once every 12-14 days. This dosing has been found to completely and consistently block terminal complement activity and has greatly inhibited the symptoms of PNH (Hillmen et al., 2004). The administered dose is able to block the effects of complement for approximately two weeks before the eculizumab is inactivated or removed from the body. Therefore, a chronic treatment of eculizumab may be, e.g., the administration of 900 mg to the allograft recipient once every two weeks for the remaining life-time of the patient. Similarly, other drugs can be delivered chronically as needed, whether this is on a daily basis or another schedule is required to maintain an effective amount of the drug in the allograft recipient. Because it is well known that graft rejection can be caused by more than just complement activation, e.g., by T cell activity, the experiments included immunosuppressants such as cyclosporin to further aid in preventing graft rejection.

[0055] A preferred method of inhibiting complement activity is to use a monoclonal antibody which binds to complement

Samsung Bioepis Exhibit No. 1002
Page 1829

C5 and prevents C5 from being cleaved. This prevents the formation of both C5a and C5b-9 while at the same time allowing the formation of C3a and C3b which are beneficial to the recipient. Such antibodies that are specific to human complement are known (U.S. Patent 6,355,245). These antibodies disclosed in U.S. Patent 6,355,245 include both a whole or full-length antibody (now named eculizumab) and a single-chain antibody (now named pexelizumab). A similar antibody against mouse C5 is called BB5.1 (Frei et al., 1987). BB5.1 was utilized in the experiments set forth below. Antibodies to inhibit complement activity need not be monoclonal antibodies. They can be, e.g., polyclonal antibodies. They may additionally be antibody fragments. An antibody fragment includes, but is not limited to, an Fab, F(ab'), $F(ab')_2$, a single-chain antibody, a domain antibody and an Fv. Furthermore, it is well known by those of skill in the art that antibodies can be humanized (Jones et al., 1986), chimerized, or deimmunized. An antibody may also comprise a mutated Fc portion, such that the mutant Fc does not activate complement. The antibodies to be used in the present disclosure may be any of these. It is preferable to use humanized antibodies when the recipient of the allograft is a human.

Administration and Formulations

[0056] Administration of the inhibitor of complement activity is performed according to methods known to those of skill in the art. These inhibitors are administered preferably before the time of allograft transplantation or at the time of transplantation with administration continuing in a chronic fashion. These inhibitors can additionally be administered during a rejection episode in the event such an episode does occur.

[0057] The present disclosure also provides uses of a drug that inhibits complement activity and an immunosuppressive agent in the manufacture of a medicament or medicament package. Such medicament or medicament package is useful in prolonging allograft survival in a recipient, in particular, chronic survival of the allograft. In preferred embodiments, the medicament or medicament package is formulated and prepared such that it is suitable for chronic administration to the recipient of the allograft, for example, stable formulations are employed. In certain embodiments, the medicament or medicament package is formulated and prepared such that it is suitable for concurrent administration of the drug that inhibits complement activity and the immunosuppressive drug to the recipient of the allograft. In certain embodiments, the medicament or medicament package is formulated and prepared such that it is suitable for sequential (in either order) administration of the drug that inhibits complement activity and the immunosuppressive drug to the recipient of the allograft.

[0058] A pharmaceutical package of the present disclosure may comprise a drug that inhibits complement activity and at least one immunosuppressive agent. The pharmaceutical package may further comprise a label for chronic administration. The pharmaceutical package may also comprise a label for self-administration by a patient, for example, a recipient of a transplant graft, or instructions for a caretaker of a recipient of a transplant graft. In certain embodiments, the drug and the agent in the pharmaceutical package are in a formulation or separate formulations that are suitable for chronic administration and/or self-administration.

[0059] The present disclosure also provides lyophilized formulations and formulations suitable for injection. Certain embodiments provide a lyophilized antibody formulation comprising an antibody that inhibits complement activity and a lyoprotectant. In preferred embodiments, the antibody formulation is suitable for chronic administration, for example, the antibody formulation stable. Alternative embodiments provide an injection system comprising a syringe; the syringe comprises a cartridge containing an antibody that inhibits complement activity and is in a formulation suitable for injection.

[0060] An antibody employed in various embodiments of the present disclosure preferably inhibits the formation of terminal complement or C5a. In certain embodiments, antibody inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment. The whole antibody or antibody fragment may be a human, humanized, chimerized or deimmunized antibody or antibody fragment. In certain embodiments, the whole antibody or antibody fragment may inhibit cleavage of complement C5. In certain embodiments, the antibody fragment is a Fab, an F(ab')2, an Fv, a domain antibody, or a single-chain antibody. In preferred embodiments, the antibody fragment is pexelizumab. In alternative preferred embodiments, the whole antibody is eculizumab.

[0061] In certain embodiments, a drug, such as an antibody, that inhibits complement activity is present in unit dosage form, which can be particularly suitable for self-administration. Similarly, an immunosuppressive agent of the present disclosure may also be present in unit dosage form. A formulated product of the present disclosure can be included within a container, typically, for example, a vial, cartridge, prefilled syringe or disposable pen. A doser such as the doser device described in U.S. Patent 6,302,855 may also be used, for example, with an injection system of the present disclosure.

[0062] A "stable" formulation is one in which the drug (e.g., an antibody) or agent therein essentially retains its physical and chemical stability and integrity upon storage. Various analytical techniques for measuring protein stability are available in the art and are reviewed in Peptide and Protein Drug Delivery, 247-301, Vincent Lee Ed., Marcel Dekker, Inc., New York, N.Y., Pubs. (1991) and Jones, A. Adv. Drug Delivery Rev. 10: 29-90 (1993). Stability can be measured at a selected temperature for a selected time period. For example, the extent of aggregation following lyophilization and storage can be used as an indicator of protein stability. For example, a "stable" formulation may be one wherein less

Samsung Bioepis Exhibit No. 1002
Page 1830

than about 10% and preferably less than about 5% of the protein is present as an aggregate in the formulation. In other embodiments, any increase in aggregate formation following lyophilization and storage of the lyophilized formulation can be determined. For example, a "stable" lyophilized formulation may be one wherein the increase in aggregate in the lyophilized formulation is less than about 5% and preferably less than about 3%, when the lyophilized formulation is stored at 2-8 °C for at least one year. In other embodiments, stability of the protein formulation may be measured using a biological activity assay.

[0063] A "reconstituted" formulation is one which has been prepared by dissolving a lyophilized protein formulation in a diluent such that the protein is dispersed in the reconstituted formulation. The reconstituted formulation in suitable for administration (e.g. parenteral administration) to a patient to be treated with the protein of interest and, in certain embodiments of the invention, may be one which is suitable for subcutaneous administration.

[0064] An isotonic reconstituted formulation is preferable in certain embodiments. By "isotonic" is meant that the formulation of interest has essentially the same osmotic pressure as human blood. Isotonic formulations will generally have an osmotic pressure from about 250 to 350 mOsm. Isotonicity can be measured using a vapor pressure or ice-freezing type osmometer, for example.

[0065] A "lyoprotectant" is a molecule which, when combined with a drug (e.g., antibody) of interest, significantly prevents or reduces chemical and/or physical instability of the drug (e.g., antibody) upon lyophilization and subsequent storage. Exemplary lyoprotectants include sugars such as sucrose or trehalose; an amino acid such as monosodium glutamate or histidine; a methylamine such as betaine; a lyotropic salt such as magnesium sulfate; a polyol such as trihydric or higher sugar alcohols, e.g. glycerin, erythritol, glycerol, arabitol, xylitol, sorbitol, and mannitol; propylene glycol; polyethylene glycol; Pluronics; and combinations thereof. The preferred lyoprotectant is a non-reducing sugar, such as trehalose or sucrose.

[0066] The lyoprotectant is added to the pre-lyophilized formulation in a "lyoprotecting amount" which means that, following lyophilization of the drug (e.g., antibody) in the presence of the lyoprotecting amount of the lyoprotectant, the drug (e.g., antibody) essentially retains its physical and chemical stability and integrity upon lyophilization and storage.

[0067] The "diluent" of interest herein is one which is pharmaceutically acceptable (safe and non-toxic for administration to a human) and is useful for the preparation of a reconstituted formulation. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (e.g. phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution.

[0068] A "preservative" is a compound which can be added to the diluent to essentially reduce bacterial action in the reconstituted formulation, thus facilitating the production of a multi-use reconstituted formulation, for example. Examples of potential preservatives include octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride (a mixture of alkylbenzyldimethylammonium chlorides in which the alkyl groups are long-chain compounds), and benzethonium chloride. Other types of preservatives include aromatic alcohols such as phenol, butyl and benzyl alcohol, alkyl parabens such as methyl or propyl parahen, catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol.

[0069] A "bulking agent" is a compound which adds mass to the lyophilized mixture and contributes to the physical structure of the lyophilized cake (e.g. facilitates the production of an essentially uniform lyophilized cake which maintains an open pore structure). Exemplary bulking agents include mannitol, glycine, polyethylene glycol and xorbitol.

[0070] Accordingly, a stable lyophilized antibody formulation can be prepared using a lyoprotectant (preferably a sugar such as sucrose or trehalose), which lyophilized formulation can be reconstituted to generate a stable reconstituted formulation having an antibody concentration which is significantly higher (e.g. from about 2-40 times higher, preferably 3-10 times higher and most preferably 3-6 times higher) than the antibody concentration in the pre-lyophilized formulation. Such high protein concentrations in the reconstituted formulation are considered to be particularly useful where the formulation is intended for subcutaneous administration. Despite the very high protein concentration in the reconstituted formulation, the reconstituted formulation can be stable (i.e. fails to display significant or unacceptable levels of chemical or physical instability of the protein) at 2-8 °C. for at least about 30 days. See U.S. Patent 6,821,515. In certain embodiments, the reconstituted formulation is isotonic.

[0071] When reconstituted with a diluent comprising a preservative (such as bacteriostatic water for injection, BWFI), the reconstituted formulation may be used as a multi-use formulation. Such a formulation is useful, for example, where a subject patient requires frequent administrations of the drug or antibody and/or agent to treat a chronic medical condition. The advantage of a multi-use formulation is that it facilitates ease of use for the patient, reduces waste by allowing complete use of vial contents, and results in a significant cost savings for the manufacturer since several doses are packaged in a single vial (lower filling and shipping costs).

[0072] The present disclosure also provides a method for preparing a formulation comprising the steps of: (a) lyophilizing a mixture of an antibody and a lyoprotectant; and (b) reconstituting the lyophilized mixture of step (a) in a diluent such that the reconstituted formulation is isotonic and stable.

[0073] An article of manufacture is also provided herein which comprises: (a) a container which holds a lyophilized mixture of an antibody and a lyoprotectant; and (b) instructions for reconstituting the lyophilized mixture with a diluent to a desirable antibody concentration in the reconstituted formulation. The article of manufacture may further comprise

Samsung Bioepis Exhibit No. 1002
Page 1831

a second container which holds a diluent (e.g. bacteriostatic water for injection (BWFI) comprising an aromatic alcohol).

[0074] An injection system of the present disclosure may employ a medication delivery pen as described in U.S. Patent 5,308,341. Medication delivery pens have been developed to facilitate the self-administration of medication. A medication of the present disclosure can be a drug that inhibits complement activity, for example an antibody specific to complement C5, and/or an immunosuppressive agent. One medication delivery pen includes a vial holder into which a vial of insulin or other medication may be received. The vial holder is an elongate generally tubular structure with proximal and distal ends. The distal end of the vial holder includes mounting means for engaging a double-ended needle cannula. The proximal end also includes mounting means for engaging a pen body which includes a driver and dose setting apparatus. A disposable medication containing vial for use with the prior art vial holder includes a distal end having a pierceable elastomeric septum that can be pierced by one end of a double-ended needle cannula. The proximal end of this vial includes a stopper slidably disposed in fluid tight engagement with the cylindrical wall of the vial. This medication delivery pen is used by inserting the vial of medication into the vial holder. A pen body then is connected to the proximal end of the vial holder. The pen body includes a dose setting apparatus for designating a dose of medication to be delivery by the pen and a driving apparatus for urging the stopper of the vial distally for a distance corresponding to the selected dose.

[0075] The user of the pen mounts a double-ended needle cannula to the distal end of the vial holder such that the proximal point of the needle cannula pierces the septum on the vial. The patient then selects a dose and operates the pen to urge the stopper distally to deliver the selected dose. The dose selecting apparatus returns to zero upon injection of the selected dose. The patient then removes and discards the needle cannula, and keeps the prior art medication delivery pen in a convenient location for the next required medication administration. The medication in the vial will become exhausted after several such administrations of medication. The patient then separates the vial holder from the pen body. The empty vial may then be removed and discarded. A new vial can be inserted into the vial holder, and the vial holder and pen body can be reassembled and used as explained above.

[0076] Accordingly, a medication delivery pen generally has a drive mechanism for accurate dosing and ease of use. A dosage mechanism such as a rotatable knob allows the user to accurately adjust the amount of medication that will be injected by the pen from a prepackaged vial of medication. To inject the dose of medication, the user inserts the needle under the skin and depresses the knob once as far as it will depress. The pen may be an entirely mechanical device or it may be combined with electronic circuitry to accurately set and/or indicate the dosage of medication that is injected into the user. See U.S. Patent 6,192,891.

[0077] The present disclosure also presents controlled-release or extended-release formulations suitable for chronic and/or self-administration of a medication.

[0078] The various formulations can be administered to a patient in need of treatment (e.g., a recipient of an allograft) with the medication (e.g., an antibody of the present disclosure and at least one immunosuppressive agent) by intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes.

[0079] In certain embodiments, a formulation is administered to the patient by subcutaneous (i.e. beneath the skin) administration. For such purposes, the formulation may be injected using a syringe. However, other devices for administration of the formulation are available such as injection devices (e.g. the Inject-ease® and Genject® devices); injector pens (such as the GenPen®); needleless devices (e.g. MediJector® and BioJector®); and subcutaneous patch delivery systems.

[0080] The present methods and uses are described with reference to the following Examples, which are offered by way of illustration and are not intended to limit the disclosure in any manner. Standard techniques well known in the art or the techniques specifically described below are utilized. The following abbreviations are used herein: ABMR, antibody-mediated rejection; ACHR, accelerated humoral rejection; ACR, acute cellular rejection; AVR, acute vascular rejection; CsA, cyclosporin; CyP, cyclophosphamide; HAR, hyperacute rejection; MCP-1, monocyte chemotactic protein 1; MST, mean survival time; POD, postoperative day.

Example 1

Methods

[0081] *Animals and Immunosuppressive Drugs* Male adult C3H (H-2^k) mice and BALB/c (H-2^d) mice (Jackson Labs, Bar Harbor, Maine) weighing 25-30 g were chosen as donors and recipients, respectively. In the groups receiving immunosuppression, the recipients were injected with CsA (15 mg/kg/day, s.c., daily from day 0 to endpoint rejection or until day 100), or with CyP (40 mg/kg/day, i.v., on day 0 and 1), or with anti-C5 mAb (clone BB5.1, Alexion Pharmaceuticals Inc., 40 mg/kg/day, i.p., day 0-2, followed by twice a week, day 0-60). Animals were housed under conventional conditions at the Animal Care Facility, University of Western Ontario, and were cared for in accordance with the guidelines established by the Canadian Council on Animal Care. Olfert et al., 1993.

[0082] *Skin Presensitization* Full-thickness skin grafts taken from C3H donors were cut into square pieces of 1×1 cm^2

Samsung Bioepis Exhibit No. 1002
Page 1832

and transplanted onto the back of the BALB/c recipients' thorax one week prior to heart transplantation from the same donors. Rejection was defined as complete necrosis of the skin grafts.

[0083] *Abdominal and Cervical Cardiac Transplantation* Seven days after skin presensitization, C3H mouse hearts were transplanted into the abdomen of presensitized BALB/c recipients by anastomosing the donor aorta and recipient aorta, and the donor pulmonary artery and recipient inferior vena cava. In the groups with re-transplantation, second heart grafts harvested from either naive C3H mice or long-term surviving presensitized BALB/c recipients were transplanted into the cervical area of the recipients carrying a long-term surviving first abdominal heart graft by anastomosing the donor aorta and recipient carotid artery, and the donor pulmonary artery and recipient external jugular vein (end-to-side). The heart grafts were monitored daily until rejection unless otherwise indicated and rejection was defined as complete cessation of pulsation.

[0084] *Experimental Groups* Presensitized recipients were randomly assigned to eight groups, each consisting of eight animals: Group 1, mice with no treatment; Group 2, mice treated with CsA; Group 3, mice treated with CyP; Group 4, mice treated with CsA plus CyP; Group 5, mice treated with anti-C5 mAb; Group 6, mice treated with anti-C5 mAb plus CsA; Group 7, mice treated with anti-C5 mAb plus CyP; Group 8, mice treated with anti-C5 mAb in combination of CsA and CyP. When cardiac impulses were no longer palpable or at POD100, the grafts were removed for routine histology, immunohistochemistry and western blot analysis, serum samples were collected for flow cytometric analysis and complement hemolytic assay. Five additional animals were placed and sacrificed in groups 6 and 8 on POD3 (MST for groups 1-5, 7) to allow for comparisons at a uniform time point. Serum samples were also collected on POD 11, 21, 28 and 60 in Group 8 for detecting the sequential changes of anti-donor antibody levels and complement activity. In addition, when triple therapy treated presensitized recipients carried a first heart graft for 100 days, they were re-transplanted with a second heart. A naive C3H heart or a 100-day surviving C3H heart from another presensitized BALB/c recipient was used as the second heart. Eight animals were included in each re-transplant group.

[0085] *Graft Histology* Tissue samples were fixed in 10% buffered formaldehyde. Specimens were then embedded in paraffin, and sectioned for H&E staining. The microscopic sections were examined in a blinded fashion for severity of rejection by a pathologist. Criteria for graft rejection included the presence of vasculitis, thrombosis, hemorrhage and lymphocyte infiltration. These changes were scored as: 0, no change; 1, minimum change; 2, mild change; 3, moderate change; or 4, marked change.

[0086] *Immunohistochemistry* Four micrometer sections were cut from tissue samples embedded in Tissue-Tek O.C.T gel (Optimum Cutting Temperature, Skura Finetek, Torrance, CA) mounted on gelatin-coated glass microscope slides and stained by a standard indirect avidin-biotin immunoperoxidase staining method using an Elite Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA). Specimens were stained for $CD4^+$ and $CD8^+$ cells with biotin-conjugated rat anti-mouse CD4 mAb (clone YTS 191.1.2, Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) and biotin-conjugated rat anti-mouse CD8 mAb (clone 53-6.7, Pharmingen, Franklin Lakes, NJ), respectively. Intragraft monocyte/macrophage infiltration was detected by staining with biotin-conjugated rat anti-mouse Mac-1 mAb (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada). Mouse IgG and IgM deposition in grafts was detected using biotin-conjugated goat anti-mouse-IgG and goat anti-mouse-IgM (Cedarlane). For identification of complement deposition, sections were serially incubated with goat anti-C3 or anti-C5 polyclonal Abs (Quidel, San Diego, CA), biotinylated rabbit anti-goat IgG (Vector Laboratories), and HRP-conjugated-streptavidin (Zymed Laboratories, South San Francisco, CA). Slides were washed with phosphate-buffered saline between steps, and examined under light microscopy. Negative controls were performed by omitting the primary antibodies. The immunostaining was scored in five high-power fields of each section, and five independent experiments were performed. The sections of immunoperoxidase staining were graded from 0 to 4+ according to the staining intensity: 0, negative; 1+, equivocal; 2+, weak staining; 3+, moderate staining; and 4+, very intensive staining.

[0087] *Flow Cytometry* The circulating anti-donor specific IgG and IgM antibodies were evaluated in the recipient serum by FACScan flow cytometry (Becton Dickinson, Mountain View, CA). Glotz et al., (1993); Tyan et al. (1994). Briefly, C3H mouse splenocytes were isolated and incubated at 37°C for 30 minutes with serum from naive control and experimental groups. To stain for total IgG, IgG1, IgG2a, IgG2b and IgM, the cells were washed and incubated with FITC-conjugated goat antibody specific for the Fc portion of mouse IgG or with phycoerythrin-conjugated goat antibody specific for the μ-chain of mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA), or with FITC-conjugated goat anti-mouse IgG1 (CALTAG Laboratories, Burlingame, CA), or with FITC-conjugated goat anti-mouse IgG2a (CALTAG), or with FITC-conjugated goat anti-mouse IgG2b (CALTAG). After 1 hour of staining at 4°C, the cells were washed with PBS, resuspended at 5×10^6/mL, and analyzed by flow cytometry for mean channel fluorescence intensity, which represents the antibody-binding reactivity.

[0088] *Complement Hemolytic Assay* The purified anti-C5 mAb was serially diluted twofold (175-0.1 μg/ml) in GVB^{2+} buffer (gelatin Veronal-buffered saline: 0.1% gelatin, 141 mM NaCl, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, and 1.8 mM sodium barbital) and added in triplicate (50 μl/well) to a 96-well plate. BALB/c mouse serum was diluted to 40% v/v with GVB^{2+} buffer and added (50 μl/ml) to the rows of the same 96-well plate such that the final concentration of BALB/c mouse serum in each well was 20%. The plate was then incubated at room temperature for approximately 30 min while chicken

Samsung Bioepis Exhibit No. 1002
Page 1833

erythrocytes were prepared. Chicken erythrocytes were washed 5×1 ml with GVB^{2+} buffer and resuspended to a final concentration of 5×10^7/ml in GVB^{2+}. Four milliliters of the chicken erythrocytes were sensitized by adding anti-chicken RBC polyclonal antibody (Intercell Technologies, Hopewell, NJ, 0.1 % v/v) and the cells were incubated at 4 °C for 15 min with frequent vortexing. The cells were then washed 2×1 ml with GVB^{2+} and resuspended to a final volume of 2.4 ml in GVB^{2+}. The chicken erythrocytes (30 μl/well, 2.5×10^6 cells) were added to the plate containing serum and anti-C5 mAb as described above, mixed well, and incubated at 37 °C for 30 min. The plate was then centrifuged at 1000×g for 2 min, and 85 μl of the supernatant was transferred to a new 96-well microtiter plate. The plate was read at OD 415 nm using a microplate reader and the percentage of hemolysis was determined using this formula:

$$\% \text{ hemolysis} = 100 \times \frac{(\text{OD sample}) - (\text{OD } GVB^{2+} \text{ control})}{(\text{OD 100\% lysed control}) - (\text{OD } GVB^{2+} \text{ control})}$$

with 100% lysed control obtained by the addition of 100 μl GVB^{2+} containing 0.1% NP-40 to the 30 μg/ml of chicken erythrocytes as prepared above.

[0089] *Western Blot Analysis* Sonication of frozen heart samples was performed in RIPA lysis buffer (Santa Cruz Biotechnology, Inc.) at 4 °C for 1 minute at 10-second intervals, followed by microcentrifugation at 13,000 rpm for 10 minutes at 4 °C. Clarified supernatants were immediately quantitated in triplicate for protein content using Detergent-compatible protein assay kit (BIO-RAD). Heart lysates (10μg protein/well) were separated on NuPAGE, 4-12% gradient Bis-Tris gels and MES buffer system (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membrane (0.45 μm pore size; Invitrogen) using a semi-dry transfer apparatus (BIO-RAD). Membranes were cut appropriately at the correct molecular weights to allow the development of the blots with two different primary antibodies per blot such that each blot was exposed to a test antibody and an internal control antibody to insure equal sample loading. The test primary antibodies including anti-Bcl-2 (N-19) rabbit polyclonal sera (Santa Cruz Biotechnology, Inc.) and anti-Bcl-XS/L (M-125) rabbit polyclonal sera (Santa Cruz Biotechnology, Inc.) were used to detect intragraft expression of Bcl-2 and Bcl-xl proteins. Anti-calsequestrin rabbit polyclonal sera (Calbiochem) were used as internal control primary antibody (Kobayashi et al., 1999). Detection of primary antibody binding was performed as previously described (Arp et al., 1996) by exposing washed incubated blots to a polyclonal goat anti-rabbit IgG fraction conjugated to horseradish peroxidase (HRP; Roche Laboratories) and then appropriately developed by exposure to enhance chemiluminescence for HRP-conjugated antibodies (Roche Laboratories).

[0090] *Statistical Analysis* The data were reported as the mean ± SD. Allograft survival among experimental groups was compared using the rank-log test. Histological and immunohistological findings were analyzed using the Mann-Whitney U test. Flow cytometric data and western blot data were analyzed using one-way ANOVA. Differences with p values less than 0.05 were considered significant.

Example 2

Presensitization with C3H donor skin graft induces antibody-mediated ACHR in heart allografts of BALB/c recipients.

[0091] To develop a suitable small animal model that mimics presensitized patients in the clinic and to study ABMR, a novel, fully MHC-mismatched mouse ABMR model has been developed through presensitization of mouse recipients. In this model, BALB/c recipients were presensitized with C3H donor skin grafts one week prior to heart transplantation from the same donor. Seven days after donor skin presensitization, serum level of anti-donor IgG, but not IgM antibody was markedly elevated and reached to a peak level in the presensitized BALB/c recipients (Figure 1A). Heart transplantation from same donor was then performed in these highly sensitized recipients. Without immunosuppression, C3H heart grafts were rapidly rejected in 3.1 ± 0.4 days by ACHR, characterized by severe thrombosis, hemorrhage and infarction (Figure 1B-a). In contrast, same heart grafts in unsensitized BALB/c recipients (with mean survival time, MST of 8.2 ± 0.8 days) show the normal histology on post-operative day (POD) 3 (Figure 1B-b). When compared to unsensitized BALB/c recipients at the same day, heart grafts in presensitized animals revealed massive IgG antibody and complement (C3 and C5) deposition, but minimal $CD4^+$ and $CD8^+$ cell infiltration (Table 1). Furthermore, circulating anti-donor IgG levels in presensitized recipients were significantly higher than those of unsensitized same recipients receiving a heart graft on POD3 ($P< 0.01$, Figure 1C). However, anti-donor IgM remained at very low levels both in circulation (Figure 1C) and in heart grafts (Table 1) and it showed no significant difference between unsensitized and presensitized recipients. In addition, normal levels of complement hemolytic activity were shown in both presensitized and unsensitized

Samsung Bioepis Exhibit No. 1002
Page 1834

heart recipients without treatment (Figure 1D). These data indicate that this is an ideal transplant model to study ABMR in presensitized recipients in which complement plays an important role in the pathogenesis.

Table 1.

Comparison of immunohistological changes of C3H heart allografts in unsensitized and presensitized BALB/c recipients on POD3

Groups	Unsensitized	Presensitized
IgG	1+	4+
IgM	1+	1+
C3	2+	3+
C5	2+	3+
CD4	0	1+
CD8	0	1+

Grades for immunoperoxidase staining: 0, negative; 1+, equivocal; 2+, weak; 3+, moderate; 4+, intense.

Anti-C5 mAb in combination with CsA and CyP prevents ABMR and achieves indefinite heart allograft survival in presensitized mouse recipients.

[0092] Complement has been shown to play an important role in ABMR. However, the inhibitory effect of functionally blocking terminal complement cascade at the C5 level in highly sensitized recipients is unknown. In the study presented herein, the presensitized model was used to study the efficacy of anti-C5 mAb either alone or combined with CsA and /or CyP in prevention of ABMR. As presented in Figure 2A, treatment with either CsA or CyP or the two drugs in combination did not prevent ABMR and grafts were rejected in 3.0 $\pm$ 0.0 days, 3.3 $\pm$ 0.5 days and 3.5 $\pm$ 0.6 days, respectively with typical pathological features of ACHR including intravascular thrombosis and interstitial hemorrhage (Figure 2B-b, c, d), which were indistinguishable from heart grafts in untreated presensitized BALB/c recipients (Figure 2B-a). Anti-C5 monotherapy or combined with CyP was not able to improve graft survival and heart grafts were rejected by ACHR (Figure 2B-e, f) in 3.5 $\pm$ 0.6 days and 3.2 $\pm$ 0.4 days, respectively (Figure 2A). Although the combination therapy of anti-C5 mAb and CsA, the protocol capable of inducing long-term heart allograft survival in unsensitized animals, marginally prolonged graft survival in this presensitized model, heart grafts were also rejected by severe humoral rejection with vasculitis, thrombosis, hemorrhage and minimal cell infiltration (Figure 2B-g) in 11.9 $\pm$ 1.8 days (Figure 2A). In contrast, triple therapy of anti-C5 mAb in combination of CsA and CyP achieved indefinite heart graft survival over 100 days (Figure 2A) in presensitized animals ($P<0.01$ vs. the animals without treatment or treated with either monotherapy or two drugs in combination) with no evidence of rejection (Figure 2B-h). In this presensitized mouse model, as shown in Table 2, only minor intragraft $CD4^+$ and $CD8^+$ cell infiltration was observed in the recipients that rejected their heart grafts within 3 days. However, the number of these T cells was slightly increased if heart grafts survived longer in anti-C5 mAb plus CsA-treated recipients at the time of rejection (POD11) and in triple therapy-treated recipients at early stages of graft survival (e.g. POD11). Furthermore, with continuous treatment of CsA in the triple therapy group, $CD4^+$ and $CD8^+$ cell infiltration was inhibited in long-term surviving heart grafts on POD60 and 100. In addition, moderate intragraft Mac-1^+ cell infiltration, including monocytes and macrophages, was found in untreated and CsA-, CyP- or CsA plus CyP-treated animals, while the infiltration of these cells was significantly reduced in anti-C5 mAb treated animals (Table 2). These results indicate that functionally blocking anti-C5 mAb enables the use and efficacy of conventional immunosuppressive agents, thereby preventing ABMR and achieving indefinite heart graft survival in presensitized recipients.

Table 2.

Grades for immunoperoxidase staining of heart allografts in presensitized mouse recipients at necropsy

Groups	Date for sample collection (POD)	C3	C5	**CD4**	CD8	Mac-1	IgG	IgM
Untreated	3	3+	3+	1+	1+	3+	4+	1+
CsA	3	3+	3+	1+	1+	3+	4+	1+
CyP	3	3+	3+	1+	1+	3+	3+	1+
CsA+CyP	3	3+	3+	1+	1+	3+	3+	1+
Anti-C5mAb	3	3+	0	1+	1+	2+	4+	1+

Samsung Bioepis Exhibit No. 1002
Page 1835

(continued)

Grades for immunoperoxidase staining of heart allografts in presensitized mouse recipients at necropsy

Groups	Date for sample collection (POD)	C3	C5	**CD4**	CD8	Mac-1	IgG	IgM
Anti-C5mAb+CsA	11	3+	0	2+	2+	2+	4+	1+
Anti-C5mAb+CyP	3	3+	0	1+	1+	2+	3+	1+
Anti-C5mAb+CsA+CyP	3	3+	0	1+	1+	2+	3+	1+
Anti-C5mAb+CsA+CyP	11	3+	0	2+	2+	1+	3+	1+
Anti-C5mAb+CsA+CyP	60	3+	0	1+	1+	0	2+	1+
Anti-C5mAb+CsA+CyP	100	3+	2+	0	0	0	2+	1+

Grades: 0, negative; 1+, equivocal; 2+, weak; 3+, moderate; 4+, intense.

Anti-C5 mAb completely inhibits total complement hemolytic activity and local C5 deposition in presensitized recipients receiving a heart allograft.

[0093] Anti-C5 mAb was previously shown to block the cleavage of complement protein C5 into the proinflammatory molecules C5a and C5b-9 (Kroshus et al., 1995), and to completely and consistently block terminal complement activity in mice (Wang et al., 1999). In the current study, terminal complement activity was measured by assessing the ability of recipient mouse sera to lyse antibody presensitized chicken erythrocytes and was compared at the same time-point (POD3). Treatment of mice with either CsA or CyP or the two drugs in combination had no effect on terminal complement activity, while treatment with anti-C5 mAb either alone or combined with CsA or/and CyP completely inhibited this activity (Figure 3; $P<0.01$, vs. naive and untreated animals, as well as CsA-, CyP-, or CsA plus CyP-treated animals). In addition, sera obtained from anti-C5 mAb treated animals at several earlier time points showed similarly diminished hemolytic activity, suggesting that serum terminal complement was inhibited throughout the treatment period. Furthermore, local C5 deposition in heart grafts was completely prevented in the anti-C5 mAb treated presensitized recipients, but not in untreated, or CsA-, CyP- and CsA plus CyP-treated presensitized animals (Table 2). As predicted, treatment with anti-C5 mAb did not prevent C3 deposition in the grafts (Table 2). These results suggest anti-C5 therapy completely blocks total complement activity after cardiac allografting in highly sensitized recipients.

Long-term surviving heart grafts in presensitized animals are resistant to humoral injury in the presence of low level of anti-donor antibodies and complement- a situation of accommodation.

[0094] To further investigate the role of anti-C5 mAb in humoral rejection, anti-donor alloantibody levels were measured in recipient sera by flow cytometry and intragraft antibody deposition by using immunostaining techniques in different groups. Figure 4A shows that on POD3 untreated presensitized BALB/c recipients had high levels of circulating anti-donor IgG antibodies. When presensitized recipients receiving either monotherapy or two drugs in combination, CsA and/or CyP partially down-regulated circulating anti-donor IgG levels, while treatment with anti-C5 mAb either alone or combined with CsA or CyP did not further affect anti-donor antibody levels at the same day. In contrast, with triple therapy of anti-C5 mAb, CsA and CyP, a high level of circulating anti-donor IgG was gradually downregulated and reached a low level on POD60, thereafter remaining at this level until day 100 (Figure 4B). Similar to levels of circulating antibodies in the different treatment groups, Table 2 shows that strong deposition of anti-mouse IgG was present in the rapidly rejected heart grafts of presensitized animals with no treatment or treated with monotherapy or two drugs in combination therapy. Interestingly, with triple therapy, IgG antibody deposition was gradually attenuated to a mild level in the long-term surviving heart grafts on POD 100 (Figure 4C-a, Table 2). In this model, IgM remained at very low levels in either circulation (Figure 4A. B) or transplanted heart grafts (Figure 4C-b, Table 2) in presensitized recipients with or without treatment. In addition, treatment with anti-C5 mAb eliminated complement activity to an undetectable level until day 60, followed by a progressive recovery to predepletion levels on POD 100 after discontinuation of anti-C5 therapy in presensitized mouse recipients receiving triple therapy (Figure 4D). Furthermore, intragraft C5 deposition was also detected in 100-day surviving presensitized animals (Table 2). These data demonstrate that ongoing transplant accommodation occurs in triple therapy treated presensitized recipients despite the presence of anti-graft antibodies and complement activation.

Samsung Bioepis Exhibit No. 1002
Page 1836

Anti-C5 mAb in combination with CsA and CyP reduces the IgG1/IgG2α ratio and leads to a shift in IgG subclass to IgG2b in recipients with accommodated grafts.

[0095] To determine whether anti-C5 mAb-based triple therapy would induce a shift in IgG subclass, which may be associated with accommodation, serum levels of anti-donor IgG subclasses of IgG1, IgG2a and IgG2b were compared between untreated recipients and the recipients with accommodated heart graft. Sera from untreated recipients contained predominant IgG1 isotype, indicated by a high ratio of IgG1/IgG2a (Figure 5A). In contrast, a significant reduction in the ratio of IgG1/IgG2a was observed in the recipients carrying accommodated grafts (Figure 5A, $P<0.01$). Furthermore, presensitized recipients with the accommodated heart grafts displayed an increased level of anti-donor IgG2b as compared to the same recipients with rejected grafts (Figure 5B, $P<0.01$). In addition, the pattern of IgG isotypes in the recipients treated with either monotherapy or two drugs in combination is indistinguishable from that of untreated animals. These data indicate that anti-donor IgG1 isotype may be associated with graft rejection, while production of anti-donor IgG2b subclass may function as a protective antibody and plays an important role in the induction of accommodation.

Anti-C5 mAb in combination with CsA and CyP induces intragraft Bcl-2 and Bcl-xl expression in highly sensitized mouse recipients.

[0096] To determine whether a causal relationship exists between intragraft expression of protective proteins and graft resistance to humoral injury in this model, western blot analysis was employed to detect proteins of interest in heart graft tissues from highly sensitized mouse recipients. Long-term surviving heart grafts were found to express high levels of Bcl-2 and Bcl-xl proteins on POD 100, and these proteins were detected as early as 12 days after heart transplantation in highly sensitized recipients receiving anti-C5 mAb-based triple therapy (Figure 6). In contrast, there were no Bcl-2 and Bcl-xl proteins expressed on heart grafts of untreated animals (Figure 6) or animals treated with either monotherapy or two drugs in combination therapy. This result suggests that graft resistance to humoral injury in indefinite surviving animals is associated with the protection provided by Bcl-2 and Bcl-xl proteins in this presensitized model.

Presensitized recipients with an accommodating first heart graft accept a second accommodated heart graft but reject a second naive heart graft from the same donors.

[0097] The ability of accommodated grafts to resist rejection has not been tested directly under pathophysiological conditions where naive grafts undergo rejection following allotransplantation. In this model, to determine whether presensitized recipients with an accommodating first heart graft will accept a second accommodated graft but reject a second naive graft, we performed re-transplantation scenarios. After the accommodated C3H heart grafts have survived to the 100-day point; the time at which low levels of alloantibodies were detected (Figure 4B) and complement activity has returned to pretreatment levels (Figure 4D), in presensitized BALB/c recipient treated with anti-C5 mAb-based triple therapy, these recipients received a second heart graft. Specifically, either a naive (Figure 7A) or a 100-day accommodated C3H heart (Figure 7B) from another presensitized BALB/c recipient was transplanted into the neck of the presensitized recipients carrying an accommodating first C3H heart. These recipients rejected a second naive heart at 6.6 ± 1.1 days (Figure 8A) with severe AVR (Figure 8B-a) while the first heart continued to survive. In contrast, when the accommodated hearts that had been already surviving for 100 days in different presensitized mice were used as second grafts, these grafts were accepted by the presensitized recipients carrying an accommodating first heart graft (Figure 8A). There was no sign of rejection in those accommodated second heart grafts 90 days after second transplantation (Figure 8B-b). These data indicate that accommodated grafts become resistant to the effects of anti-donor antibodies and complement that normally mediate allograft rejection in these presensitized recipients. Furthermore, the fact that the host of the accommodated graft rejected a new graft suggests that accommodation involves changes to the graft.

Presensitized recipients being treated with CsA reject accommodated heart grafts.

[0098] Another re-transplantation was performed to determine whether accommodation in this presensitized model would be caused by the changes in the grafts and/or the recipients. Specifically, after C3H heart grafts have been accommodated in presensitized BALB/c mice for 100 days, the accommodated heart graft will then be re-transplanted into a second presensitized BALB/c recipient being treated with CsA alone (Figure 7C), a therapy that can prevent cellular rejection but cannot prevent accelerated humoral rejection of a fresh C3H heart in presensitized recipients. The accommodated C3H heart grafts were rapidly rejected in CsA treated presensitized BALB/c recipients. After re-transplantation, the pathology in accommodated heart grafts was changed from normal (Figure 9A) to severe ACHR with massive interstitial hemorrhage but few cell infiltrates (Figure 9B). In addition, high levels of anti-donor IgG and normal levels of complement hemolytic activity in these recipients receiving an accommodated C3H heart were similar to those of CsA treated presensitized recipients receiving a naive C3H heart. This result further indicates that accommodation

Samsung Bioepis Exhibit No. 1002
Page 1837

induced by anti-C5 mAb-based triple therapy can originate from mechanisms involving changes not only to the graft, but also to the recipient.

Example 3

Acute Vascular Rejection in a Heart Transplantation Model

[0099] Experiments were performed to determine whether inclusion of an inhibitor of formation of terminal complement would attenuate acute vascular rejection and whether the use of such an inhibitor in conjunction with an immunosuppressant would achieve long-term allograft survival. In this set of experiments an anti-C5 monoclonal antibody was used in conjunction with cyclosporin. The model used was an allograft heterotopic heart transplant from C3H mice into BALB/c mice. This model is a stringent acute vascular rejection model with the C3H and BALB/c mice being strongly MHC mismatched. The transplantations and other methods were performed as described in Wang et al. (2003).

Heterotopic Cardiac Transplantation

[0100] Intra-abdominal heterotopic cardiac transplantation was performed as previously described by Wang et al. (2003). Briefly, a median sternotomy was performed on the donor, and the heart graft was slowly perfused in situ with 1.0 ml of cold heparinized Ringer's lactate solution through the inferior vena cava and aorta before the superior vena cava and pulmonary veins were ligated and divided. The ascending aorta and pulmonary artery were transected, and the graft was removed from the donor. The graft was then revascularized with end-to-side anastomoses between the donor's pulmonary artery and the recipient's inferior vena cava as well as the donor's aorta and the recipient's abdominal aorta using 11-0 nylon suture. The beating of the grafted heart was monitored daily by direct abdominal palpation. The degree of pulsation was scored as: A, beating strongly; B, noticeable decline in the intensity of pulsation; or C, complete cessation of cardiac impulses. When cardiac impulses were no longer palpable, the graft was removed for routine histology. In certain instances, mice in which the graft was still functioning were sacrificed to perform histology.

Results

[0101] Mice (male 8-12 week old mice weighing 25 - 30 g) were split into six experimental groups with six to eight mice per group. Transplant occurred on day 0. Histological changes were checked at the endpoint (the endpoint being graft failure) or in some cases a mouse was sacrificed prior to graft failure. The dosage of BB5.1 which was administered (40 mg/kg body weight three times per week) was known from prior studies to completely inhibit terminal complement activity.

Group 1' (control) - mice were administered 0.75 mL of saline intraperitoneally on days -1, 0, 1 and 2. Subsequently these mice were treated with 0.75 mL of saline intraperitoneally three times per week (Monday, Wednesday, Friday) until the endpoint.

Group 2' (cyclosporin A alone) - mice were administered 15 mg/kg body weight of cyclosporin A subcutaneously on a daily basis beginning at day 0 (day of transplant) until the endpoint.

Group 3' (anti-complement antibody alone) - mice were administered the anti-mouse C5 antibody BB5.1 (Frei et al., 1987) at 40 mg/kg body weight intraperitoneally on days -1, 0, 1 and 2 followed by 40 mg/kg body weight administered three times per week (Monday, Wednesday, Friday) until the endpoint.

Group 4' (anti-complement antibody until day 14 post-transplant plus cyclosporin A) - mice were administered the anti-mouse C5 antibody BB5.1 at 40 mg/kg body weight intravenously on days -1 through day 14 and were also administered cyclosporin A at 15 mg/kg of body weight on a daily basis beginning at day 0 until the endpoint. Note that this differs from the other groups in that the BB5.1 was administered intravenously and on a daily basis.

Group 5' (anti-complement antibody until day 28 post-transplant plus cyclosporin A) - mice were administered the anti-mouse C5 antibody BB5.1 at 40 mg/kg body weight intraperitoneally on days -1,0,1 and 2 followed by 40 mg/kg body weight administered three times per week (Monday, Wednesday, Friday) until day 28 and were also administered cyclosporin A at 15 mg/kg of body weight on a daily basis beginning at day 0 until the endpoint.

Group 6' (anti-complement antibody chronically until 100 days plus cyclosporin) - mice were administered the anti-mouse C5 antibody BB5.1 at 40 mg/kg body weight intraperitoneally on days -1,0, 1 and 2 followed by 40 mg/kg

Samsung Bioepis Exhibit No. 1002
Page 1838

body weight administered three times per week (Monday, Wednesday, Friday) until 100 days and were also administered cyclosporin A at 15 mg/kg of body weight on a daily basis beginning at day 0 until 100 days.

[0102] The results of this experiment are shown in Tables 3 and 4. Table 3 shows the survival time for the grafts. Table 4 sets forth the histological scores.

Table 3

Allograft Survival		
Group (Treatment)	Individual Survival (days)	Mean Survival Time (days)
1'. Saline	8, 8, 8, 8, 8, 9	8.3 ± 0.5
2'. Cyclosporin A	14, 15, 15, 16, 16, 16, 17	15.5 ± 1.1
3'. BB5.1	7, 8, 8, 8, 8, 9	8.0 ± 0.6
4'. BB5.1 until day 14 + cyclosporin A	35, 38, 43, 45, 46, 47	42.3 ± 4.8
5'. BB5.1 until day 28 + cyclosporin A	77, 80, 80, 81, 82	80 ±1.9
6'. BB5.1 until day 100 + cyclosporin A	>100 days (7 mice; one sacrificed for histology)	> 100 days

Table 4

Median Scores of Histological Changes of Heart Allografts at Necropsy							
Groups	Vasc*	Infarc	Lymph	Throm	Hemo	Fibrin	PMN
1'. Saline (endpoint)	3.0	3.0	1.0	4.0	3.0	3.0	3.0
2'. Cyclosporin A (endpoint)	2.0	1.0	2.0	3.0	2.0	2.0	3.0
3'. BB5.1 (endpoint)	2.0	1.0	2.0	2.0	1.0	0.0	0.0
4'. BB5.1 until day 14 + cyclosporin A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
5'. BB5.1 28 days + Cyclosporin A (post-operative day 8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5'. BB5.1 28 days + Cyclosporin A (endpoint)	0.0	0.0	1.0	1.0	2.0	1.0	0.0
6'. BB5.1 until day 100 + Cyclosporin A (post-operative day 100)	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Median scores: 0 - normal; 1- minimum change; 2 - mild change; 3 - moderate change; 4 - marked change. N/A - not available. *Vasc - vasculitis; Infar - infarction; Lymph - lymphocyte infiltration; Throm - thrombosis; Hemo - hemorrhage; Fibrin - fibrin deposition; PMN - polymorphonuclear cell infiltrate							

[0103] The results indicate the synergistic effects of using a complement inhibiting drug in addition to an immunosuppressant. In untreated mice the grafts were rejected in about 8 davs. Use of the immunosuppressant cyclosporin A alone on a daily, chronic basis resulted in an increase in graft survival until approximately 15 days post-transplant. The use of the anti-C5 antibody BB5.1 to inhibit formation of terminal complement had no effect on its own, graft rejection occurring at 8 days post-transplant as in the control group (Group 1'). The combination of BB5.1 through day 28 post-transplant plus cyclosporin A showed a synergistic effect with graft survival being extended until approximately day 80. A more surprising result is that of Group 5' in which BB5.1 and cyclosporin A were each administered chronically post-transplant. In this case the graft survival was for more than 100 days (as much data as presently available). Additionally, the histological results shown in Table 4 indicate that the administration of both BB5.1 and cyclosporin A protected the graft from changes much better than either BB5.1 or cyclosporin A alone, and that the chronic administration of BB5.1 and cyclosporin A protected the graft to such an extent that even at 100 days post-transplant there were no histological changes seen in the engrafted hearts. A survival time of 100 days in these models is considered to be the gold standard. A survival of 100 days in the model is believed to indicate that there will be an indefinite survival of the allograft. When BB5.1 administration was stopped after 28 days, the grafts were protected but they did begin to show some minimal to mild histological changes by about day 80 which was the time at which graft failure occurred.

Samsung Bioepis Exhibit No. 1002
Page 1839

[0104] The Group 4' mice were treated differently in that they were administered BB5.1 on a daily basis by an intravenous administration. These animals became ill, showing weight loss and urine retention and were sacrificed at a time at which the grafted hearts were still beating although their function had declined. This was the first group of mice studied and it is unknown why these ill effects were seen. These ill effects were not seen when the BB5.1 was administered intraperitoneally with a schedule of three times per week. As seen below in Example 4, daily administration of BB5.1 via an intraperitoneal route did not cause ill effects. Also, intravenous administration was not necessarily the cause of the illness in these animals. Intravenous administration of eculizumab (a human equivalent antibody to BB5.1 in that it binds to human C5) has been successfully administered intravenously without ill effects to humans in a study of PNH (Hillmen et al., 2004). Complement inhibitors may be administered by other routes in addition to intravenous and intraperitoneal, with all such routes being well known by those skilled in the art.

Example 4

Accelerated Rejection in a Presensitized Heart Transplantation Model

[0105] A second set of experiments similar to those of Example 3 was performed but the recipient mouse was presensitized to the donor organ. In these experiments, the presensitization was brought about by prior transplantation of a skin graft. In general, presensitization can occur not only as a result of having received an earlier allograft, but can also be caused by having received multiple blood transfusions or in women who have been pregnant. Besides such presensitization methods, allografts with an ABO mismatch will be rapidly attacked and rejected because of preformed antibodies to the ABO antigens unless steps are taken to prevent such an attack.

[0106] Some mice in these studies were administered cyclophosphamide in addition to BBS.1 and/or cyclosporin A. For these experiments BALB/c recipient mice were presensitized with C3H skin grafts one week prior to heart transplantation from the same donor (using the method of Pruitt and Bollinger, 1991). This model is designed to mimic presensitized transplantation in humans, especially in relation to accelerated humoral rejection. Recipient mice were split into eight groups of six to eight mice each. The treatments were as follow.

Group 1" (control) - mice (male 8-12 week old mice weighing 25 - 30 g) were administered 0.75 mL saline intraperitoneally on a daily basis beginning at day -1 and continuing until the endpoint (graft rejection).

Group 2" (cyclosporin A alone) - mice were administered cyclosporin A subcutaneously at a dose of 15 mg/kg body weight beginning on day 0 (day of transplant) until the endpoint.

Group 3" (BB5.1 alone) - mice were administered the anti-mouse complement monoclonal antibody BB5.1 at a dose of 40 mg/kg body weight delivered intraperitoneally on a daily basis beginning at day -1 and continuing until the endpoint.

Group 4" (cyclophosphamide alone) - mice were administered cyclophosphamide intravenously at a dose of 40 mg/kg body weight on each of days 0 and 1.

Group 5" (BB5.1 plus cyclosporin A) - mice were administered BB5.1 intraperitoneally at a dose of 40 mg/kg body weight on a daily basis beginning at day -1 and continuing until the endpoint. These mice were additionally administered cyclosporin A subcutaneously at a dose of 15 mg/kg body weight on a daily basis from day 0 until the endpoint.

Group 6" (BB5.1 plus cyclophosphamide) - mice were administered BB5.1 intraperitoneally at a dose of 40 mg/kg body weight on a daily basis beginning at day -1 and continuing until the endpoint. These mice were additionally administered cyclophosphamide intravenously at a dose of 40 mg/kg body weight on each of days 0 and 1.

Group 7" (cyclosporin A plus cyclophosphamide) - mice were administered cyclosporin A subcutaneously at a dose of 15 mg/kg body weight on a daily basis from day 0 until the endpoint. These mice were additionally administered cyclophosphamide intravenously at a dose of 40 mg/kg body weight on each of days 0 and 1.

Group 8" (BB5.1 plus cyclosporin A plus cyclophosphamide) - mice were administered BB5.1 intraperitoneally at a dose of 40 mg/kg body weight on a daily basis beginning at day -1 and continuing until 100 days. These mice were also administered cyclosporin A subcutaneously at a dose of 15 mg/kg body weight on a daily basis from day 0 until 100 days. These mice were additionally administered cyclophosphamide intravenously at a dose of 40 mg/kg body weight on each of days 0 and 1. Two mice in this group were sacrificed at day 60 for histological studies (no rejection had yet occurred) and the four remaining mice still had not rejected their grafts by day 100.

Samsung Bioepis Exhibit No. 1002
Page 1840

[0107] Additionally a control group of mice which was not presensitized and received only the saline treatment as for Group 1" was tested.

[0108] The results of these experiments are shown in Tables 5 and 6. Table 5 lists survival times for the grafts and Table 6 summarizes the histological results.

Table 5

Allograft Survival		
Groups (Treatment)	Individual survival (days)	Mean survival time (days)
No presensitization	8, 8, 8, 8, 8, 9	8.3 ± 0.5*
1". One skin presensitization	3, 3, 3, 3, 3, 3, 3, 4	3.1 ± 0.4
2". Cyclosporin A	3, 3, 3, 3	3.0 ± 0.0
3". BB5.1	3, 3, 4, 4	3.5 ± 0.6
4". Cyclophosphamide	3, 3, 3, 4	3.3 ± 0.5
5". BB5.1 + Cyclosporin A	10, 10, 11, 11, 12, 12, 14, 15	11.9 ± 1.8**
6". BB5.1 + Cyclophosphamide	3, 3, 3, 3, 3, 4	3.2 ± 0.4
7". Cyclosporin A + Cyclophosphamide	3, 3, 3, 4, 4, 4	3.5 ± 0.6
8". BB5.1 + Cyclosporin A + Cyclophosphamide	> 100 days (4 mice)	> 100***
*P < 0.01 group 1" vs. no presensitization **P < 0.01 group 5" vs. groups 1"-4" and 6"-7". ***P<0.01 group 8" vs. groups 1"-7".		

Table 6

Median Scores of Histological Changes of Heart Allografts at Necropsy							
Groups	Vasc*	Infar	Lymph	Throm	Hemo	Fibrin	PMN
No presensitization (endpoint)	3.0	3.0	1.0	4.0	3.0	3.0	3.0
1". One skin presensitization (endpoint)	0.0	4.0	1.0	4.0	3.0	0.0	0.0
2". Cyclosporin A (endpoint)	0.0	4.0	0.0	3.0	3.0	N/A	N/A
3". BB5.1 (endpoint)	2.0	2.0	2.0	2.0	3.0	N/A	N/A
4". Cyclophosphamide (endpoint)	2.0	4.0	0.0	3.0	2.0	N/A	N/A
5". BB5.1 + Cyclosporin A (post-operative day 3)	0.0	1.0	1.0	0.0	0.0	0.0	0.0
5". BB5.1 + Cyclosporin A (endpoint)	2.0	2.0	2.0	2.0	2.0	0.0	0.0
6". BB5.1 + Cyclophosphamide (endpoint)	1.0	3.0	0.0	3.0	2.0	N/A	N/A
7". Cyclosporin A + Cyclophosphamide (endpoint)	0.0	1.0	1.0	2.0	3.0	N/A	N/A
8". BB5.1 + Cyclosporin A + Cyclophosphamide (post-operative day 3)	0.0	0.0	1.0	0.0	0.0	N/A	N/A
8. BB5.1 + Cyclosporin A + Cyclophosphamide (post-operative day 12)	0.0	0.0	1.0	0.0	0.0	N/A	N/A
8". BB5.1 + Cyclosporin A + Cyclophosphamide (post-operative day 60)	0.0	0.0	1.0	0.0	0.0	N/A	N/A

Samsung Bioepis Exhibit No. 1002
Page 1841

(continued)

Median Scores of Histological Changes of Heart Allografts at Necropsy							
Groups	Vasc*	Infar	Lymph	Throm	Hemo	Fibrin	PMN
8". BB5.1 + Cyclosporin A + Cyclophosphamide (post-operative day 100)	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Median scores: 0 - normal; 1- minimum change; 2 - mild change; 3 - moderate change; 4 - marked change. N/A - not available. *Vasc - vasculitis; Infar - infarction; Lymph - lymphocyte infiltration; Throm - thrombosis; Hemo - hemorrhage; Fibrin - fibrin deposition; PMN - polymorphonuclear cell infiltrate							

[0109] The results shown in Table 5 indicate a difference between the presensitized mouse model and the nonpresensitized mouse model as used in Example 3. The results indicate that in the absence of presensitization, grafts are rejected in approximately 8 days in the absence of treatment with any drugs. Presensitizing the animals causes a more rapid rejection, the rejection of the graft in the presensitized animals being in approximately 3 days in the absence of any drug treatment. Treatment with either BB5.1, cyclosporin A or cyclophosphamide had no effect upon graft survival, with the grafts being rejected in approximately 3-4 days in each of these groups of animals. The combination of BB5.1 and cyclosporin A showed some effect with rejection occurring about day 12. The combination of BB5.1 and cyclophosphamide had no protective effect with rejection occurring about day 3. Similarly the combination of cyclosporin A and cyclophosphamide had essentially no protective effect with rejection occurring at 3-4 days. Very surprisingly, the combination of all three drugs (chronic administration of BB5.1 and cyclosporin plus administration of cyclophosphamide at the time of transplant) showed a highly synergistic effect with all of the mice surviving for more than 100 days. Again, a survival of 100 days in this model is considered to be the gold standard and assumes an indefinite survival.

[0110] These results as well as the histological results as shown in Table 6 indicate that the combination of chronic treatment with a complement inhibitor and an immunosuppressant such as cyclosporin A in treating a presensitized mouse results in some attenuation of accelerated rejection. Treatment of these animals additionally with cyclophosphamide at the time of transplant and on the first day after transplant results in a much greater time of survival, no rejection having been seen by at least day 100.

[0111] It will be appreciated that the methods and compositions of the instant disclosure can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent to the artisan that other embodiments exist and do not depart from the spirit of the disclosure. Thus, the described embodiments are illustrative and should not be construed as restrictive.

LIST OF REFERENCES

[0112] The publications and other materials used herein to illuminate the background of the disclosure, and in particular, cases to provide additional details respecting the practice, are incorporated herein by reference in their entirety, and for convenience, are referenced by author and date in the text and respectively grouped in the following List of References.

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U.S. Patent 6,534,058

U.S. Patent 6,821,515

[0113] The contents of all references cited herein are incorporated by reference in their entirety.

[0114] The invention furthermore comprises the following items:

1. A method to prolong survival of an MHC mismatched allograft in a recipient mammal, said method comprising administering to said mammal a) a drug which inhibits complement activity and b) at least one immunosuppressive drug, wherein said drug which inhibits complement activity is administered chronically.

2. The method of item 1 wherein said mammal is a human.

3. The method of item 2 wherein said MHC mismatched allograft is an HLA mismatched allograft.

4. The method of item 1 wherein said drug which inhibits complement activity inhibits the formation of terminal complement or C5a.

5. The method of item 4 wherein said drug which inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment.

6. The method of item 5 wherein said whole antibody or antibody fragment is a human, humanized, chimerized or deimmunized antibody or antibody fragment.

7. The method of item 5 wherein said whole antibody or antibody fragment inhibits cleavage of complement C5.

8. The method of item 5 wherein said antibody fragment is selected from the group consisting of an Fab, an $F(ab')_2$, an Fv, a domain antibody, and a single-chain antibody.

Samsung Bioepis Exhibit No. 1002
Page 1845

9. The method of item 5 wherein said antibody fragment is pexelizumab.

10. The method of item 5 wherein said whole antibody is eculizumab.

11. The method item 10 wherein said eculizumab is administered once every 2 weeks.

12. The method of item 1 wherein said inhibitor of complement activity is selected from the group consisting of a i) soluble complement receptor, ii) CD59, iii) CD55, iv) CD46, and v) an antibody to C5, C6, C7, C8, or C9.

13. The method of item 1 wherein said immunosuppressive drug inhibits T-cell activity or B-cell activity.

14. The method of item 1 wherein said immunosuppressive drug inhibits T-cell activity and B-cell activity.

15. The method of item 1 wherein said immunosuppressive drug is selected from the group consisting of cyclosporin A, tacrolimus, sirolimus, OKT3, a corticosteroid, daclizumab, basiliximab, azathioprene, mycophenolate mofetil, methotrexate, 6-mercaptopurine, anti-T cell antibodies, cyclophosphamide, leflunamide, brequinar, ATG, ALG, 15-deoxyspergualin, and bredinin.

16. The method of item 1 wherein more than one immunosuppressive drug is administered.

17. The method of item 1 wherein said method comprises administering i) a drug which inhibits complement activity and ii) cyclosporin A.

18. The method of item 17 wherein said drug which inhibits complement activity is an antibody which inhibits cleavage of complement C5.

19. The method of item 1 wherein said allograft is selected from the group consisting of i) heart, ii) kidney, iii) lung, iv) pancreas, v) liver, vi) vascular tissue, vii) eye, viii) cornea, ix) lens, x) skin, xi) bone marrow, xii) muscle, xiii) connective tissue, xiv) gastrointestinal tissue, xv) nervous tissue, xvi) bone, xvii) stem cells, xviii) islets, xix) cartilage, xx) hepatocytes, and xxi) hematopoietic cells.

20. The method of item 1 wherein said allograft survives for a time at least 20% longer than would occur if said method were to be performed without said drug which inhibits complement activity.

21. The method of item 1 wherein said allograft survives for a time at least 40% longer than would occur if said method were to be performed without said drug which inhibits complement activity.

22. The method of item 1 wherein said allograft survives for the remaining life-time of said mammal.

23. The method of item 2 wherein said allograft survives for at least six months.

24. The method of item 2 wherein said allograft survives for at least one year.

25. The method of item 2 wherein said allograft survives for at least five years.

26. The method of item 2 wherein said allograft survives for the remaining life-time of said human.

27. The method of item 1 wherein said drug which inhibits complement activity is administered chronically for at least 14 days.

28. The method of item 1 wherein said drug which inhibits complement activity is administered chronically for at least 28 days.

29. The method of item 1 wherein said drug which inhibits complement activity is administered chronically for at least 3 months.

30. The method of item 1 wherein said drug which inhibits complement activity is administered chronically for at least 6 months.

Samsung Bioepis Exhibit No. 1002
Page 1846

31. The method of item 1 wherein said drug which inhibits complement activity is administered chronically for at least 1 year.

32. The method of item 1 wherein said drug which inhibits complement activity is administered chronically for at least 5 years.

33. The method of item 1 wherein said drug which inhibits complement activity is administered chronically for the remaining life-time of said mammal.

34. The method of item 1 wherein at least one immunosuppressive drug is administered chronically for the remaining life-time of said mammal.

35. The method of item 34 wherein at least cyclosporin A is administered chronically for the remaining life-time of said mammal.

36. A method to prolong survival of an allograft in a recipient mammal presensitized to said allograft, said method comprising administering to said mammal a) a drug which inhibits complement activity and b) at least one immunosuppressive drug, wherein said drug which inhibits complement activity is administered chronically.

37. The method of item 36 wherein said mammal is a human.

38. The method of item 36 wherein said drug which inhibits complement activity inhibits the formation of terminal complement or C5a.

39. The method of item 38 wherein said drug that inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment.

40. The method of item 39 wherein said whole antibody or antibody fragment is a human, humanized, chimerized or deimmunized antibody or antibody fragment.

41. The method of item 39 wherein said whole antibody or antibody fragment inhibits cleavage of complement C5.

42. The method of item 39 wherein said antibody fragment is selected from the group consisting of an Fab, an $F(ab')_2$, an Fv, a domain antibody, and a single-chain antibody.

43. The method of item 39 wherein said antibody fragment is pexelizumab.

44. The method of item 39 wherein said whole antibody is eculizumab.

45. The method item 44 wherein said eculizumab is administered once every 2 weeks.

46. The method of item 36 wherein said inhibitor of complement activity is selected from the group consisting of a i) soluble complement receptor, ii) CD59, iii) CD55, iv) CD46, and v) an antibody to C5, C6, C7, C8, or C9.

47. The method of item 36 wherein said immunosuppressive drug inhibits T-cell activity or B-cell activity.

48. The method of item 36 wherein said immunosuppressive drug inhibits T-cell activity and B-cell activity.

49. The method of item 36 wherein said immunosuppressive drug is selected from the group consisting of cyclosporin A, tacrolimus, sirolimus, OKT3, a corticosteroid, daclizumab, basiliximab, azathioprene, mycophenolate mofetil, methotrexate, 6-mercaptopurine, anti-T cell antibodies, cyclophosphamide, leflunamide, brequinar, ATG, ALG, 15-deoxyspergualin, and bredinin.

50. The method of item 36 wherein more than one immunosuppressive drug is administered.

51. The method of item 36 wherein said method comprises administering i) a drug which inhibits complement activity and ii) cyclosporin A.

Samsung Bioepis Exhibit No. 1002
Page 1847

52. The method of item 51 wherein said method comprises administering i) a drug which inhibits complement activity, ii) cyclosporin A, and iii) cyclophosphamide.

53. The method of item 51 wherein said drug which inhibits complement activity is an antibody which inhibits cleavage of complement C5.

54. The method of item 36 wherein said allograft is selected from the group consisting of i) heart, ii) kidney, iii) lung, iv) pancreas, v) liver, vi) vascular tissue, vii) eye, viii) cornea, ix) lens, x) skin, xi) bone marrow, xii) muscle, xiii) connective tissue, xiv) gastrointestinal tissue, xv) nervous tissue, xvi) bone, xvii) stem cells, xviii) islets, xix) cartilage, xx) hepatocytes, and xxi) hematopoietic cells.

55. The method of item 36 wherein said allograft survives for a time at least 20% longer than would occur if said method were to be performed without said drug which inhibits complement activity.

56. The method of item 36 wherein said allograft survives for a time at least 40% longer than would occur if said method were to be performed without said drug which inhibits complement activity.

57. The method of item 36 wherein said allograft survives for the remaining life-time of said mammal.

58. The method of item 37 wherein said allograft survives for at least six months.

59. The method of item 37 wherein said allograft survives for at least one year.

60. The method of item 37 wherein said allograft survives for at least five years.

61. The method of item 37 wherein said allograft survives for the remaining life-time of said human.

62. The method of item 36 wherein said drug which inhibits complement activity is administered chronically for at least 14 days.

63. The method of item 36 wherein said drug which inhibits complement activity is administered chronically for at least 28 days.

64. The method of item 36 wherein said drug which inhibits complement activity is administered chronically for at least 3 months.

65. The method of item 36 wherein said drug which inhibits complement activity is administered chronically for at least 6 months.

66. The method of item 36 wherein said drug which inhibits complement activity is administered chronically for at least 1 year.

67. The method of item 36 wherein said drug which inhibits complement activity is administered chronically for at least 5 years.

68. The method of item 36 wherein said drug which inhibits complement activity is administered chronically for the remaining life-time of said mammal.

69. The method of item 36 wherein at least one immunosuppressive drug is administered chronically for the remaining life-time of said mammal.

70. The method of item 69 wherein at least cyclosporin A is administered chronically for the remaining life-time of said mammal.

71. A method to prolong survival of an allograft in a recipient mammal which is an ABO mismatch to said allograft, said method comprising administering to said mammal a) a drug which inhibits complement activity and b) at least one immunosuppressive drug, wherein said drug which inhibits complement activity is administered chronically.

Samsung Bioepis Exhibit No. 1002
Page 1848

72. The method of item 71 wherein said mammal is a human.

73. The method of item 71 wherein said drug which inhibits complement activity inhibits the formation of terminal complement or C5a.

74. The method of item 73 wherein said drug which inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment.

75. The method of item 74 wherein said whole antibody or antibody fragment is a human, humanized, chimerized or deimmunized antibody or antibody fragment.

76. The method of item 74 wherein said whole antibody or antibody fragment inhibits cleavage of complement C5.

77. The method of item 74 wherein said antibody fragment is selected from the group consisting of an Fab, an $F(ab')_2$, an Fv, a domain antibody, and a single-chain antibody.

78. The method of item 74 wherein said antibody fragment is pexelizumab.

79. The method of item 74 wherein said whole antibody is eculizumab.

80. The method item 79 wherein said eculizumab is administered once every 2 weeks.

81. The method of item 71 wherein said inhibitor of complement activity is selected from the group consisting of a i) soluble complement receptor, ii) CD59, iii) CD55, iv) CD46, and v) an antibody to C5, C6, C7, C8, or C9.

82. The method of item 71 wherein said immunosuppressive drug inhibits T-cell activity or B-cell activity.

83. The method of item 71 wherein said immunosuppressive drug inhibits T-cell activity and B-cell activity.

84. The method of item 71 wherein said immunosuppressive drug is selected from the group consisting of cyclosporin A, tacrolimus, sirolimus, OKT3, a corticosteroid, daclizumab, basiliximab, azathioprene, mycophenolate mofetil, methotrexate, 6-mercaptopurine, anti-T cell antibodies, cyclophosphamide, leflunamide, brequinar, ATG, ALG, 15-deoxyspergualin, and bredinin.

85. The method of item 71 wherein more than one immunosuppressive drug is administered.

86. The method of item 71 wherein said method comprises administering i) a drug which inhibits complement activity and ii) cyclosporin A.

87. The method of item 86 wherein said method comprises administering i) a drug which inhibits complement activity, ii) cyclosporin A, and iii) cyclophosphamide.

88. The method of item 86 wherein said drug that inhibits complement activity is an antibody that inhibits cleavage of complement C5.

89. The method of item 71 wherein said allograft is selected from the group consisting of i) heart, ii) kidney, iii) lung, iv) pancreas, v) liver, vi) vascular tissue, vii) eye, viii) cornea, ix) lens, x) skin, xi) bone marrow, xii) muscle, xiii) connective tissue, xiv) gastrointestinal tissue, xv) nervous tissue, xvi) bone, xvii) stem cells, xviii) islets, xix) cartilage, xx) hepatocytes, and xxi) hematopoietic cells.

90. The method of item 71 wherein said allograft survives for a time at least 20% longer than would occur if said method were to be performed without said drug that inhibits complement activity.

91. The method of item 71 wherein said allograft survives for a time at least 40% longer than would occur if said method were to be performed without said drug that inhibits complement activity.

92. The method of item 71 wherein said allograft survives for the remaining life-time of said mammal.

Samsung Bioepis Exhibit No. 1002
Page 1849

93. The method of item 72 wherein said allograft survives for at least six months.

94. The method of item 72 wherein said allograft survives for at least one year.

95. The method of item 72 wherein said allograft survives for at least five years.

96. The method of item 72 wherein said allograft survives for the remaining life-time of said human.

97. The method of item 71 wherein said drug which inhibits complement activity is administered chronically for at least 14 days.

98. The method of item 71 wherein said drug which inhibits complement activity is administered chronically for at least 28 days.

99. The method of item 71 wherein said drug which inhibits complement activity is administered chronically for at least 3 months.

100. The method of item 71 wherein said drug which inhibits complement activity is administered chronically for at least 6 months.

101. The method of item 71 wherein said drug which inhibits complement activity is administered chronically for at least 1 year.

102. The method of item 71 wherein said drug which inhibits complement activity is administered chronically for at least 5 years.

103. The method of item 71 wherein said drug which inhibits complement activity is administered chronically for the remaining life-time of said mammal.

104. The method of item 71 wherein at least one immunosuppressive drug is administered chronically for the remaining life-time of said mammal.

105. The method of item 104 wherein at least cyclosporin A is administered chronically for the remaining life-time of said mammal.

106. Use of a drug that inhibits complement activity and an immunosuppressive drug in the manufacture of a medicament or medicament package for prolong survival of an allograft in a mammal.

107. The use of item 106, wherein more than one immunosuppressive drug is included in said medicament or medicament package.

108. The use of item 106, wherein said drug that inhibits complement activity and said immunosuppressive drug are in a formulation suitable for concurrent administration to said mammal.

109. The use of item 106, wherein said drug that inhibits complement activity and said immunosuppressive drug are in a formulation or formulations suitable for sequential administration to said mammal.

110. The use of item 106, wherein said drug that inhibits complement activity is in a formulation suitable for chronic administration to said mammal.

111. The use of item 106, wherein said immunosuppressive drug is in a formulation suitable for chronic administration to said mammal.

112. A method to prolong survival of allograft in a recipient mammal, comprising preparing an allograft from a first allograft accommodated by a first recipient mammal, wherein said first recipient mammal, after receiving said first allograft, has been treated with a drug that inhibits complement activity and at least one immunosuppressive agent.

113. An allograft that has prolonged survival in a recipient mammal, wherein said allograft is prepared from a first

Samsung Bioepis Exhibit No. 1002
Page 1850

recipient mammal and was transplanted to the first recipient mammal that has been treated with a drug that inhibits complement activity and at least one immunosuppressive agent.

114. A pharmaceutical package comprising a drug that inhibits complement activity and at least one immunosuppressive agent, wherein said drug and said agent are formulated for chronic administration.

115. The pharmaceutical package of item 114, wherein said drug is an antibody in a lyophilized formulation comprising the antibody and a lyoprotectant.

116. The pharmaceutical package of item 114, wherein said agent is in a lyophilized formulation comprising the agent and a lyoprotectant.

117. The pharmaceutical package of item 114, wherein said drug and said agent are in the same lyophilized formulation comprising said drug, said agent, and a lyoprotectant.

118. The pharmaceutical package of item 114, wherein said drug is in an injection system comprising a syringe that comprises a cartridge, wherein said cartridge contains said drug in a formulation suitable for injection.

119. The pharmaceutical package of item 114, wherein said agent is in an injection system comprising a syringe that comprises a cartridge, wherein said cartridge contains said agent in a formulation suitable for injection.

120. The pharmaceutical package of item 114, wherein said drug and said agent are in an injection system comprising a syringe that comprises a cartridge, wherein said cartridge contains said drug and said agent in a formulation suitable for injection.

121. The pharmaceutical package of item 114, wherein said drug is present in unit dosage form.

122. The pharmaceutical package of item 114, wherein said agent is present in unit dosage form.

123. A lyophilized antibody formulation comprising an antibody that inhibits complement activity and a lyoprotectant, wherein said antibody formulation is suitable for chronic administration.

124. The lyophilized antibody formulation of item 123, wherein the antibody inhibits the formation of terminal complement or C5a.

125. The lyophilized antibody formulation of item 123, wherein the antibody is a whole antibody or an antibody fragment.

126. The lyophilized antibody formulation of item 125, wherein the antibody wherein said whole antibody or antibody fragment is a human, humanized, chimerized or deimmunized antibody or antibody fragment.

127. The lyophilized antibody formulation of item 125, wherein said whole antibody or antibody fragment inhibits cleavage of complement C5.

128. The lyophilized antibody formulation of item 125, wherein said antibody fragment is selected from the group consisting of an Fab, an $F(ab')_2$, an Fv, a domain antibody, and a single-chain antibody.

129. The lyophilized antibody formulation of item 125, wherein said antibody fragment is pexelizumab.

130. The lyophilized antibody formulation of item 125, wherein said whole antibody is eculizumab.

131. An injection system comprising a syringe, wherein said syringe comprises a cartridge containing an antibody that inhibits complement activity and is formulated suitable for injection.

Claims

1. A composition for use in prolonging survival of an allograft organ in a human recipient, wherein the composition

Samsung Bioepis Exhibit No. 1002
Page 1851

comprises an inhibitor of human complement component C5, and wherein the inhibitor is to be administered to the human recipient in conjunction with at least one immunosuppressive drug.

2. The composition according to claim 1, wherein the inhibitor inhibits cleavage of C5 into fragments C5a and C5b

3. The composition according to claim 2, wherein the inhibitor is a whole antibody or an antigen-binding fragment thereof that binds to C5.

4. The composition according to claim 3, wherein the whole antibody or antigen-binding fragment thereof is a human, humanized, chimerized, or de-immunized antibody or antigen-binding fragment thereof.

5. The composition according to claim 3, wherein the antigen-binding fragment is selected from the group consisting of an Fab, an $F(ab')_2$, an Fv, and a single chain antibody.

6. The composition according to claim 3, wherein the whole antibody is eculizumab.

7. The composition according to claim 3, wherein the antigen-binding fragment is pexelizumab.

8. The composition according to any one of claims 1 to 7, wherein the allograft is selected from the group consisting of i) heart, ii) kidney, iii) lung, iv) pancreas, v) liver, vi) vascular tissue, vii) eye, viii) cornea, ix) lens, x) skin, xi) bone marrow, xii) muscle, xiii) connective tissue, xiv) gastrointestinal tissue, xv) nervous tissue, xvi) bone, xvii) stem cells, xviii) islets, xix) cartilage, xx) hepatocytes, and xxi) hematopoietic cells.

9. The composition according to any one of claims 1 to 8, wherein the at least one immunosuppressive drug is selected from the group consisting of cyclosporin A, tacrolimus, sirolimus, OKT3, a corticosteroid, daclizumab, basiliximab, azathioprene, mycophenolate mofetil, methotrexate, 6-mercaptopurine, anti-T cell antibodies, cyclophosphamide, leflunamide, brequinar, ATG, ALG, 15-deoxyspergualin, and bredinin.

10. The composition according to any one of claims 1 to 9, wherein the inhibitor is to be used in conjunction with at least two immunosuppressive drugs.

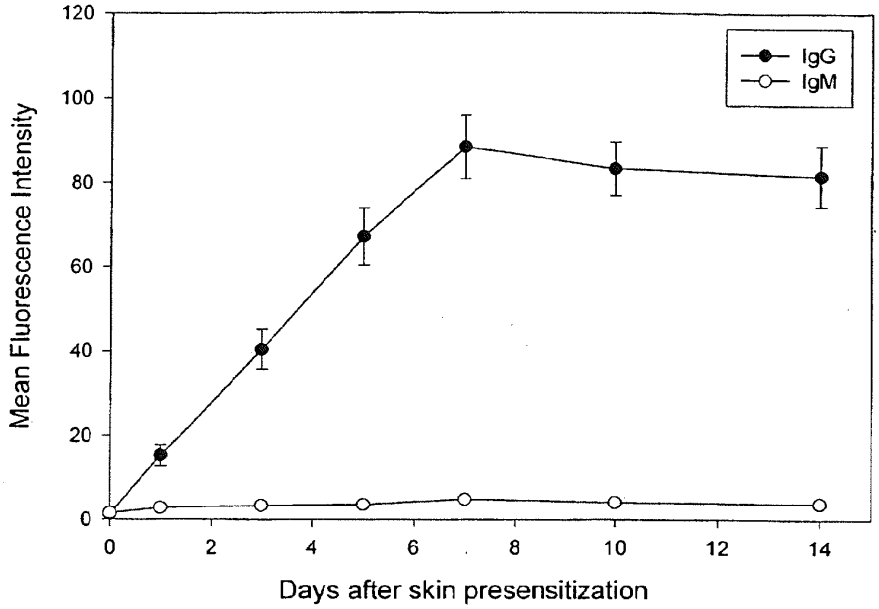
11. The composition according to any one of claims 1 to 10, wherein cyclosporin A is to be administered to the human recipient.

12. The composition according to any one of claims 1 to 11, wherein the human recipient is an MHC mismatch to the allograft, presensitized to the allograft, or an ABO mismatch to the allograft.

13. A composition for use in prolonging survival of an allograft organ in a human recipient, wherein the composition comprises an inhibitor of human complement component C5, and wherein the inhibitor is to be administered to the human recipient in conjunction with at least two immunosuppressive drugs and wherein the human recipient is an MHC mismatch to the allograft, presensitized to the allograft, or an ABO mismatch to the allograft.

Samsung Bioepis Exhibit No. 1002
Page 1852

A



B

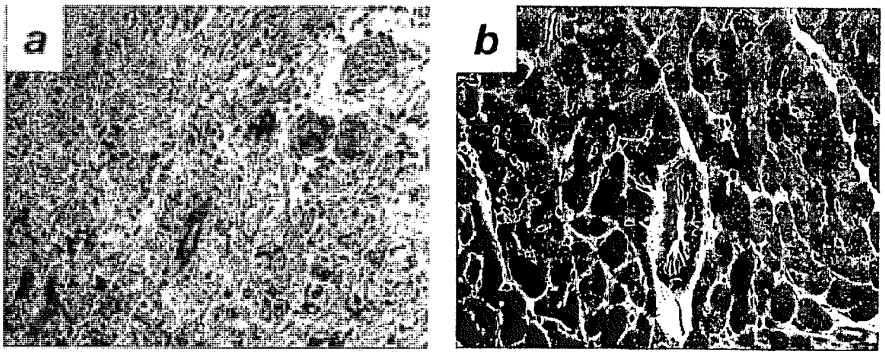


Figure 1A, B

Samsung Bioepis Exhibit No. 1002
Page 1853

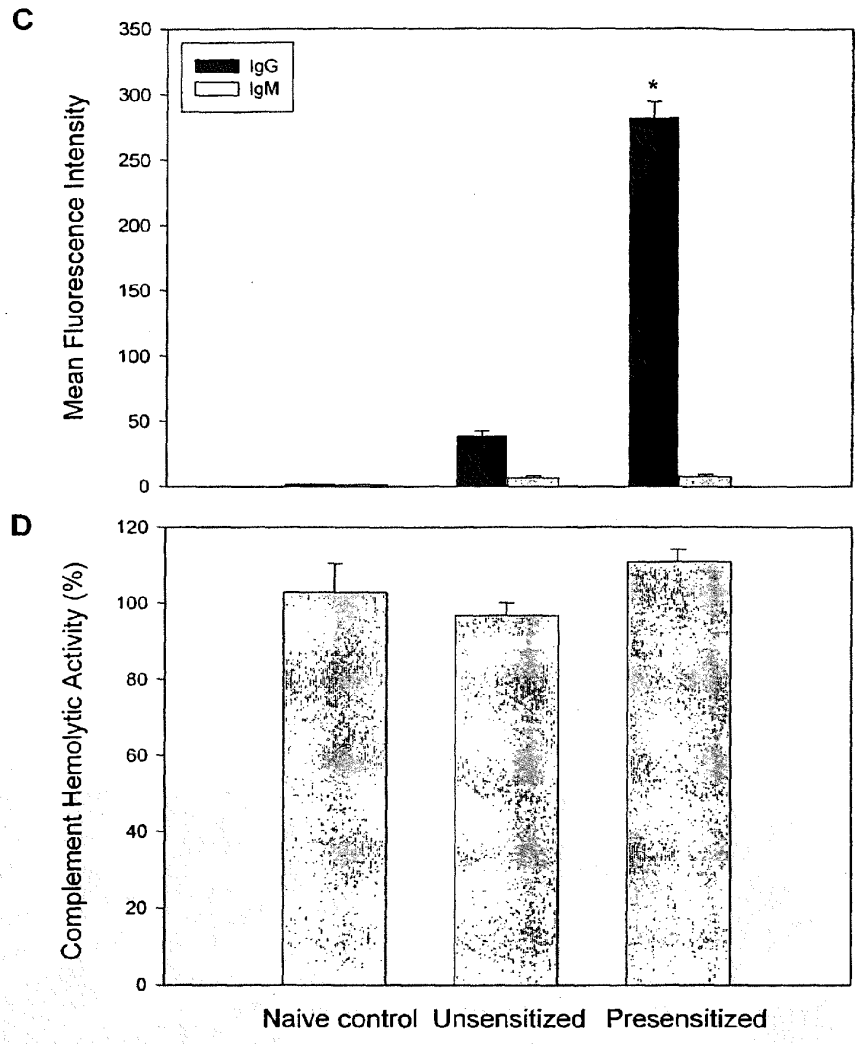


Figure 1C, D

Samsung Bioepis Exhibit No. 1002
Page 1854

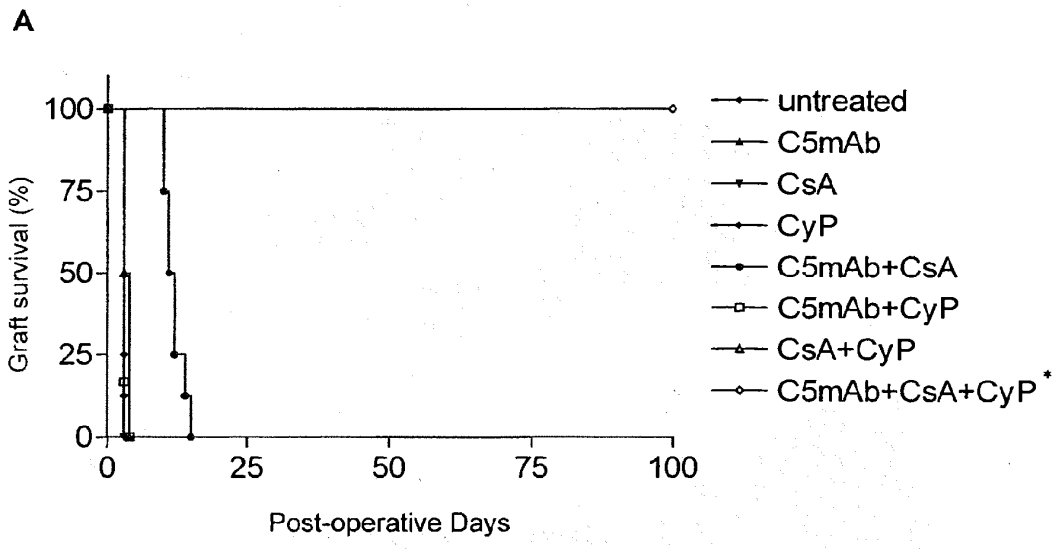


Figure 2A

Samsung Bioepis Exhibit No. 1002
Page 1855

B

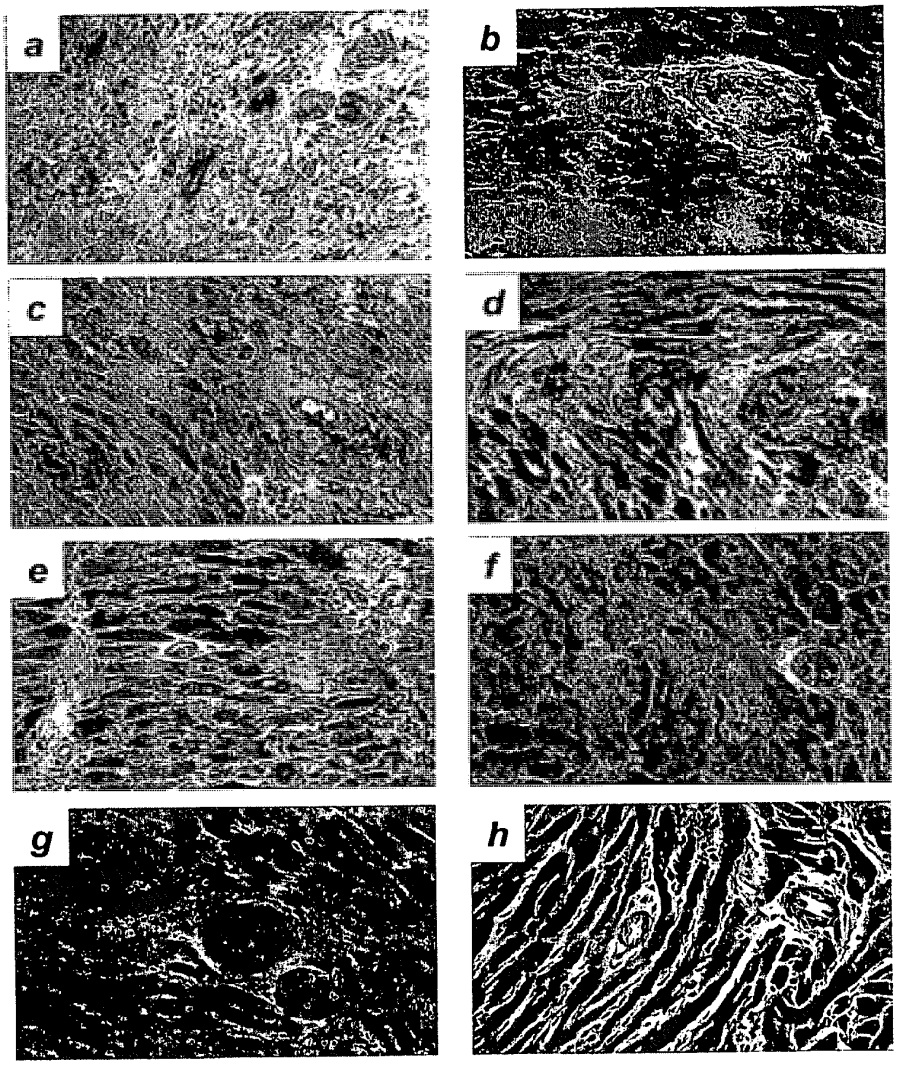


Figure 2B

Samsung Bioepis Exhibit No. 1002
Page 1856

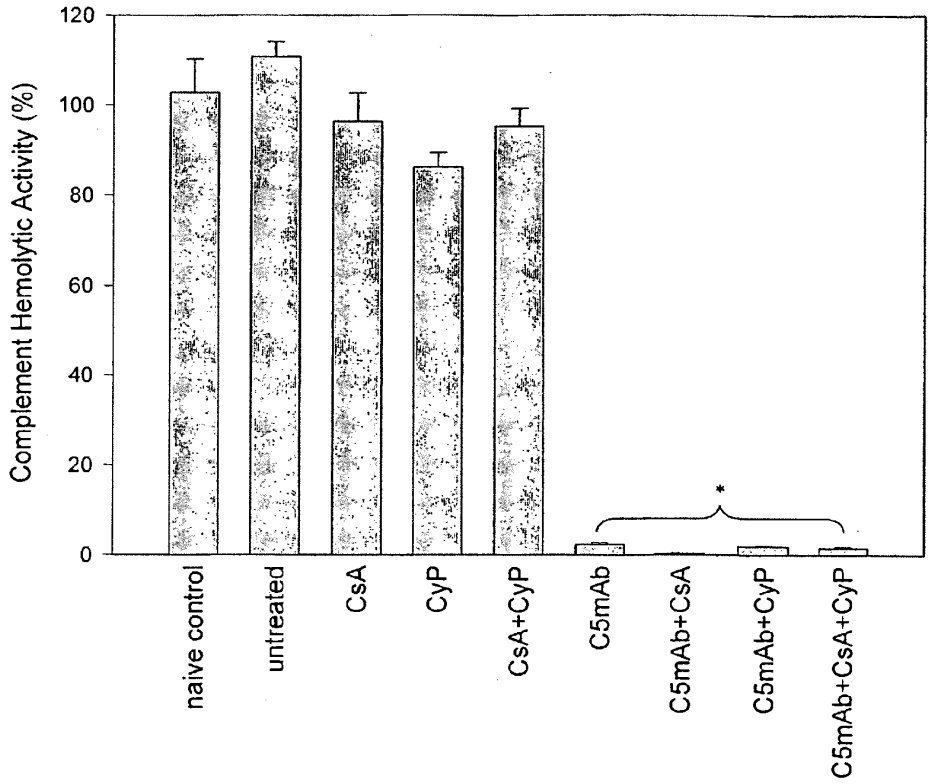


Figure 3

Samsung Bioepis Exhibit No. 1002
Page 1857

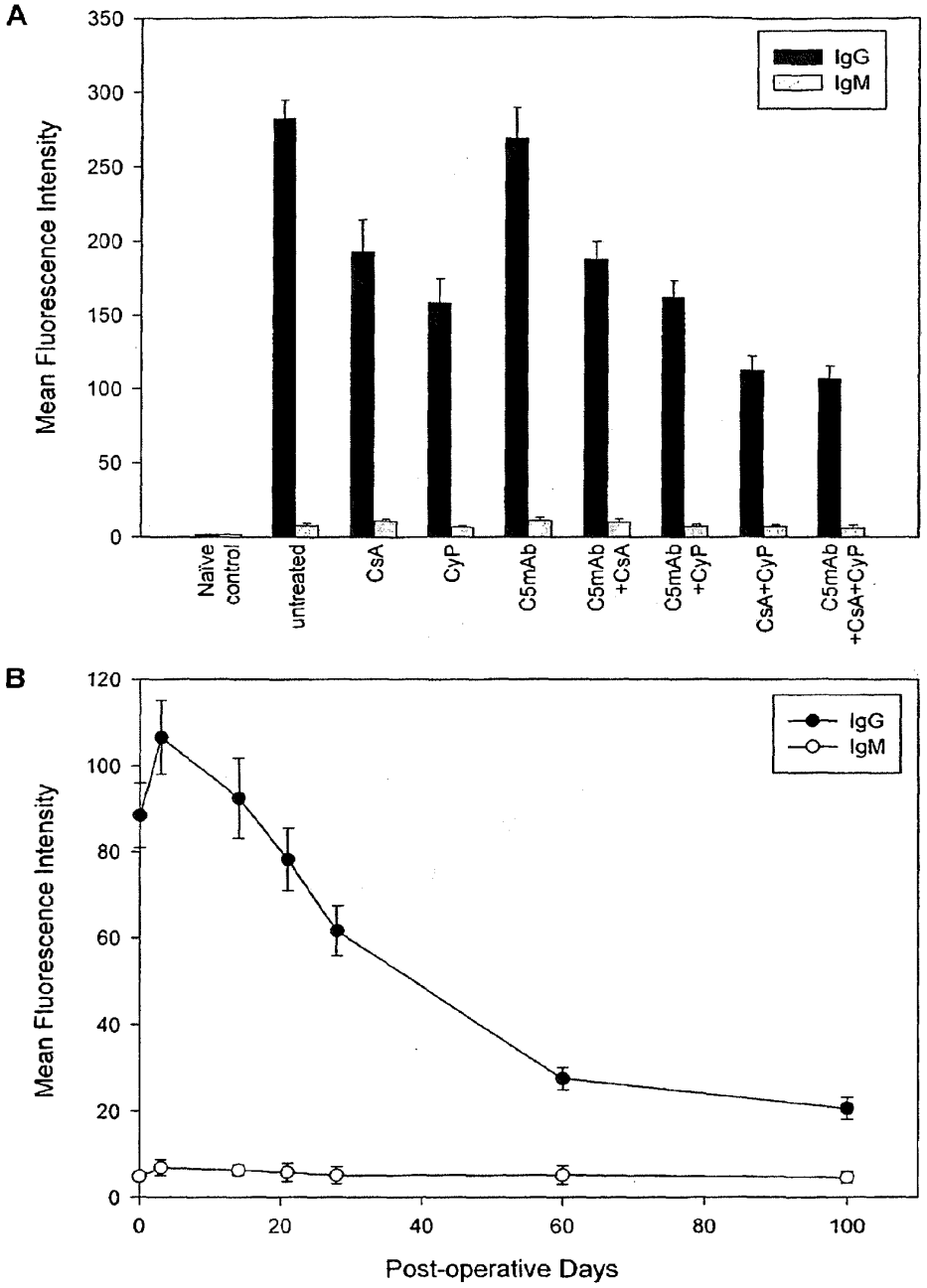
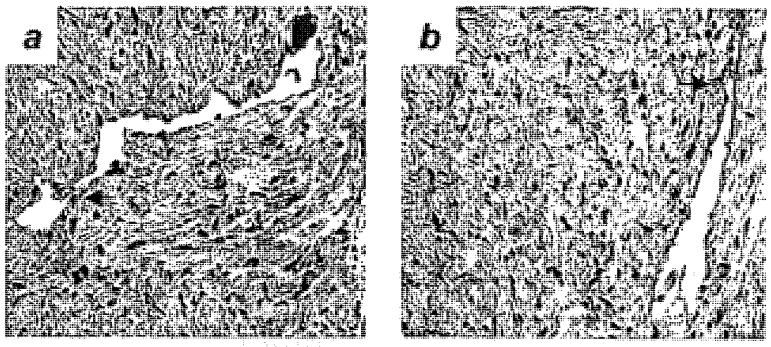


Figure 4A, B

Samsung Bioepis Exhibit No. 1002
Page 1858

C



D

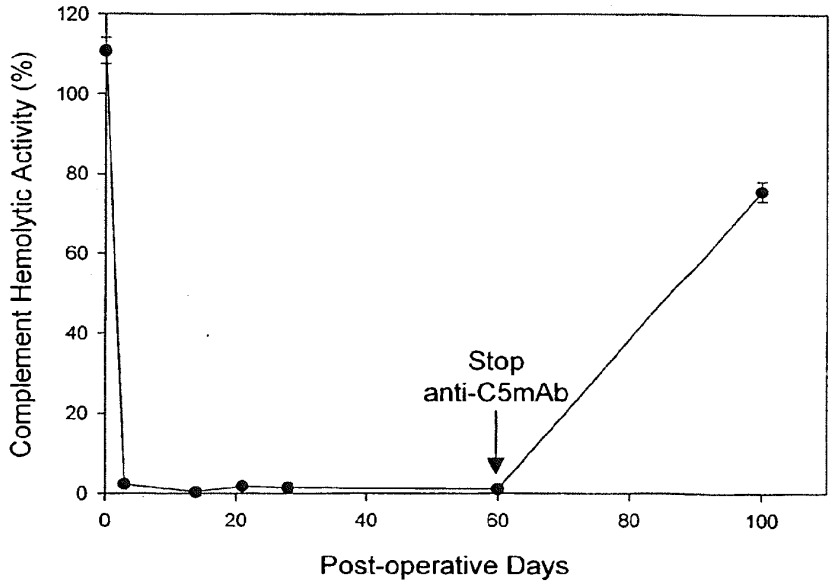


Figure 4C, D

Samsung Bioepis Exhibit No. 1002
Page 1859

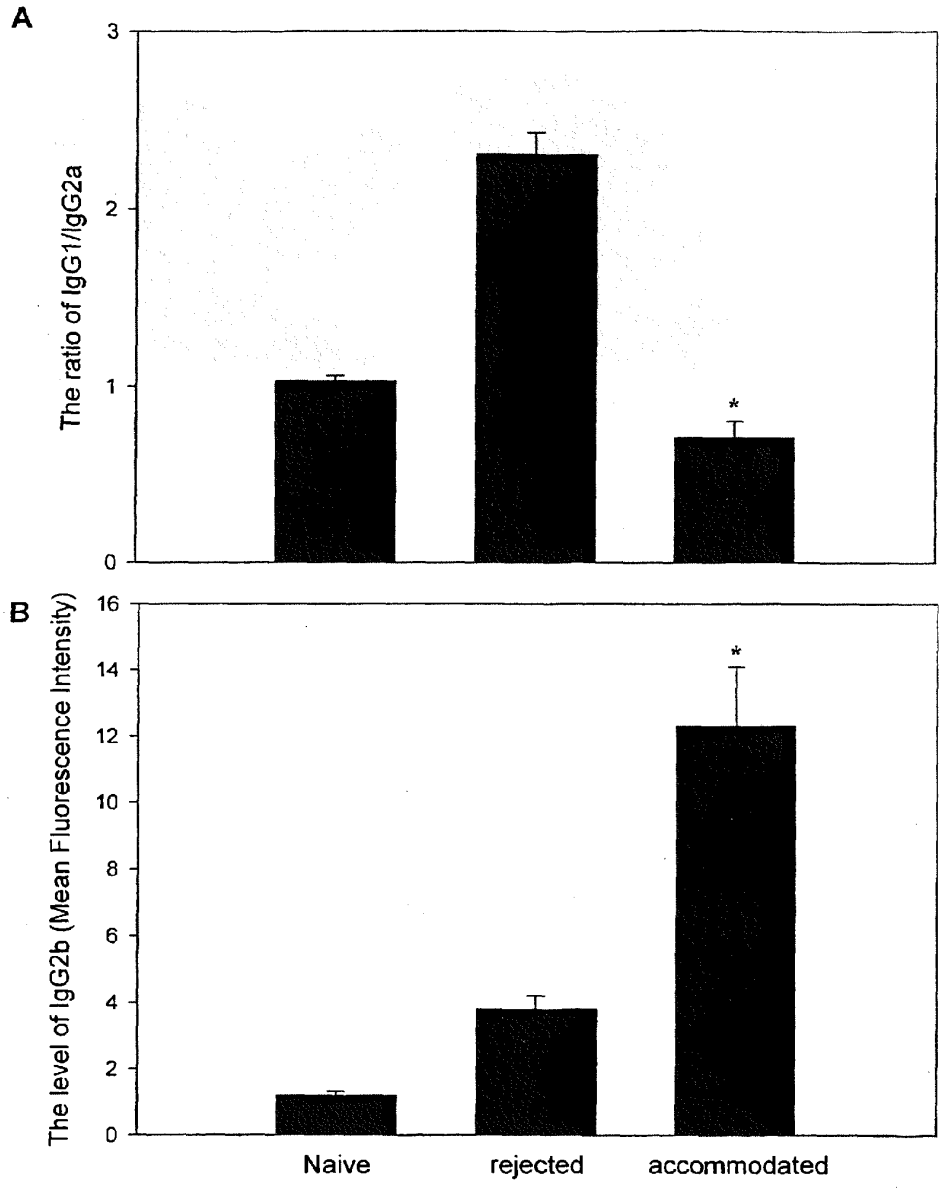


Figure 5A, B

Samsung Bioepis Exhibit No. 1002
Page 1860

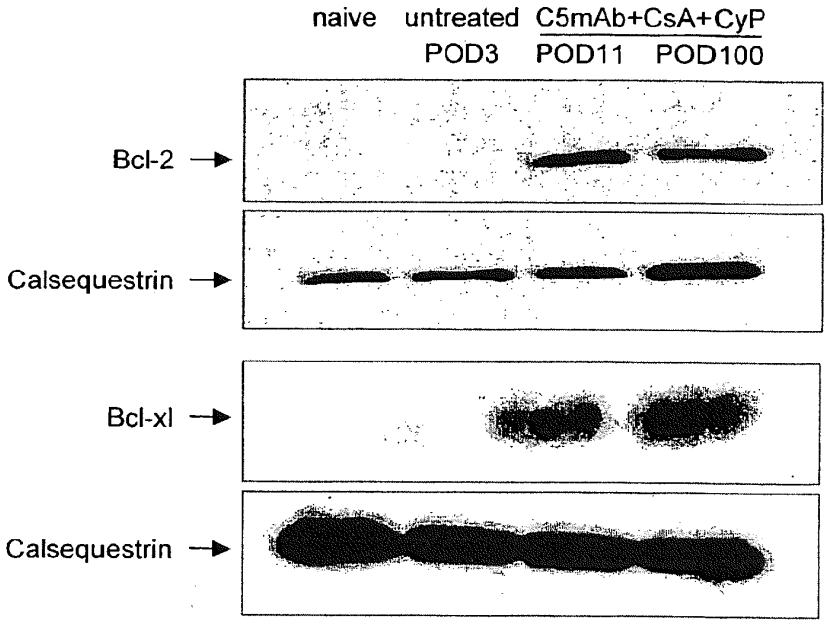


Figure 6

Samsung Bioepis Exhibit No. 1002
Page 1861

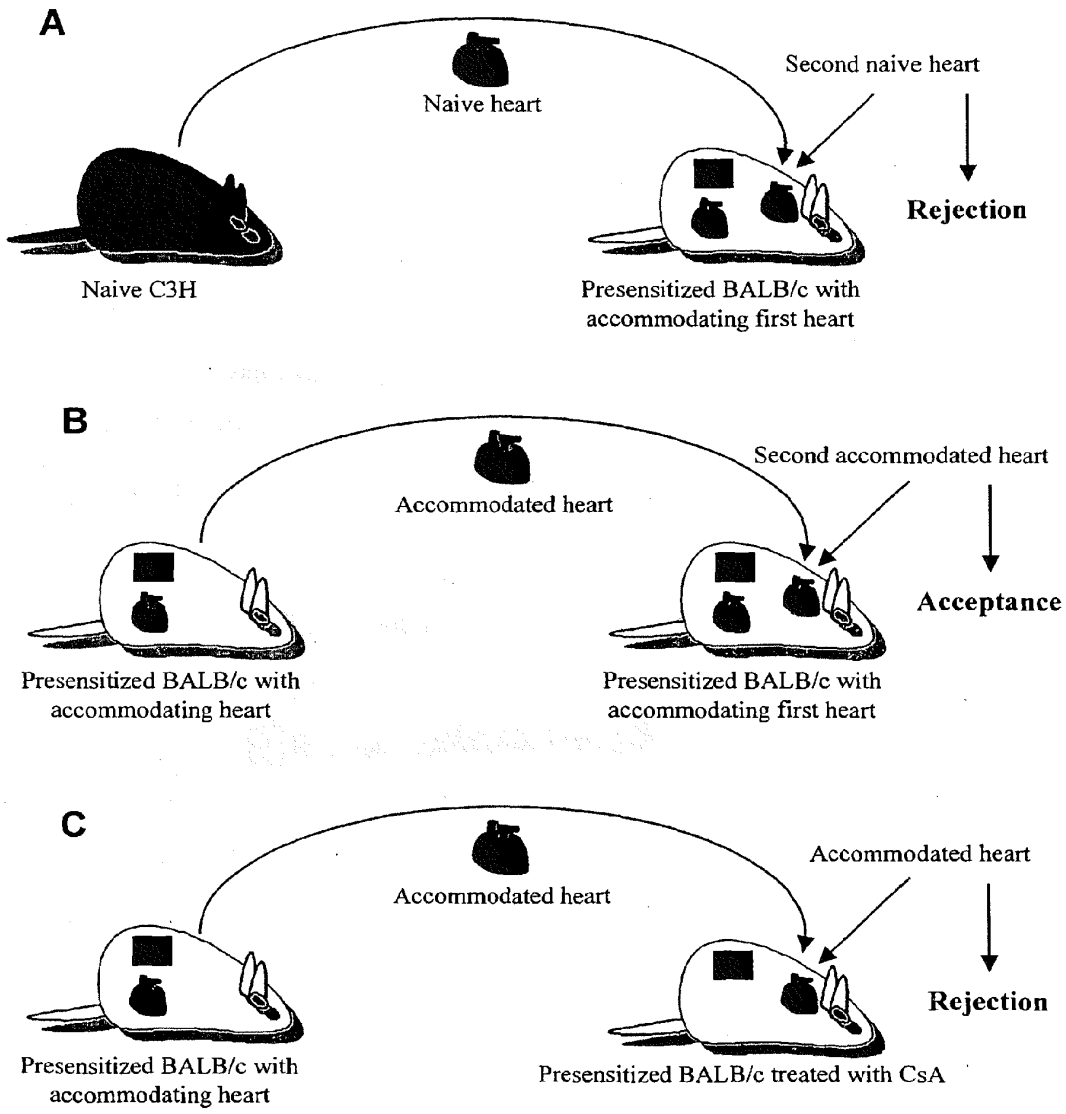


Figure 7

Samsung Bioepis Exhibit No. 1002
Page 1862

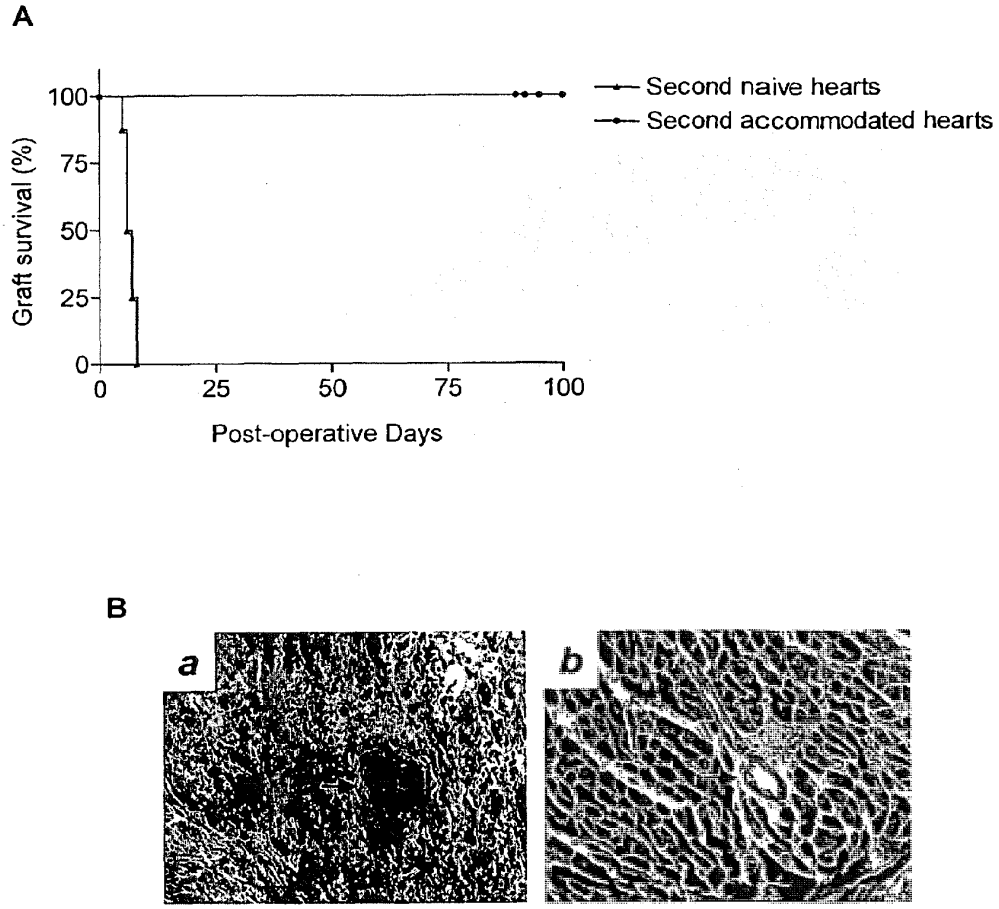


Figure 8

Samsung Bioepis Exhibit No. 1002
Page 1863

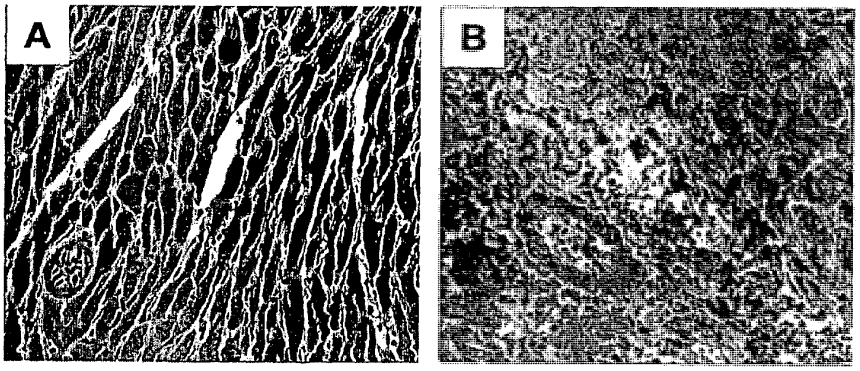


Figure 9

Samsung Bioepis Exhibit No. 1002
Page 1864

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EUROPEAN SEARCH REPORT

Application Number
EP 14 17 7646

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	4 November 2014	Rojo Romeo, Elena

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Samsung Bioepis Exhibit No. 1002
Page 1865

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EUROPEAN SEARCH REPORT

Application Number
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
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Samsung Bioepis Exhibit No. 1002
Page 1866

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Samsung Bioepis Exhibit No. 1002
Page 1867

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Samsung Bioepis Exhibit No. 1002
Page 1868

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Samsung Bioepis Exhibit No. 1002
Page 1869

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(71) Applicant: **ALEXION PHARMACEUTICALS, INC.**
Cheshire, CT 06410 (US)

(72) Inventors:
- **Bell, Leonard**
  Woodbridge, CT 06525 (US)
- **Rother, Russel P.**
  Oklahoma City, OK 73151 (US)

(74) Representative: **Vossius & Partner**
Patentanwälte Rechtsanwälte mbB
Siebertstrasse 3
81675 München (DE)

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(54) **Method of treating hemolytic disease**

(57) Paroxysmal nocturnal hemoglobinuria or other hemolytic diseases are treating using a compound which binds to or otherwise blocks the generation and/or the activity of one or more complement components, such as, for example, a complement-inhibiting antibody.

EP 2 910 253 A1

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Description

RELATED APPLICATIONS

[0001] This application claims priority to, and is a continuation-in-part of, U.S. Patent Application Serial No. 10/771,552, filed February 3, 2004, the entire disclosure of which is incorporated herein by this reference.

BACKGROUND

Technical Field

[0002] This disclosure relates to a method of treating a hemolytic disease such as, for example, paroxysmal nocturnal hemoglobinuria ("PNH"), by administering a compound which binds to, or otherwise blocks, the generation and/or activity of one or more complement components.

Background of Related Art

[0003] Paroxysmal nocturnal hemoglobinuria ("PNH") is an uncommon blood disorder wherein red blood cells are compromised and are thus destroyed more rapidly than normal red blood cells. PNH results from a mutation of bone marrow cells resulting in the generation of abnormal blood cells. More specifically, PNH is believed to be a disorder of hematopoietic stem cells, which give rise to distinct populations of mature blood cells. The basis of the disease appears to be somatic mutations leading to the inability to synthesize the glycosyl-phosphatidylinositol ("GPI") anchor that is responsible for binding proteins to cell membranes. The mutated gene, PIG-A (phosphatidylinositol glycan class A) resides in the X chromosome and can have several different mutations, varying from deletions to point mutations.

[0004] PNH causes a sensitivity to complement proteins and this sensitivity occurs in the cell membrane. PNH cells are deficient in a number of proteins, particularly essential complement-regulating surface proteins. These complement-regulating surface proteins include the decay-accelerating factor ("DAF") or CD55 and membrane inhibitor of reactive lysis ("MIRL") or CD59.

[0005] PNH is characterized by hemolytic anemia (a decreased number of red blood cells), hemoglobinuria (the presence of hemoglobin in the urine particularly evident after sleeping), and hemoglobinemia (the presence of hemoglobin in the bloodstream). PNH-afflicted individuals are known to have paroxysms, which are defined here as incidences of dark-colored urine. Hemolytic anemia is due to intravascular destruction of red blood cells by complement components. Other known symptoms include dysphagia, fatigue, erectile dysfunction, thrombosis and recurrent abdominal pain.

[0006] Hemolysis resulting from hemolytic diseases causes local and systemic nitric oxide (NO) deficiency through the release of free hemoglobin. Free hemoglobin is a very efficient scavenger of NO, due in part to the accessibility of NO in the non-erythrocyte compartment and a 10^6 times greater affinity of the heme moiety for NO than that for oxygen. The occurrence of intravascular hemolysis often generates sufficient free hemoglobin to completely deplete haptoglobin. Once the capacity of this hemoglobin scavenging protein is exceeded, consumption of endogenous NO ensues. For example, in a setting of intravascular hemolysis such as PNH, where LDH levels can easily exceed 2 - 3 times their normal levels, free hemoglobin would likely obtain concentrations of 0.8 - 1.6 g/l. Since haptoglobin can only bind somewhere between 0.7 to 1.5 g/l of hemoglobin depending on the haptoglobin allotype, a large excess of free hemoglobin would be generated. Once the capacity of hemoglobin reabsorption by the kidney proximal tubules is exceeded, hemoglobinuria ensues. The release of free hemoglobin during intravascular hemolysis results in excessive consumption of NO with subsequent enhanced smooth muscle contraction, vasoconstriction and platelet activation and aggregation. PNH-related morbidities associated with NO scavenging by hemoglobin include abdominal pain, erectile dysfunction, esophageal spasm, and thrombosis.

[0007] The laboratory evaluation of hemolysis normally includes hematologic, serologic, and urine tests. Hematologic tests include an examination of the blood smear for morphologic abnormalities of RBCs (to determine causation), and the measurement of the reticulocyte count in whole blood (to determine bone marrow compensation for RBC loss). Serologic tests include lactate dehydrogenase (LDH; widely performed), and free hemoglobin (not widely performed) as a direct measure of hemolysis. LDH levels, in the absence of tissue damage in other organs, can be useful in the diagnosis and monitoring of patients with hemolysis. Other serologic tests include bilirubin or haptoglobin, as measures of breakdown products or scavenging reserve, respectively. Urine tests include bilirubin, hemosiderin, and free hemoglobin, and are generally used to measure gross severity of hemolysis and for differentiation of intravascular vs. extravascular etiologies of hemolysis rather than routine monitoring of hemolysis. Further, RBC numbers, RBC (i.e. cell-bound) hemoglobin, and hematocrit are generally performed to determine the extent of any accompanying anemia rather than as a measure of hemolytic activity per se.

[0008] Steroids have been employed as a therapy for hemolytic diseases and may be effective in suppressing hemolysis

Samsung Bioepis Exhibit No. 1002
Page 1871

in some patients, although long term use of steroid therapy carries many negative side effects. Afflicted patients may require blood transfusions, which carry risks of infection. Anticoagulation therapy may also be required to prevent blood clot formation. Bone marrow transplantation has been known to cure PNH, however, bone marrow matches are often very difficult to find and mortality rates are high with such procedure.

[0009] It would be advantageous to provide a treatment which safely and reliably eliminates and/or limits hemolytic diseases, such as PNH, and their effects.

Summary

[0010] Paroxysmal nocturnal hemoglobinuria ("PNH") and other hemolytic diseases are treated in accordance with this disclosure using a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Suitable compounds include, for example, antibodies which bind to or otherwise block the generation and/or activity of one or more complement components, such as, for example, an antibody specific to complement component C5. In particularly useful embodiments, the compound is an anti-C5 antibody selected from the group consisting of h5G1.1-mAb (eculizumab), h5G1.1-scFv (pexelizumab) and other functional fragments of h5G1.1. It has surprisingly been found that the present methods provide improvements in the PNH subject within 24 hours of administration of the compound. For example, hemolysis is significantly reduced within 24 hours of administration of the compound as indicated by resolution of hemoglobinuria.

[0011] The complement-inhibiting compound can be administered prophylactically in individuals known to have a hemolytic disease to prevent, or help prevent the onset of symptoms. Alternatively, the complement-inhibiting compound can be administered as a therapeutic regimen to an individual experiencing symptoms of a hemolytic disease.

[0012] In another aspect, a method of increasing the proportion of complement sensitive type III red blood cells and therefore the total red blood cell count in a patient afflicted with a hemolytic disease is contemplated. The method comprises administering a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components to a patient afflicted with a hemolytic disease. By increasing type III red blood cell count, symptoms such as fatigue and anemia also can be alleviated in a patient afflicted with a hemolytic disease.

[0013] In yet another aspect, the present disclosure contemplates a method of rendering a subject afflicted with a hemolytic disease less dependent on transfusions or transfusion-independent by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. It has surprisingly been found that patients can be rendered transfusion-independent in accordance with the present methods. Unexpectedly, transfusion-independence can be maintained in some embodiments for twelve months or more, long beyond the 120 day life cycle of red blood cells. In other embodiments, transfusion-independence can be maintained for two years or more. Treatment for six months or more is required for the evaluation of transfusion independence given the long half life of red blood cells.

[0014] In another aspect, the present disclosure contemplates a method of treating a nitric oxide (NO) imbalance in a subject by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of nitric oxide (NO). In particularly useful embodiments, NO homeostasis is restored wherein there is a resolution of symptoms attributable to NO deficiency.

[0015] In another aspect, the present disclosure contemplates a method of treating thrombosis in a subject by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

[0016] In another aspect, the present disclosure contemplates a method of treating fatigue in a subject afflicted with a hemolytic disease by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

[0017] In another aspect, the present disclosure contemplates a method of treating erectile dysfunction in a subject afflicted with a hemolytic disease by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

[0018] In another aspect, the present disclosure contemplates a method of treating abdominal pain in a subject afflicted with a hemolytic disease by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation

Samsung Bioepis Exhibit No. 1002
Page 1872

of one or more complement components and compounds which block the activity of one or more complement components.

[0019] In yet another aspect, the present disclosure contemplates a method of treating a subject afflicted with a hemolytic disease by administering: 1) one or more compounds known to increase hematopoiesis (for example, either by boosting production, eliminating stem cell destruction or eliminating stem cell inhibition) in combination with 2) a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. Suitable compounds known to increase hematopoiesis include, for example, steroids, immunosuppressants (such as, cyclosporin), anti-coagulants (such as, warfarin), folic acid, iron and the like, erythropoietin (EPO) and antithymocyte globulin (ATG), antilymphocyte globulin (ALG), EPO derivatives, and darbepoetin alfa (commercially available as Aranesp® from Amgen, Inc., Thousand Oaks, CA (Aranesp® is a man-made form of EPO produced in Chinese hamster ovary (CHO) cells by recombinant DNA technology)). In particularly useful embodiments, erythropoietin (EPO) (a compound known to increase hematopoiesis), EPO derivatives, or darbepoetin alfa may be administered in combination with an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1.

[0020] In yet another aspect, the present disclosure contemplates a method of treating one or more symptoms of hemolytic diseases in a subject where the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10% before or during treatment, by administering a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components, said compound being administered alone or in combination with one or more compounds known to increase hematopoiesis, such as EPO, EPO derivatives, or darbepoetin alfa.

[0021] In yet another aspect, the present disclosure contemplates a method of treating one or more symptoms of hemolytic diseases in a subject having a platelet count above 40,000 per microliter, by administering a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components, said compound being administered alone or in combination with one or more compounds known to increase hematopoiesis, such as EPO, EPO derivatives, or darbepoetin alfa.

[0022] In yet another aspect, the present disclosure contemplates a method of treating one or more symptoms of a hemolytic diseases in a subject having a reticulocyte count above 80 x 10^9 per liter, by administering a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components, said compound being administered alone or in combination with one or more compounds known to increase hematopoiesis, such as EPO, EPO derivatives, or darbepoetin alfa.

Brief Description of the Drawings

[0023]

Figure 1A reports biochemical parameters of hemolysis measured during treatment of PNH patients with an anti-C5 antibody.

Figure 1B graphically depicts the effect of treatment with an anti-C5 antibody on lactate dehydrogenase (LDH) levels.

Figure 2 shows a urine color scale devised to monitor the incidence of paroxysm of hemoglobinuria in PNH patients.

Figure 3 is a graph of the effects of eculizumab treatments on patient paroxysm rates, as compared to pre-treatment rates.

Figure 4 shows urine samples of PNH patients and measurements of hemoglobinuria, dysphagia, LDH, AST, pharmacokinetics (PK) and pharmacodynamics (PD) reflecting the immediate and positive effects of the present methods on hemolysis, symptoms and pharmacodynamics suitable to completely block complement.

Figure 5 graphically depicts the effect of anti-C5 antibody dosing schedule on hemoglobinuria over time.

Figures 6a and 6b are graphs comparing the number of transfusion units required per patient per month, prior to and during treatment with an anti-C5 antibody: Figure 6a depicts cytopenic patients; and Figure 6b depicts non-cytopenic patients.

Figure 7 shows the management of a thrombocytopenic patient by administering an anti-C5 antibody and erythropoietin (EPO).

Figure 8 graphically depicts the pharmacodynamics of an anti-C5 antibody.

Figure 9 is a chart of the results of European Organization for Research and Treatment of Cancer questionnaires ("EORTC QLC-C30") completed during the anti-C5 therapy regimen addressing quality of life issues.

Figure 10 is a chart depicting the effects of anti-C5 antibody treatments on adverse symptoms associated with PNH.

Samsung Bioepis Exhibit No. 1002
Page 1873

Detailed Description

[0024] The present disclosure relates to a method of treating paroxysmal nocturnal hemoglobinuria ("PNH") and other hemolytic diseases in mammals. Specifically, the methods of treating hemolytic diseases, which are described herein, involve using compounds which bind to or otherwise block the generation and/or activity of one or more complement components. The present methods have been found to provide surprising results. For instance, hemolysis rapidly ceases upon administration of the compound which binds to or otherwise blocks the generation and/or activity of one or more complement components, with hemoglobinuria being significantly reduced after treatment. Also, hemolytic patients can be rendered less dependent on transfusions or transfusion-independent for extended periods (twelve months or more), well beyond the 120 day life cycle of red blood cells. In addition, type III red blood cell count can be increased dramatically in the midst of other mechanisms of red blood cell lysis (non-complement mediated and/or earlier complement component mediated e.g., Cb3). Another example of a surprising result is that symptoms resolved, indicating that NO serum levels were increased enough even in the presence of other mechanisms of red blood cell lysis. These and other results reported herein are unexpected and could not be predicted from prior treatments of hemolytic diseases.

[0025] Any compound which binds to or otherwise blocks the generation and/or activity of one or more complement components can be used in the present methods. A specific class of such compounds which is particularly useful includes antibodies specific to a human complement component, especially anti-C5 antibodies. The anti-C5 antibody inhibits the complement cascade and, ultimately, prevents red blood cell ("RBC") lysis by the complement protein complex C5b-9. By inhibiting and/or reducing the lysis of RBCs, the effects of PNH and other hemolytic diseases (including symptoms such as hemoglobinuria, anemia, hemoglobinemia, dysphagia, fatigue, erectile dysfunction, recurrent abdominal pain and thrombosis) are eliminated or decreased.

[0026] In another embodiment, soluble forms of the proteins CD55 and CD59, singularly or in combination with each other, can be administered to a subject to inhibit the complement cascade in its alternative pathway. CD55 inhibits at the level of C3, thereby preventing the further progression of the cascade. CD59 inhibits the C5b-8 complex from combining with C9 to form the membrane attack complex (see discussion below).

[0027] The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of bacterial and viral pathogens. There are at least 25 proteins involved in the complement cascade, which are found as a complex collection of plasma proteins and membrane cofactors. Complement components achieve their immune defensive functions by interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions. A concise summary of the biologic activities associated with complement activation is provided, for example, in The Merck Manual, 16th Edition.

[0028] The complement cascade progresses via the classical pathway, the alternative pathway or the lectin pathway. These pathways share many components, and while they differ in their initial steps, they converge and share the same "terminal complement" components (C5 through C9) responsible for the activation and destruction of target cells. The classical complement pathway is typically initiated by antibody recognition of and binding to an antigenic site on a target cell. The alternative pathway is usually antibody independent, and can be initiated by certain molecules on pathogen surfaces. Additionally, the lectin pathway is typically initiated with binding of mannose-binding lectin ("MBL") to high mannose substrates. These pathways converge at the point where complement component C3 is cleaved by an active protease to yield C3a and C3b.

[0029] C3a is an anaphylatoxin (see discussion below). C3b binds to bacteria and other cells, as well as to certain viruses and immune complexes, and tags them for removal from the circulation. (C3b in this role is known as opsonin.) The opsonic function of C3b is generally considered to be the most important anti-infective action of the complement system. Patients with genetic lesions that block C3b function are prone to infection by a broad variety of pathogenic organisms, while patients with lesions later in the complement cascade sequence, i.e., patients with lesions that block C5 functions, are found to be more prone only to Neisseria infection, and then only somewhat more prone (Fearon, in Intensive Review of Internal Medicine, 2nd Ed. Fanta and Minaker, eds. Brigham and Women's and Beth Israel Hospitals, 1983).

[0030] C3b also forms a complex with other components unique to each pathway to form classical or alternative C5 convertase, which cleaves C5 into C5a and C5b. C3 is thus regarded as the central protein in the complement reaction sequence since it is essential to all three activation pathways (Wurzner, et al., Complement Inflamm. 8:328-340,1991). This property of C3b is regulated by the serum protease Factor I, which acts on C3b to produce iC3b (inactive C3b). While still functional as an opsonin, iC3b can not form an active C5 convertase.

[0031] The pro-C5 precursor is cleaved after amino acid 655 and 659, to yield the beta chain as an amino terminal fragment (amino acid residues +1 to 655 of the sequence) and the alpha chain as a carboxyl terminal fragment (amino acid residues 660 to 1658 of the sequence), with four amino acids (amino acid residues 656-659 of the sequence) deleted between the two. C5 is glycosylated, with about 1.5-3 percent of its mass attributed to carbohydrate. Mature C5 is a heterodimer of a 999 amino acid 115 kDa alpha chain that is disulfide linked to a 655 amino acid 75 kDa beta chain.

Samsung Bioepis Exhibit No. 1002
Page 1874

C5 is found in normal serum at approximately 75 μg/ml (0.4 μM). C5 is synthesized as a single chain precursor protein product of a single copy gene (Haviland et al. J. Immunol. 1991, 146:362-368). The cDNA sequence of the transcript of this gene predicts a secreted pro-C5 precursor of 1658 amino acids along with an 18 amino acid leader sequence (see, U.S. patent 6,355,245).

[0032] Cleavage of C5 releases C5a, a potent anaphylatoxin and chemotactic factor, and leads to the formation of the lytic terminal complement complex, C5b-9. C5a is cleaved from the alpha chain of C5 by either alternative or classical C5 convertase as an amino terminal fragment comprising the first 74 amino acids of the alpha chain (i.e., amino acid residues 660-733 of the sequence). Approximately 20 percent of the 11 kDa mass of C5a is attributed to carbohydrate. The cleavage site for convertase action is at, or immediately adjacent to, amino acid residue 733 of the sequence. A compound that binds at, or adjacent, to this cleavage site would have the potential to block access of the C5 convertase enzymes to the cleavage site and thereby act as a complement inhibitor.

[0033] C5b combines with C6, C7, and C8 to form the C5b-8 complex at the surface of the target cell. Upon binding of several C9 molecules, the membrane attack complex ("MAC", C5b-9, terminal complement complex-TCC) is formed. When sufficient numbers of MACs insert into target cell membranes, the openings they create (MAC pores) mediate rapid osmotic lysis of the target cells. Lower, non-lytic concentrations of MACs can produce other proinflammatory effects. In particular, membrane insertion of small numbers of the C5b-9 complexes into endothelial cells and platelets can cause deleterious cell activation. In some cases activation may precede cell lysis.

[0034] C5a and C5b-9 also have pleiotropic cell activating properties, by amplifying the release of downstream inflammatory factors, such as hydrolytic enzymes, reactive oxygen species, arachidonic acid metabolites and various cytokines. C5 can also be activated by means other than C5 convertase activity. Limited trypsin digestion (Minta and Man, J. Immunol. 1977, 119:1597-1602; Wetsel and Kolb, J. Immunol. 1982, 128:2209-2216) and acid treatment (Yammamoto and Gewurz, J. Immunol. 1978, 120:2008; Damerau et al., Molec. Immunol. 1989, 26:1133-1142) can also cleave C5 and produce active C5b.

[0035] As mentioned above, C3a and C5a are anaphylatoxins. These activated complement components can trigger mast cell degranulation, which releases histamine and other mediators of inflammation, resulting in smooth muscle contraction, increased vascular permeability, leukocyte activation, and other inflammatory phenomena including cellular proliferation resulting in hypercellularity. C5a also functions as a chemotactic peptide that serves to attract pro-inflammatory granulocytes to the site of complement activation.

[0036] Any compounds which bind to or otherwise block the generation and/or activity of any of the human complement components may be utilized in accordance with the present disclosure. In some embodiments, antibodies specific to a human complement component are useful herein. Some compounds include antibodies directed against complement components C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, Factor D, Factor B, Factor P, MBL, MASP-1, and MASP-2, thus preventing the generation of the anaphylatoxic activity associated with C5a and/or preventing the assembly of the membrane attack complex associated with C5b.

[0037] Also useful in the present methods are naturally occurring or soluble forms of complement inhibitory compounds such as CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH. Other compounds which may be utilized to bind to or otherwise block the generation and/or activity of any of the human complement components include, but are not limited to, proteins, protein fragments, peptides, small molecules, RNA aptamers including ARC187 (which is commercially available from Archemix Corp., Cambridge, MA), L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, molecules which may be utilized in RNA interference (RNAi) such as double stranded RNA including small interfering RNA (siRNA), locked nucleic acid (LNA) inhibitors, peptide nucleic acid (PNA) inhibitors, etc.

[0038] Functionally, one suitable class of compounds inhibits the cleavage of C5, which blocks the generation of potent proinflammatory molecules C5a and C5b-9 (terminal complement complex). Preferably, the compound does not prevent the formation of C3b, which subserves critical immunoprotective functions of opsonization and immune complex clearance.

[0039] While preventing the generation of these membrane attack complex molecules, inhibition of the complement cascade at C5 preserves the ability to generate C3b, which is critical for opsonization of many pathogenic microorganisms, as well as for immune complex solubilization and clearance. Retaining the capacity to generate C3b appears to be particularly important as a therapeutic factor in complement inhibition for hemolytic diseases, where increased susceptibility to thrombosis, infection, fatigue, lethargy and impaired clearance of immune complexes are pre-existing clinical features of the disease process.

[0040] Particularly useful compounds for use herein are antibodies that reduce, directly or indirectly, the conversion of complement component C5 into complement components C5a and C5b. One class of useful antibodies are those having at least one antigen binding site and exhibiting specific binding to human complement component C5. Particularly useful complement inhibitors are compounds which reduce the generation of C5a and/or C5b-9 by greater than about 30%. Anti-C5 antibodies that have the desirable ability to block the generation of C5a have been known in the art since at least 1982 (Moongkarndi et al. Immunobiol. 1982, 162:397; Moongkarndi et al. Immunobiol. 1983, 165:323). Antibodies

Samsung Bioepis Exhibit No. 1002
Page 1875

known in the art that are immunoreactive against C5 or C5 fragments include antibodies against the C5 beta chain (Moongkarndi et al. Immunobiol. 1982, 162:397; Moongkarndi et al. Immunobiol. 1983, 165:323; Wurzner et al. 1991, supra; Mollnes et al. Scand. J. Immunol. 1988, 28:307-312); C5a (see for example, Ames et al. J. Immunol. 1994, 152:4572-4581, U.S. Pat. No. 4,686,100, and European patent publication No. 0 411 306); and antibodies against non-human C5 (see for example, Giclas et al. J. Immunol. Meth. 1987, 105:201-209). Particularly useful anti-C5 antibodies are h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1. Methods for the preparation of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1 are described in U.S. Patent No. 6,355,245 and "Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chain Fv", Thomas et al., Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401, 1996, the disclosures of which are incorporated herein in their entirety by this reference. The antibody h5G1.1-mAb is currently undergoing clinical trials under the tradename eculizumab.

[0041] Hybridomas producing monoclonal antibodies reactive with complement component C5 can be obtained according to the teachings of Sims, et al., U.S. Pat. No. 5,135,916. Antibodies are prepared using purified components of the complement C5 component as immunogens according to known methods. In accordance with this disclosure, complement component C5, C5a or C5b is preferably used as the immunogen. In accordance with particularly preferred useful embodiments, the immunogen is the alpha chain of C5.

[0042] Particularly useful antibodies share the required functional properties discussed in the preceding paragraph and have any of the following characteristics:

(1) they compete for binding to portions of C5 that are specifically immunoreactive with 5G1.1;
(2) they specifically bind to the C5 alpha chain -- such specific binding, and competition for binding can be determined by various methods well known in the art, including the plasmon surface resonance method (Johne et al., J. Immunol. Meth. 1993, 160:191-198); and
(3) they block the binding of C5 to either C3 or C4 (which are components of the C5 convertases).

[0043] The compound that inhibits the production and/or activity of at least one complement component can be administered in a variety of unit dosage forms. The dose will vary according to the particular compound employed. For example, different antibodies may have different masses and/or affinities, and thus require different dosage levels. Antibodies prepared as fragments (e.g., Fab, $F(ab')_2$, scFv) will also require differing dosages than the equivalent intact immunoglobulins, as they are of considerably smaller mass than intact immunoglobulins, and thus require lower dosages to reach the same molar levels in the patient's blood.

[0044] The dose will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician.

[0045] Administration of the compound that inhibits the production and/or activity of at least one complement component will generally be in an aerosol form with a suitable pharmaceutical carrier, via intravenous infusion by injection, subcutaneous injection, orally, or sublingually. Other routes of administration may be used if desired.

[0046] It is further contemplated that a combination therapy can be used wherein a complement-inhibiting compound is administered in combination with a regimen of known therapy for hemolytic disease. Such regimens include administration of 1) one or more compounds known to increase hematopoiesis (for example, either by boosting production, eliminating stem cell destruction or eliminating stem cell inhibition) in combination with 2) a compound selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. Suitable compounds known to increase hematopoiesis include, for example, steroids, immunosuppressants (such as, cyclosporin), anti-coagulants (such as, warfarin), folic acid, iron and the like, erythropoietin (EPO) and antithymocyte globulin (ATG), antilymphocyte globulin (ALG), EPO derivatives, and darbepoetin alfa (commercially available as Aranesp® from Amgen, Inc., Thousand Oaks, CA (Aranesp® is a man-made form of EPO produced in Chinese hamster ovary (CHO) cells by recombinant DNA technology)). In particularly useful embodiments, erythropoietin (EPO) (a compound known to increase hematopoiesis), EPO derivatives, or darbepoetin alfa may be administered in combination with an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and other functional fragments of h5G1.1.

[0047] Formulations suitable for injection are found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, Pa., 17th ed. (1985). Such formulations must be sterile and non-pyrogenic, and generally will include a pharmaceutically effective carrier, such as saline, buffered (e.g., phosphate buffered) saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions, and the like. The formulations may contain pharmaceutically acceptable auxiliary substances as required, such as, tonicity adjusting agents, wetting agents, bactericidal agents, preservatives, stabilizers, and the like.

[0048] The present disclosure contemplates methods of reducing hemolysis in a patient afflicted with a hemolytic disease by administering one or more compounds which bind to or otherwise block the generation and/or activity of one or more complement components. Reducing hemolysis means that the duration of time a person suffers from hemolysis

Samsung Bioepis Exhibit No. 1002
Page 1876

is reduced by about 25% or more. The effectiveness of the treatment can be evaluated in any of the various manners known to those skilled in the art for determining the level of hemolysis in a patient. One qualitative method for detecting hemolysis is to observe the occurrences of hemoglobinuria. Quite surprisingly, treatment in accordance with the present methods reduces hemolysis as determined by a rapid reduction in hemoglobinuria.

[0049] A more qualitative manner of measuring hemolysis is to measure lactate dehydrogenase (LDH) levels in the patient's bloodstream. LDH catalyzes the interconversion of pyruvate and lactate. Red blood cells metabolize glucose to lactate, which is released into the blood and is taken up by the liver. LDH levels are used as an objective indicator of hemolysis. As those skilled in the art will appreciate, measurements of "upper limit of normal" levels of LDH will vary from lab to lab depending on a number of factors including the particular assay employed and the precise manner in which the assay is conducted. Generally speaking, however, the present methods can reduce hemolysis in a patient afflicted with a hemolytic disease as reflected by a reduction of LDH levels in the patients to within 20% of the upper limit of normal LDH levels. Alternatively, the present methods can reduce hemolysis in a patient afflicted with a hemolytic disease as reflected by a reduction of LDH levels in the patients of greater than 50% of the patient's pre-treatment LDH level, preferably greater than 65% of the patient's pre-treatment LDH level, most preferably greater than 80% of the patient's pre-treatment LDH level.

[0050] Another quantitative measurement of a reduction in hemolysis is the presence of GPI-deficient red blood cells (Type III red blood cells). As those skilled in the art will appreciate, Type III red blood cells have no GPI-anchor protein expression on the cell surface. The proportion of GPI-deficient cells can be determined by flow cytometry using, for example, the technique described in Richards, et al., Clin. Appl. Immunol. Rev., vol. 1, pages 315-330, 2001. The present methods can reduce hemolysis in a patient afflicted with a hemolytic disease as reflected by an increase in Type III red blood cells. Preferably an increase in Type III red blood cell levels in the patient of greater than 25% of the total red blood cell count is achieved, more preferably an increase in Type III red blood cell levels in the patients greater than 50% of the total red blood cell count is achieved, most preferably an increase in Type III red blood cell levels in the patients greater than 75% of the total red blood cell count is achieved.

[0051] Methods of reducing one or more symptoms associated with PNH or other hemolytic diseases are also within the scope of the present disclosure. Such symptoms include, for example, abdominal pain, fatigue, dyspnea and insomnia. Symptoms can be the direct result of lysis of red blood cells (e.g., hemoglobinuria, anemia, fatigue, low red blood cell count, etc.) or the symptoms can result from low nitric oxide (NO) levels in the patient's bloodstream (e.g., abdominal pain, erectile dysfunction, dysphagia, thrombosis, etc.). It has recently been reported that almost all patients with greater than 40% PNH type III granulocyte clone have thrombosis, abdominal pain, erectile dysfunction and dysphagia, indicating a high hemolytic rate (see Moyo et al., British J. Haematol. 126:133-138 (2004)).

[0052] In particularly useful embodiments, the present methods provide a reduction in one or more symptoms associated with PNH or other hemolytic diseases in a patient having a platelet count in excess of 40,000 per microliter (a hypoplastic patient), preferably in excess of 75,000 per microliter, most preferably in excess of 150,000 per microliter. In other embodiments, the present methods provide a reduction in one or more symptoms associated with PNH or other hemolytic diseases in a patient where the proportion of PNH type III red blood cells of the subject's total red blood cell content is greater than 10%, preferably greater than 25%, most preferably in excess of 50%. In yet other embodiments, the present methods provide a reduction in one or more symptoms associated with PNH or other hemolytic diseases in a patient having a reticulocyte count in excess of 80×10^9 per liter, more preferably in excess of 120×10^9 per liter, most preferably in excess of 150×10^9 per liter. Patients in the most preferable ranges recited above have active bone marrow and will produce adequate numbers of red blood cells. While in a patient afflicted with PNH or other hemolytic disease the red blood cells may be defective in one or more ways (e.g., GPI deficient), the present methods are particularly useful in protecting such cells from lysis resulting from complement activation. Thus, patients within the preferred ranges benefit most from the present methods.

[0053] In one aspect, a method of reducing fatigue is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing fatigue means the duration of time a person suffers from fatigue is reduced by about 25% or more. Fatigue is a symptom believed to be associated with intravascular hemolysis as the fatigue relents when hemoglobinuria resolves even in the presence of anemia. By reducing the lysis of red blood cells, the present methods reduce fatigue. Patients within the above-mentioned preferred ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

[0054] In another aspect, a method of reducing abdominal pain is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing abdominal pain means the duration of time a person suffers from abdominal pain is reduced by about 25% or more. Abdominal pain is a symptom resulting from the inability of a patient's natural levels of haptoglobin to process all the free hemoglobin released into the bloodstream as a result of intravascular hemolysis, resulting in the scavenging of NO and intestinal dystonia and spasms. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream,

Samsung Bioepis Exhibit No. 1002
Page 1877

thereby reducing abdominal pain. Patients within the above-mentioned preferred ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

[0055] In another aspect, a method of reducing dysphagia is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing dysphagia means the duration of time a person has dysphagia attacks is reduced by about 25% or more. Dysphagia is a symptom resulting from the inability of a patient's natural levels of haptoglobin to process all the free hemoglobin released into the bloodstream as a result of intravascular hemolysis, resulting in the scavenging of NO and esophageal spasms. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby reducing dysphagia. Patients within the above-mentioned preferred ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

[0056] In yet another aspect, a method of reducing erectile dysfunction is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing erectile dysfunction means the duration of time a person suffers from erectile dysfunction is reduced by about 25% or more. Erectile dysfunction is a symptom believed to be associated with scavenging of NO by free hemoglobin released into the bloodstream as a result of intravascular hemolysis. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of NO and reducing erectile dysfunction. Patients within the above-mentioned preferred ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

[0057] In yet another aspect, a method of reducing hemoglobinuria is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing hemoglobinuria means a reduction in the number of times a person has red, brown, or darker urine, wherein the reduction is typically about 25% or more. Hemoglobinuria is a symptom resulting from the inability of a patient's natural levels of haptoglobin to process all the free hemoglobin released into the bloodstream as a result of intravascular hemolysis. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream and urine thereby reducing hemoglobinuria. Quite surprisingly, the reduction in hemoglobinuria occurs rapidly. Patients within the above-mentioned preferred ranges of type III red blood cells, reticulocytes and platelets benefit most from the present methods.

[0058] In still another aspect, a method of reducing thrombosis is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing thrombosis means the duration of time a person has thrombosis attacks is reduced by about 25% or more. Thrombosis is a symptom believed to be associated with scavenging of NO by free hemoglobin released into the bloodstream as a result of intravascular hemolysis and/or the lack of CD59 on the surface of platelets resulting in terminal complement mediated activation of the platelet. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of NO and reducing thrombosis. In addition, blockade of complement will prevent terminal complement-mediated activation of platelets and thrombosis. C5a will also be inhibited by this method which can induce platelet aggregation through C5a receptors on platelets and endothelial cells.

[0059] Thrombosis is thought to be multi-factorial in etiology including NO scavenging by free hemoglobin, the absence of terminal complement inhibition on the surface of circulating platelets and changes in the endothelium surface by cell free heme. The intravascular release of free hemoglobin may directly contribute to small vessel thrombosis. NO has been shown to inhibit platelet aggregation, induce disaggregation of aggregated platelets and inhibit platelet adhesion. Conversely, NO scavenging by hemoglobin or the reduction of NO generation by the inhibition of arginine metabolism results in an increase in platelet aggregation. PNH platelets also lack the terminal complement inhibitor CD59 and multiple studies have shown that deposition of terminal complement (C5b-9) on platelets causes membrane vesiculation and the generation of microvesicles. The microvesicles act as a site for the generation of the clotting components factor Va, Xa or the prothrombinase complex. It is thought that these particles may also contribute to the genesis of thrombosis in PNH. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of NO and reducing thrombosis. In addition, inhibiting complement at C5 will prevent C5b-9 and C5a mediated activation of platelets and/or endothelial cells.

[0060] In particularly useful embodiments, the present methods reduce thrombosis, especially patients having a platelet count in excess of 40,000 per microliter, preferably in excess of 75,000 per microliter, most preferably in excess of 150,000 per microliter. In other embodiments, the present methods reduce thrombosis in patients where the proportion of PNH type III red blood cells of the subject's total red blood cell content is greater than 10%, preferably greater than 25%, more preferably in excess of 50%, most preferably in excess of 75%. In yet other embodiments, the present methods reduce transfusion thrombosis in patients having a reticulocyte count in excess of 80 x 10^9 per liter, more preferably in excess of 120 x 10^9 per liter, most preferably in excess of 150 x 10^9 per liter.

Samsung Bioepis Exhibit No. 1002
Page 1878

[0061] In still another aspect, a method of reducing anemia is contemplated, the method including the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Reducing anemia means the duration of time a person has anemia is reduced by about 25% or more. Anemia in hemolytic diseases results from the blood's reduced capacity to carry oxygen due to the loss of red blood cell mass. By reducing the lysis of red blood cells, the present methods assist red blood cell levels to increase thereby reducing anemia.

[0062] In another aspect, a method of increasing the proportion of complement sensitive type III red blood cells and therefore the total red blood cell count in a patient afflicted with a hemolytic disease is contemplated. By increasing the patient's RBC count, fatigue, anemia and the patient's need for blood transfusions is reduced. The reduction in transfusions can be in frequency of transfusions, amount of blood units transfused, or both.

[0063] The method of increasing red blood cell count in a patient afflicted with a hemolytic disease includes the step of administering a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components to a patient afflicted with a hemolytic disease. In particularly useful embodiments, the present methods increase red blood cell count in a patient afflicted with a hemolytic disease, especially patients having a platelet count in excess of 40,000 per microliter, preferably in excess of 75,000 per microliter, most preferably in excess of 150,000 per microliter. In other embodiments, the present methods increase red blood cell count in a patient afflicted with a hemolytic disease where the proportion of PNH type III red blood cells of the subject's total red blood cell content is greater than 10%, preferably greater than 25%, more preferably in excess of 50%, most preferably in excess of 75%. In yet other embodiments, the present methods increase red blood cell count in a patient afflicted with a hemolytic disease having a reticulocyte count in excess of 80 x 10^9 per liter, more preferably in excess of 120 x 10^9 per liter, most preferably in excess of 150 x 10^9 per liter. In some embodiments, the methods of the present disclosure may result in a decrease in the frequency of transfusions by about 50%, typically a decrease in the frequency of transfusions by about 70%, more typically a decrease in the frequency of transfusions by about 90%.

[0064] In yet another aspect, the present disclosure contemplates a method of rendering a subject afflicted with a hemolytic disease less dependent on transfusions or transfusion-independent by administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components. As those skilled in the art will appreciate, the normal life cycle for a red blood cell is about 120 days. Treatment for six months or more is required for the evaluation of transfusion independence given the long half life of red blood cells. It has unexpectedly been found that in some patients transfusion-independence can be maintained for twelve months or more, in some cases more than two years, long beyond the 120 day life cycle of red blood cells. In particularly useful embodiments, the present methods provide decreased dependence on transfusions or transfusion-independence in a patient afflicted with a hemolytic disease, especially patients having a platelet count in excess of 40,000 per microliter, preferably in excess of 75,000 per microliter, most preferably in excess of 150,000 per microliter. In other embodiments, the present methods provide decreased dependence on transfusions or transfusion-independence in a patient afflicted with a hemolytic disease where the proportion of PNH type III red blood cells of the subject's total red blood cell content is greater than 10%, preferably greater than 25%, more preferably in excess of 50%, most preferably in excess of 75%. In yet other embodiments, the present methods provide decreased dependence on transfusions or transfusion-independence in a patient afflicted with a hemolytic disease having a reticulocyte count in excess of 80 x 10^9 per liter, more preferably in excess of 120 x 10^9 per liter, most preferably in excess of 150 x 10^9 per liter.

[0065] Methods of increasing the nitric oxide (NO) levels in a patient having PNH or some other hemolytic disease are also within the scope of the present disclosure. These methods of increasing NO levels include the step of administering to a subject having or susceptible to a hemolytic disease a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components. Low NO levels arise in patients afflicted with PNH or other hemolytic diseases as a result of scavenging of NO by free hemoglobin released into the bloodstream as a result of intravascular hemolysis. By reducing the lysis of red blood cells, the present methods reduce the amount of free hemoglobin in the bloodstream, thereby increasing serum levels of NO. In particularly useful embodiments, NO homeostasis is restored as evidenced by a resolution of symptoms attributable to NO deficiencies.

EXAMPLES

[0066] Eleven patients participated in therapy trials to evaluate the effects of anti-C5 antibody on PNH and symptoms associated therewith. PNH patients were transfusion-dependent and hemolytic. Patients were defined as transfusion dependent with a history of four or more transfusions within twelve months. The median number of transfusions within the patient pool was nine in the previous twelve months. The median number of transfusion units used in the previous twelve months was twenty-two for the patient pool.

[0067] Over the course of four weeks, each of 11 patients received a weekly 600 mg intravenous infusion of anti-C5

Samsung Bioepis Exhibit No. 1002
Page 1879

antibody for approximately thirty minutes. The specific anti-C5 antibody used in the study was eculizumab. Patients received 900 mg of eculizumab 1 week later then 900 mg on a biweekly basis. The first twelve weeks of the study constituted the pilot study. Following completion of the initial acute phase twelve week study, all patients participated in an extension study conducted to a total of 64 weeks. Ten of the eleven patients participated in an extension study conducted to a total of two years.

[0068] The effect of anti-C5 antibody treatments on PNH type III red blood cells ("RBCs") was tested. "PNH Type" refers to the density of GPI-anchored proteins expressed on the cell surface. Type I is normal expression, Type II is intermediate expression, and Type III has no GPI-anchor protein expression on the cell surface. The proportion of GPI-deficient cells is determined by flow cytometry in the manner described in Richards, et al., Clin. Appl. Immunol. Rev., vol. 1, pages 315-330, 2001. As compared to pre-therapy conditions, PNH Type III red blood cells increased more than 50% during the extension study. The increase from a pre-study mean value of 36.7% of all red blood cells to a 64 week mean value of 58.4% of Type III red blood cells indicated that hemolysis had decreased sharply. See Table 1, below. Eculizumab therapy protected PNH type III RBCs from complement-mediated lysis, prolonging the cells survival. This protection of the PNH-affected cells reduced the need for transfusions, paroxysms and overall hemolysis in all patients in the trial.

Table 1

PNH Cell Populations Pre- and Post- Eculizumab Treatment in All Patients

	Proportion of PNH Cells (%)			
PNH Cell Type	baseline	12 weeks	64 weeks	p-value[a]
Type III RBCs	36.7 +/- 5.9	59.2 +/- 8.0	58.4 +/- 8.5	0.005
Type II RBCs	5.3 +/- 1.4	7.5 +/- 2.1	13.2+/-2.4	0.013
Type III WBCs	92.1 +/- 4.6	89.9 +/- 6.6	91.1 +/- 5.8	N.S.
Type III Platelets	92.4 +/- 2.4	93.3 +/- 2.8	92.8+/- 2.6	N.S.

[a]comparison of mean change from baseline to 64 weeks

[0069] During the course of the two year extension study, it was found that PNH red cells with a complete deficiency of GPI-linked proteins (Type III red cells) progressively increased during the treatment period from a mean of 36.7% to 58.9% (p=0.001) while partially deficient PNH red cells (Type II) increased from 5.3% to 8.7% (p=0.01). There was no concomitant change in the proportion of PNH neutrophils in any of the patients during eculizumab therapy, indicating that the increase in the proportion of PNH red cells was due to a reduction in hemolysis and transfusions rather than a change in the PNH clone(s) themselves.

[0070] The effect of anti-C5 antibody treatments on lactate dehydrogenase levels ("LDH") was measured on all eleven patients. LDH catalyzes the interconversion of pyruvate and lactate. Red blood cells metabolize glucose to lactate, which is released into the blood and is taken up by the liver. LDH levels are used as an objective indicator of hemolysis. The LDH levels were decreased by greater than 80% as compared to pre-treatment levels. The LDH levels were lowered from a pre-study mean value of 3111 U/L to a mean value of 594 U/L during the pilot study and a mean value of 622 U/L after 64 weeks (p = 0.002 for 64 week comparison; see Figures 1A and 1B).

[0071] Similarly, aspartate aminotransferase (AST) levels, another marker of red blood cell hemolysis, decreased from a mean baseline value of 76 IU/L to 26 IU/L and 30 IU/L during the 12 and 64 weeks of treatment, respectively (p=0.02 for 64 week comparison). Levels of haptoglobin, hemoglobin and bilirubin, and numbers of reticulocytes, did not change significantly from prestudy values during the 64 week treatment period.

[0072] Paroxysm rates were measured and compared to pre-treatment levels. Paroxysm as used in this disclosure is defined as incidences of dark-colored urine with a colorimetric level of 6 of more on a scale of 1-10. Figure 2 shows the urine color scale devised to monitor the incidence of paroxysm of hemoglobinuria in patients with PNH before and during treatment. As compared to pre-treatment levels, the paroxysm percentage rate was reduced by 93% (see, Figure 3) from 3.0 paroxysms per patient per month before eculizumab treatment to 0.1 paroxysms per patient per month during the initial 12 weeks and 0.2 paroxysms per patient per month during the 64 week treatment (Figure 3 ($p < 0.001$)).

[0073] Serum hemolytic activity in nine of the eleven patients was completely blocked throughout the 64 week treatment period with trough levels of eculizumab at equilibrium ranging from approximately 35 μg/ml to 350 μg/ml. During the extension study, 2 patients did not sustain levels of eculizumab necessary to consistently block complement. This breakthrough in serum hemolytic activity occurred in the last 2 days of the 14 day dosing interval, a pattern that was repeated between multiple doses. In one of the patients, as seen in Figure 4, break-through of complement blockade resulted in hemoglobinuria, dysphagia, and increased LDH and AST, which correlated with the return of serum hemolytic

Samsung Bioepis Exhibit No. 1002
Page 1880

activity. At the next dose, symptoms resolved (Figure 5) and reduction in the dosing interval from 900 mg every 14 days to 900 mg every 12 days resulted in a regain of complement control which was maintained over the extension study to 64 weeks in both patients. This patient showed a 24 hour resolution of dysphagia and hemoglobinuria. A reduction in the dosing interval from 14 to 12 days was sufficient to maintain levels of eculizumab above 35 μg/ml and effectively and consistently blocked serum hemolytic activity for the remainder of the extension study for both patients.

[0074] The patients' need for transfusions was also reduced by the treatment with eculizumab. Figure 6a compares the number of transfusion units required per patient per month, prior to and during treatment with an anti-C5 antibody for cytopenic patients, while Figure 6b compares the number of transfusion units required per patient per month, prior to and during treatment with an anti-C5 antibody for non-cytopenic patients. A significant reduction in the need for transfusion was also noted in the entire group (mean transfusion rates decreased from 2.1 units per patient per month during a 1 year period prior to treatment to 0.6 units per patient per month during the initial 12 weeks and 0.5 units per patient per month during the combined 64 week treatment period), with non-cytopenic patients benefiting the most. In fact, four of the non-thrombocytopenic patients with normal platelet counts (≥150,000 per microliter) became transfusion-independent during the 64 week treatment.

[0075] The effect of eculizumab administered in combination with erythropoietin (EPO) was also evaluated in a thrombocytopenic patient. EPO (NeoRecormon®, Roche Pharmaceuticals, Basel, Switzerland) was administered in an amount of 18,000 I.U. three times per week beginning in week 23 of the study. As shown in Figure 7, the frequency of transfusions required for this patient was significantly reduced, and soon halted.

[0076] For the two year extension study, 10 of the 11 patients from the initial 3 month study continued to receive 900 mg of eculizumab every other week. (One patient discontinued eculizumab therapy after 23 months.) Six of the 11 patients had normal platelets (no clinical evidence of marrow failure) whereas 5 of the 11 had low platelets. For the patient who discontinued eculizumab therapy after 23 months, intravascular hemolysis was successfully controlled by eculizumab, but the patient continued to be transfused even after erythropoietin therapy. This patient had the most severe hypoplasia at the start of eculizumab therapy with a platelet count below $30x10^9/l$, suggesting that the ongoing transfusions were likely a result of the underlying bone marrow failure.

[0077] Results of the two year extension study also demonstrated that there was a statistically significant decrease in transfusion requirements for the patients. Three patients remained transfusion independent during the entire two year treatment period, and four cytopenic patients became transfusion independent, three following treatment with EPO (NeoRecormon®). The reduction in transfusion requirements was found to be most pronounced in patients with a good marrow reserve.

[0078] Pharmacodynamic levels were measured and recorded according to eculizumab doses. The pharmacodynamic analysis of eculizumab was determined by measuring the capacity of patient serum samples to lyse chicken erythrocytes in a standard total human serum complement hemolytic assay. Briefly, patient samples or human control serum (Quidel, San Diego CA) was diluted to 40% vol/vol with gelatin veronal-buffered saline (GVB2+, Advanced Research technologies, San Diego, CA) and added in triplicate to a 96-well plate such that the final concentrations of serum in each well was 20%. The plate was then incubated at room temperature while chicken erythrocytes (Lampire Biologics, Malvern, PA) were washed. The chicken erythrocytes were sensitized by the addition of anti-chicken red blood cell polyclonal antibody (0.1 % vol/vol). The cells were then washed and resuspended in GVB2+ buffer. Chicken erythrocytes ($2.5 x10^6$ cells/30 μL) were added to the plate containing human control serum or patient samples and incubated at 37°C for 30 min. Each plate contained six additional wells of identically prepared chicken erythrocytes of which four wells were incubated with 20% serum containing 2 mM EDTA as the blank and two wells were incubated with GVB2+ buffer alone as a negative control for spontaneous hemolysis. The plate was then centrifuged and the supernatant transferred to a new flat bottom 96-well plate. Hemoglobin release was determined at OD 415 nm using a microplate reader. The percent hemolysis was determined using the following formula:

$$\text{Percent Hemolysis} = 100 \times \frac{\text{(OD patient sample-OD blank)}}{\text{(OD human serum control-OD blank)}}$$

The graph of the pharmacodynamics (Figure 8), the study of the physiological effects, shows the percentage of serum hemolytic activity (i.e. the percentage of cell lysis) over time. Cell lysis was dramatically reduced in the majority of the patients to below 20% of normal serum complement activity while under eculizumab treatment. Two patients exhibited a breakthrough in complement activity, but complement blockade was permanently restored by reducing the dosing interval to 12 days (See, Figure 4).

[0079] Improvement of quality of life issues was also evaluated using the European Organization for Research and Treatment of Cancer Core (http://www.eortc.be) questionnaires ("EORTC QLC-C30"). Each of the participating patients completed the QLC-30 questionnaire before and during the eculizumab therapy. Overall improvements were observed in global health status, physical functioning, role functioning, emotional functioning, cognitive functioning, fatigue, pain,

Samsung Bioepis Exhibit No. 1002
Page 1881

dyspnea and insomnia. (See Figure 9).

[0080] Patients in the two year study experienced a reduction in adverse symptoms associated with PNH. For example, as set forth in Figure 10, there was a demonstrated decrease of abdominal pain, dysphagia, and erectile dysfunction after administration of eculizumab in those patients reporting those symptoms before administration of eculizumab.

[0081] Although preferred and other embodiments of the invention have been described herein, further embodiments may be perceived by those skilled in the art without departing from the scope of the invention.

The present invention furthermore relates to following items:

1. A method of treating a hemolytic disease in a subject comprising administering an anti-C5 antibody to a subject having a hemolytic disease, wherein there is a reduction in hemoglobinuria in the subject.
2. A method as in item 1 wherein the anti-C5 antibody is selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.
3. A method as in item 1 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.
4. A method as in item 1 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.
5. A method as in item 1 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.
6. A method as in item, 1 wherein the subject's platelet count is greater than 40,000 per microliter.
7. A method as in item 1 wherein the subject's platelet count is greater than 75,000 per microliter.
8. A method as in item 1 wherein the subject's platelet count is greater than 150,000 per microliter.
9. A method as in item 1 wherein the subject's reticulocyte count is greater than 80 x 10^9 per liter.
10. A method as in item 1 wherein the subject's reticulocyte count is greater than 120 x 10^9 per liter.
11. A method as in item 1 wherein the subject's reticulocyte count is greater than 150 x 10^9 per liter.
12. A method as in item 1 wherein said subject has a greater than 40% PNH type III granulocyte clone.
13. A method as in item 1 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.
14. A method of treating a hemolytic disease in a subject comprising administering to a subject having a hemolytic disease a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors, wherein there is a reduction in hemoglobinuria in the subject.
15. A method as in item 14 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.
16. A method as in item 14 wherein said subject has a greater than 40% PNH type III granulocyte clone.
17. A method as in item 14 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.
18. A method of restoring nitric oxide (NO) homeostasis comprising administering an anti-C5 antibody to a subject having a hemolytic disease.
19. A method as in item 18 wherein the anti-C5 antibody is selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.
20. A method as in item 18 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.
21. A method as in item 18 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.
22. A method as in item 18 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.
23. A method as in item 18 wherein the subject's platelet count is greater than 40,000 per microliter.
24. A method as in item 18 wherein the subject's platelet count is greater than 75,000 per microliter.
25. A method as in item 18 wherein the subject's platelet count is greater than 150,000 per microliter.
26. A method as in item 18 wherein the subject's reticulocyte count is greater than 80 x 10^9 per liter.
27. A method as in item 18 wherein the subject's reticulocyte count is greater than 120 x 10^9 per liter.
28. A method as in item 18 wherein the subject's reticulocyte count is greater than 150 x 10^9 per liter.
29. A method as in item 18 wherein said subject has a greater than 40% PNH type III granulocyte clone.
30. A method as in item 18 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.
31. A method of restoring nitric oxide (NO) homeostasis comprising administering to a subject having a hemolytic disease a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds,

Samsung Bioepis Exhibit No. 1002
Page 1882

proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

32. A method as in item 31 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

33. A method as in item 31 wherein said subject has a greater than 40% PNH type III granulocyte clone.

34. A method as in item 31 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

35. A method of reducing hemolysis in a subject having a hemolytic disease comprising administering an anti-C5 antibody, wherein hemolysis is reduced as evidenced by a greater than 50% reduction in lactate dehydrogenase (LDH) levels in the subject's bloodstream.

36. A method as in item 35 wherein hemolysis is reduced as evidenced by a greater than 65% reduction in lactate dehydrogenase (LDH) levels in the subject's bloodstream.

37. A method as in item 35 wherein hemolysis is reduced as evidenced by a greater than 80% reduction in lactate dehydrogenase (LDH) levels in the subject's bloodstream.

38. A method as in item 35 wherein the anti-C5 antibody is selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

39. A method as in item 35 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.

40. A method as in item 35 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.

41. A method as in item 35 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.

42. A method as in item 35 wherein the subject's platelet count is greater than 40,000 per microliter.

43. A method as in item 35 wherein the subject's platelet count is greater than 75,000 per microliter.

44. A method as in item 35 wherein the subject's platelet count is greater than 150,000 per microliter.

45. A method as in item 35 wherein the subject's reticulocyte count is greater than 80 x 10^9 per liter.

46. A method as in item 35 wherein the subject's reticulocyte count is greater than 120 x 10^9 per liter.

47. A method as in item 35 wherein the subject's reticulocyte count is greater than 150 x 10^9 per liter.

48. A method as in item 35 wherein said subject has a greater than 40% PNH type III granulocyte clone.

49. A method as in item 35 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

50. A method of reducing hemolysis in a subject having a hemolytic disease comprising administering to the subject a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors, wherein hemolysis is reduced as evidenced by a greater than 50% reduction in lactate dehydrogenase (LDH) levels in the subject's bloodstream.

51. A method as in item 50 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

52. A method as in item 50 wherein said subject has a greater than 40% PNH type III granulocyte clone.

53. A method as in item 50 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

54. A method of reducing hemoglobinuria in a subject comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

55. A method as in item 54 wherein the step of administering comprises administering an anti-C5 antibody.

56. A method as in item 54 wherein the compound is an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

57. A method as in item 54 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

58. A method as in item 54 wherein the step of administering comprises administering a compound selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

59. A method as in item 54 wherein the proportion of type III red blood cells of the subject's total red blood cell

Samsung Bioepis Exhibit No. 1002
Page 1883

content is greater than 10%.

60. A method as in item 54 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.

61. A method as in item 54 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.

62. A method as in item 54 wherein the subject's platelet count is greater than 40,000 per microliter.

63. A method as in item 54 wherein the subject's platelet count is greater than 75,000 per microliter.

64. A method as in item 54 wherein the subject's platelet count is greater than 150,000 per microliter.

65. A method as in item 54 wherein the subject's reticulocyte count is greater than 80 x 10^9 per liter.

66. A method as in item 54 wherein the subject's reticulocyte count is greater than 120 x 10^9 per liter.

67. A method as in item 54 wherein the subject's reticulocyte count is greater than 150 x 10^9 per liter.

68. A method as in item 54 wherein said subject has a greater than 40% PNH type III granulocyte clone.

69. A method as in item 54 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

70. A method of reducing dysphagia in a subject comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components and compounds which block the generation of one or more complement components.

71. A method as in item 70 wherein the step of administering comprises administering an anti-C5 antibody.

72. A method as in item 70 wherein the compound is an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

73. A method as in item 70 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

74. A method as in item 70 wherein the step of administering comprises administering a compound selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

75. A method as in item 70 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.

76. A method as in item 70 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.

77. A method as in item 70 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.

78. A method as in item 70 wherein the subject's platelet count is greater than 40,000 per microliter.

79. A method as in item 70 wherein the subject's platelet count is greater than 75,000 per microliter.

80. A method as in item 70 wherein the subject's platelet count is greater than 150,000 per microliter.

81. A method as in item 70 wherein the subject's reticulocyte count is greater than 80 x 10^9 per liter.

82. A method as in item 70 wherein the subject's reticulocyte count is greater than 120 x 10^9 per liter.

83. A method as in item 70 wherein the subject's reticulocyte count is greater than 150 x 10^9 per liter.

84. A method as in item 70 wherein said subject has a greater than 40% PNH type III granulocyte clone.

85. A method as in item 70 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

86. A method of reducing erectile dysfunction in a subject comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components and compounds which block the generation of one or more complement components.

87. A method as in item 86 wherein the step of administering comprises administering an anti-C5 antibody.

88. A method as in item 86 wherein the compound is an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

89. A method as in item 86 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

90. A method as in item 86 wherein the step of administering comprises administering a compound selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

91. A method as in item 86 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.

92. A method as in item 86 wherein the proportion of type III red blood cells of the subject's total red blood cell

Samsung Bioepis Exhibit No. 1002
Page 1884

content is greater than 25%.

93. A method as in item 86 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.

94. A method as in item 86 wherein the subject's platelet count is greater than 40,000 per microliter.

95. A method as in item 86 wherein the subject's platelet count is greater than 75,000 per microliter.

96. A method as in item 86 wherein the subject's platelet count is greater than 150,000 per microliter.

97. A method as in item 86 wherein the subject's reticulocyte count is greater than 80 x 10^9 per liter.

98. A method as in item 86 wherein the subject's reticulocyte count is greater than 120 x 10^9 per liter.

99. A method as in item 86 wherein the subject's reticulocyte count is greater than 150 x 10^9 per liter.

100. A method as in item 86 wherein said subject has a greater than 40% PNH type III granulocyte clone.

101. A method as in item. 86 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

102. A method of reducing thrombosis in a subject comprising administering a compound to a subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

103. A method as in item 102 wherein the step of administering comprises administering an anti-C5 antibody.

104. A method as in item 102 wherein the compound is an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

105. A method as in item 102 wherein the step of administering comprises administering an anti-C5a receptor antibody.

106. A method as in item 102 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

107. A method as in item 102 wherein the step of administering comprises administering a compound selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

108. A method as in item 102 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.

109. A method as in item 102 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.

110. A method as in item 102 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.

111. A method as in item 102 wherein the subject's platelet count is greater than 40,000 per microliter.

112. A method as in item 102 wherein the subject's platelet count is greater than 75,000 per microliter.

113. A method as in item 102 wherein the subject's platelet count is greater than 150,000 per microliter.

114. A method as in item 102 wherein the subject's reticulocyte count is greater than 80 x 10^9 per liter.

115. A method as in item 102 wherein the subject's reticulocyte count is greater than 120 x 10^9 per liter.

116. A method as in item 102 wherein the subject's reticulocyte count is greater than 150 x 10^9 per liter.

117. A method as in item 102 wherein said subject has a greater than 40% PNH type III granulocyte clone.

118. A method as in item 102 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

119. A method of increasing proportion of type III red blood cells of a subject's total red blood cell content comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

120. A method as in item 119 wherein the step of administering comprises administering an anti-C5 antibody.

121. A method as in item 119 wherein the compound is an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

122. A method as in item 119 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

123. A method as in item 119 wherein the step of administering comprises administering a compound selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

124. A method as in item 119 wherein the amount of PNH type III red blood cells is greater than about 10% of the

Samsung Bioepis Exhibit No. 1002
Page 1885

subject's total red blood cell count.

125. A method as in item 119 wherein the amount of PNH type III red blood cells is greater than about 25% of the subject's total red blood cell count.

126. A method as in item 119 wherein the amount of PNH type III red blood cells is greater than about 50% of the subject's total red blood cell count.

127. A method as in item 119 wherein the amount of PNH type III red blood cells is greater than about 75% of the subject's total red blood cell count.

128. A method as in item 119 wherein the subject's platelet count is greater than 40,000 per microliter.

129. A method as in item 119 wherein the subject's platelet count is greater than 75,000 per microliter.

130. A method as in item 119 wherein the subject's platelet count is greater than 150,000 per microliter.

131. A method as in item 119 wherein the subject's reticulocyte count is greater than 80 x 10^9 per liter.

132. A method as in item 119 wherein the subject's reticulocyte count is greater than 120 x 10^9 per liter.

133. A method as in item 119 wherein the subject's reticulocyte count is greater than 150 x 10^9 per liter.

134. A method as in item 119 wherein said subject has a greater than 40% PNH type III granulocyte clone.

135. A method as in item 119 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

136. A method of reducing hemolysis in a subject comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

137. A method as in item 136 wherein the step of administering comprises administering an anti-C5 antibody.

138. A method as in item 136 wherein the compound is an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

139. A method as in item 136 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

140. A method as in item 136 wherein the step of administering comprises administering a compound selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

141. A method as in item 136 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.

142. A method as in item 136 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.

143. A method as in item 136 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.

144. A method as in item 136 wherein the subject's platelet count is greater than 40,000 per microliter.

145. A method as in item 136 wherein the subject's platelet count is greater than 75,000 per microliter.

146. A method as in item 136 wherein the subject's platelet count is greater than 150,000 per microliter.

147. A method as in item 136 wherein the subject's reticulocyte count is greater than 80 x 10^9 per liter.

148. A method as in item 136 wherein the subject's reticulocyte count is greater than 120 x 10^9 per liter.

149. A method as in item 136 wherein the subject's reticulocyte count is greater than 150 x 10^9 per liter.

150. A method as in item 136 wherein said subject has a greater than 40% PNH type III granulocyte clone.

151. A method as in item 136 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

152. A method of treating a nitric oxide (NO) deficiency in a subject afflicted with a hemolytic disease comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

153. A method as in item 152 wherein the step of administering comprises administering an anti-C5 antibody.

154. A method as in item 152 wherein the compound is an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

155. A method as in item 152 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

156. A method as in item 152 wherein the step of administering comprises administering a compound selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin,

Samsung Bioepis Exhibit No. 1002
Page 1886

and K76 COOH.

157. A method as in item 152 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.

158. A method as in item. 152 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.

159. A method as in item 152 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.

160. A method as in item 152 wherein the subject's platelet count is greater than 40,000 per microliter.

161. A method as in item 152 wherein the subject's platelet count is greater than 75,000 per microliter.

162. A method as in item 152 wherein the subject's platelet count is greater than 150,000 per microliter.

163. A method as in item 152 wherein the subject's reticulocyte count is greater than 80 x 10^9 per liter.

164. A method as in item 152 wherein the subject's reticulocyte count is greater than 120 x 10^9 per liter.

165. A method as in item 152 wherein the subject's reticulocyte count is greater than 150 x 10^9 per liter.

166. A method as in item 152 wherein said subject has a greater than 40% PNH type III granulocyte clone.

167. A method as in item 152 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

168. A method of rendering a subject afflicted with a hemolytic disease transfusion independent comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.

169. A method as in item 168 wherein the step of administering comprises administering an anti-C5 antibody.

170. A method as in item 168 wherein the compound is an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

171. A method as in item 168 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

172. A method as in item 168 wherein the step of administering comprises administering a compound selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

173. A method as in item 168 further comprising the step of administering one or more compounds that increase hematopoiesis in combination with said compound.

174. A method as in item 173 wherein the one or more compounds that increase hematopoiesis is selected from the group consisting of steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin (EPO), antithymocyte globulin (ATG) and antilymphocyte globulin (ALG).

175. A method as in item 173 wherein EPO is administered in combination with an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

176. A method as in item 168 wherein the subject is transfusion independent for over six months from the first administration of said compound.

177. A method as in item 168 wherein the subject is transfusion independent for over twelve months from the first administration of said compound.

178. A method as in item 168 wherein the subject is transfusion independent for over two years from the first administration of said compound.

179. A method as in item 168 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.

180. A method as in item 168 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.

181. A method as in item 168 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.

182. A method as in item 168 wherein the subject's platelet count is greater than 40,000 per microliter.

183. A method as in item 168 wherein the subject's platelet count is greater than 75,000 per microliter.

184. A method as in item 168 wherein the subject's platelet count is greater than 150,000 per microliter.

185. A method as in item 168 wherein the subject's reticulocyte count is greater than 80 x 10^9 per liter.

186. A method as in item 168 wherein the subject's reticulocyte count is greater than 120 x 10^9 per liter.

187. A method as in item 168 wherein the subject's reticulocyte count is greater than 150 x 10^9 per liter.

188. A method as in item 168 wherein said subject has a greater than 40% PNH type III granulocyte clone.

189. A method as in item 168 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

Samsung Bioepis Exhibit No. 1002
Page 1887

190. A method of treating a subject afflicted with a hemolytic disease comprising administering: 1) one or more compounds selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components; in combination with 2) one or more compounds that increase hematopoiesis.

191. A method as in item 190 wherein the one or more compounds that increase hematopoiesis are selected from the group consisting of steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin (EPO), antithymocyte globulin (ATG) and antilymphocyte globulin (ALG).

192. A method as in item 190 wherein EPO is administered in combination with an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

193. A method as in item 190 wherein EPO is administered in combination with a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

194. A method as in item 190 wherein EPO is administered in combination with a compound selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

195. A method as in item 190 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.

196. A method as in item 190 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.

197. A method as in item 190 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.

198. A method as in item 190 wherein the subject's platelet count is greater than 40,000 per microliter.

199. A method as in item 190 wherein the subject's platelet count is greater than 75,000 per microliter.

200. A method as in item 190 wherein the subject's platelet count is greater than 150,000 per microliter.

201. A method as in item 190 wherein the subject's reticulocyte count is greater than 80 x 10^9 per liter.

202. A method as in item 190 wherein the subject's reticulocyte count is greater than 120 x 10^9 per liter.

203. A method as in item 190 wherein the subject's reticulocyte count is greater than 150 x 10^9 per liter.

204. A method as in item 190 wherein said subject has a greater than 40% PNH type III granulocyte clone.

205. A method as in item 190 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

206. A method of reducing hemolysis in a subject having a hemolytic disease comprising administering an anti-C5 antibody, wherein hemolysis is reduced as evidenced by a reduction in lactate dehydrogenase (LDH) levels in the subject's bloodstream to within 20% of the upper limit of normal.

207. A method as in item 206 wherein the anti-C5 antibody is selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.

208. A method as in item 206 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.

209. A method as in item 206 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.

210. A method as in item 206 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.

211. A method as in item 206 wherein the subject's platelet count is greater than 40,000 per microliter.

212. A method as in item 206 wherein the subject's platelet count is greater than 75,000 per microliter.

213. A method as in item 206 wherein the subject's platelet count is greater than 150,000 per microliter.

214. A method as in item 206 wherein the subject's reticulocyte count is greater than 80 x 10^9 per liter.

215. A method as in item 206 wherein the subject's reticulocyte count is greater than 120 x 10^9 per liter.

216. A method as in item 206 wherein the subject's reticulocyte count is greater than 150 x 10^9 per liter.

217. A method as in item 206 wherein said subject has a greater than 40% PNH type III granulocyte clone.

218. A method as in item 206 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

219. A method of reducing hemolysis in a subject having a hemolytic disease comprising administering to the subject a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors, wherein hemolysis is reduced as evidenced by a reduction in lactate dehydrogenase (LDH) levels in the subject's bloodstream to within 20% of the upper limit of normal.

Samsung Bioepis Exhibit No. 1002
Page 1888

220. A method as in item 219 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.
221. A method as in item 219 wherein said subject has a greater than 40% PNH type III granulocyte clone.
222. A method as in item 219 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.
223. A method of reducing hemolysis in a subject having a hemolytic disease comprising administering an anti-C5 antibody, wherein serum complement hemolytic activity is reduced at least 80% as evidenced by a serum hemolytic assay.
224. A method as in item 223 wherein the anti-C5 antibody is selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.
225. A method as in item 223 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.
226. A method as in item 223 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.
227. A method as in item 223 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.
228. A method as in item 223 wherein the subject's platelet count is greater than 40,000 per microliter.
229. A method as in item 223 wherein the subject's platelet count is greater than 75,000 per microliter.
230. A method as in item 223 wherein the subject's platelet count is greater than 150,000 per microliter.
231. A method as in item 223 wherein the subject's reticulocyte count is greater than 80 x 10^9 per liter.
232. A method as in item 223 wherein the subject's reticulocyte count is greater than 120 x 10^9 per liter.
233. A method as in item 223 wherein the subject's reticulocyte count is greater than 150 x 10^9 per liter.
234. A method as in item 223 wherein said subject has a greater than 40% PNH type III granulocyte clone.
235. A method as in item 223 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.
236. A method of reducing hemolysis in a subject having a hemolytic disease comprising administering to the subject a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors, wherein serum complement hemolytic activity is reduced at least 80% as evidenced by a serum hemolytic assay.
237. A method as in item 236 wherein the compound is selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.
238. A method as in item 236 wherein said subject has a greater than 40% PNH type III granulocyte clone.
239. A method as in item 236 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.
240. A method of reducing abdominal pain in a subject comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.
241. A method as in item 240 wherein the step of administering comprises administering an anti-C5 antibody.
242. A method as in item 240 wherein the compound is an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.
243. A method as in item 240 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.
244. A method as in item 240 wherein the step of administering comprises administering a compound selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.
245. A method as in item 240 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.
246. A method as in item 240 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.
247. A method as in item 240 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.
248. A method as in item 240 wherein the subject's platelet count is greater than 40,000 per microliter.
249. A method as in item 240 wherein the subject's platelet count is greater than 75,000 per microliter.

Samsung Bioepis Exhibit No. 1002
Page 1889

250. A method as in item 240 wherein the subject's platelet count is greater than 150,000 per microliter.
251. A method as in item 240 wherein the subject's reticulocyte count is greater than 80 x 10^9 per liter.
252. A method as in item 240 wherein the subject's reticulocyte count is greater than 120 x 10^9 per liter.
253. A method as in item 240 wherein the subject's reticulocyte count is greater than 150 x 10^9 per liter.
254. A method as in item 240 wherein said subject has a greater than 40% PNH type III granulocyte clone.
255. A method as in item 240 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.
256. A method of decreasing the frequency of transfusions required by a subject afflicted with a hemolytic disease comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components and compounds which block the activity of one or more complement components.
257. The method of item 256 wherein the frequency of transfusions is decreased by about 50%.
258. The method of item 256 wherein the frequency of transfusions is decreased by about 70%.
259. The method of item 256 wherein the frequency of transfusions is decreased by about 90%.
260. A method as in item 256 wherein the step of administering comprises administering an anti-C5 antibody.
261. A method as in item 256 wherein the step of administering comprises administering an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.
262. A method as in item 256 wherein the step of administering comprises administering a compound selected from the group consisting of antibodies, soluble complement inhibitory compounds, proteins, protein fragments, peptides, small molecules, RNA aptamers, L-RNA aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.
263. A method as in item 256 wherein the step of administering comprises administering a compound selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.
264. A method as in item 256 further comprising the step of administering one or more compounds that increase hematopoiesis in combination with said compound.
265. A method as in item 264 wherein the one or more compounds that increase hematopoiesis is selected from the group consisting of steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin (EPO), antithymocyte globulin (ATG) and antilymphocyte globulin (ALG).
266. A method as in item 264 wherein EPO is administered in combination with an anti-C5 antibody selected from the group consisting of h5G1.1-mAb, h5G1.1-scFv and functional fragments of h5G1.1.
267. A method as in item 256 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 10%.
268. A method as in item 256 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 25%.
269. A method as in item 256 wherein the proportion of type III red blood cells of the subject's total red blood cell content is greater than 50%.
270. A method as in item 256 wherein the subject's platelet count is greater than 40,000 per microliter.
271. A method as in item 256 wherein the subject's platelet count is greater than 75,000 per microliter.
272. A method as in item 256 wherein the subject's platelet count is greater than 150,000 per microliter.
273. A method as in item 256 wherein the subject's reticulocyte count is greater than 80 x 10^9 per liter.
274. A method as in item 256 wherein the subject's reticulocyte count is greater than 120 x 10^9 per liter.
275. A method as in item 256 wherein the subject's reticulocyte count is greater than 150 x 10^9 per liter.
276. A method as in item 256 wherein said subject has a greater than 40% PNH type III granulocyte clone.
277. A method as in item 256 wherein said subject has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

Claims

1. An anti-C5 antibody for use in the treatment of paroxysmal nocturnal hemoglobinuria (PNH).

2. The anti-C5 antibody for use according to claim 1, wherein the anti-C5 antibody is eculizumab.

3. The anti-C5 antibody for use according to claim 2, wherein eculizumab is to be administered by intravenous infusion to the patient under the following dosing schedule:

   (i) 600 mg each week for four weeks;

Samsung Bioepis Exhibit No. 1002
Page 1890

(ii) 900 mg 1 week later;
(iii) 900 mg on a biweekly basis; and thereafter
(iv) 900 mg every 12 days,
wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii).

4. The anti-C5 antibody for use according to claim 3, wherein cell lysis is reduced to below 20% of normal serum complement activity when treated under a dosing schedule according to items (i) to (iv).

5. The anti-C5 antibody for use according to any one of claims 1 to 4, wherein the anti-C5 antibody is to be administered in combination with one or more compounds that increase hematopoiesis.

6. The anti-C5 antibody for use according to claim 5, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin, antithymocyte globulin, and antilymphocyte globulin.

7. The anti-C5 antibody for use according to any one of claims 1 to 6, wherein the patient is **characterized by** one or more characteristics selected from the group consisting of:

(a) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;
(b) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 25%;
(c) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 50%;
(d) the patient's platelet count is greater than 40,000 per microliter;
(e) the patient's platelet count is greater than 75,000 per microliter;
(f) the patient's platelet count is greater than 150,000 per microliter;
(g) the patient's reticulocyte count is greater than 80 x 10^9 per liter;
(h) the patient's reticulocyte count is greater than 120 x 10^9 per liter;
(i) the patient's reticulocyte count is greater than 150 x 10^9 per liter;
(j) the patient has a greater than 40% type III granulocyte clone; and
(k) the patient has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

8. Use of an anti-C5 antibody in the preparation of a medicament for treating a patient with paroxysmal nocturnal hemoglobinuria (PNH).

9. The use according to claim 8, wherein the anti-C5 antibody is eculizumab.

10. The use according to claim 9, wherein eculizumab is to be administered by intravenous infusion to the patient under the following dosing schedule:

(i) 600 mg each week for four weeks;
(ii) 900 mg 1 week later;
(iii) 900 mg on a biweekly basis; and thereafter
(iv) 900 mg every 12 days,
wherein said patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii).

11. The use according to claim 10, wherein cell lysis is reduced to below 20% of normal serum complement activity when treated under a dosing schedule according to items (i) to (iv).

12. The use according to any one of claims 8 to 11, wherein the anti-C5 antibody is to be administered in combination with one or more compounds that increase hematopoiesis.

13. The use according to claim 12, wherein said one or more compounds that increase hematopoiesis are selected from the group consisting of: steroids, immunosuppressants, anti-coagulants, folic acid, iron, erythropoietin, antithymocyte globulin, and antilymphocyte globulin.

14. The use according to any one of claims 8 to 13, wherein the patient is **characterized by** one or more characteristics selected from the group consisting of:

Samsung Bioepis Exhibit No. 1002
Page 1891

(l) the proportion of type III red blood cells of the patient's total red blood cell count is greater than 10%;
(m) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 25%;
(n) the proportion of type III red blood cells of the patient's total red blood cell content is greater than 50%;
(o) the patient's platelet count is greater than 40,000 per microliter;
(p) the patient's platelet count is greater than 75,000 per microliter;
(q) the patient's platelet count is greater than 150,000 per microliter;
(r) the patient's reticulocyte count is greater than 80 x 10^9 per liter;
(s) the patient's reticulocyte count is greater than 120 x 10^9 per liter;
(t) the patient's reticulocyte count is greater than 150 x 10^9 per liter;
(u) the patient has a greater than 40% type III granulocyte clone; and
(v) the patient has an LDH level greater than or equal to 1.5 times the upper limit of a normal LDH level in a person.

Samsung Bioepis Exhibit No. 1002
Page 1892

Figure 1A.
Biochemical Parameters of Hemolysis During Eculizumab Treatment

Biochemical Marker	Normal range	Time of Analysis			p-value[a]
		Pre-study[b]	12 weeks	64 weeks	
LDH (IU/L)	150 - 480	3110.7 +/- 598.4	594.0 +/- 31.7	622.4 +/-41.1	0.002
AST (IU/L)	10 - 40	76.2 +/- 16.0	26.2 +/- 2.3	30.1 +/- 3.2	0.02
Haptoglobin (g/L)	0.5 - 2	<0.06	<0.06	0.14 +/- 0.07[c]	N.S.[d]
Hemoglobin (g/dL)	11.5 - 18	10.0 +/- 0.4	10.3 +/- 0.4	10.4 +/- 0.4	N.S.
Bilirubin	3 - 15	25.9 +/- 4.3	28.2 +/- 4.4	28.7 +/- 4.0	N.S.
Reticulocytes ($x10^{-3}/mm^3$)	20 - 80	161.4 +/- 25.9	191.2 +/- 23.6	189.6 +/- 21.8	N.S.

[a]from comparisons of mean change from pre-study to 64 weeks
[b]values represent means during 52 week period prior to treatment except for AST which represents the baseline mean
[c]10 of 11 patients were below the detectable limit of haptoglobin (<0.06 g/L); 1 patient had a value of 0.69 g/L.
[d]not significant

Samsung Bioepis Exhibit No. 1002
Page 1893

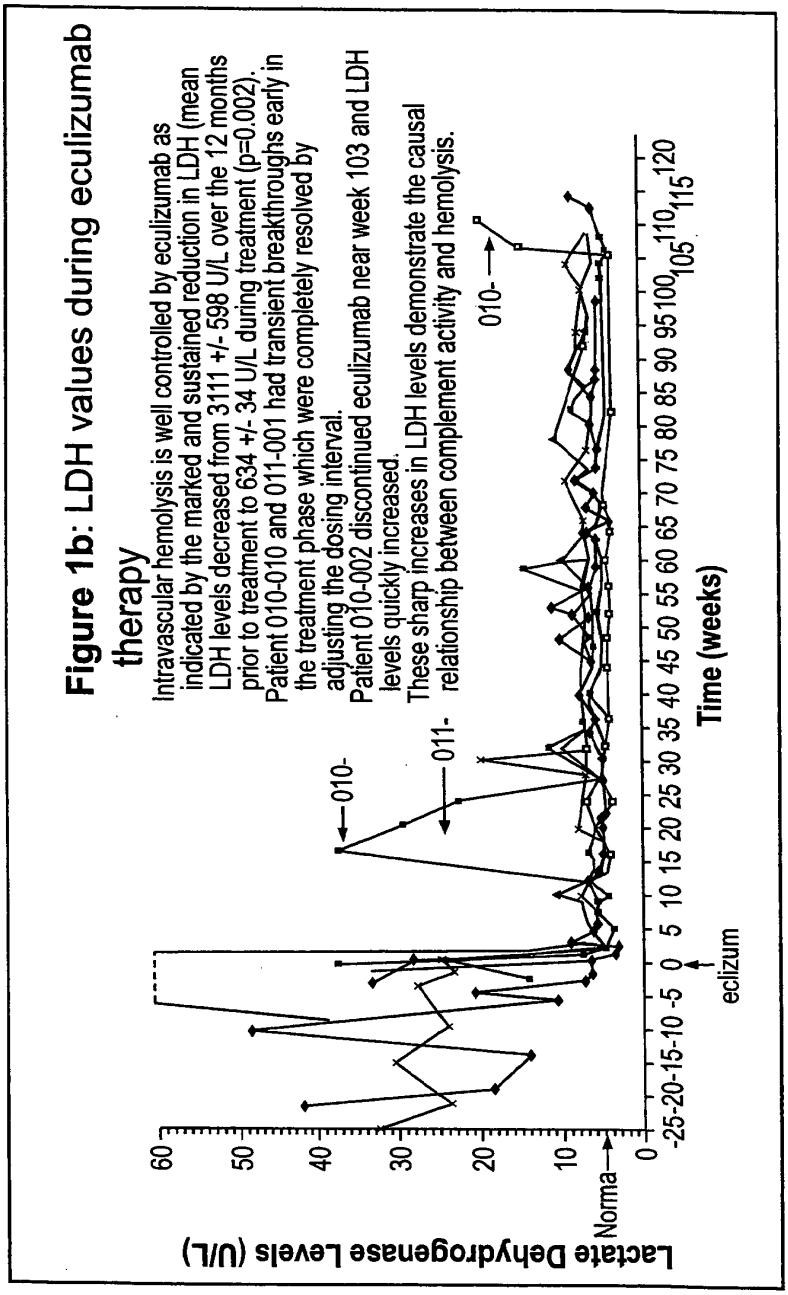


Figure 1b: LDH values during eculizumab therapy

Intravascular hemolysis is well controlled by eculizumab as indicated by the marked and sustained reduction in LDH (mean LDH levels decreased from 3111 +/- 598 U/L over the 12 months prior to treatment to 634 +/- 34 U/L during treatment (p=0.002). Patient 010-010 and 011-001 had transient breakthroughs early in the treatment phase which were completely resolved by adjusting the dosing interval. Patient 010-002 discontinued eculizumab near week 103 and LDH levels quickly increased. These sharp increases in LDH levels demonstrate the causal relationship between complement activity and hemolysis.

Samsung Bioepis Exhibit No. 1002
Page 1894

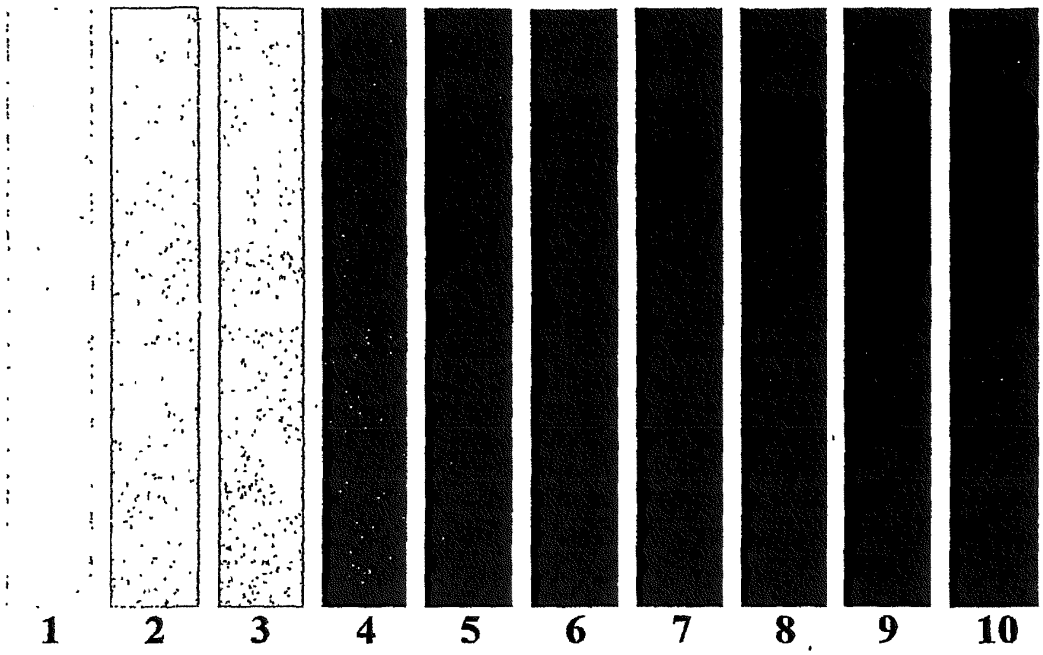
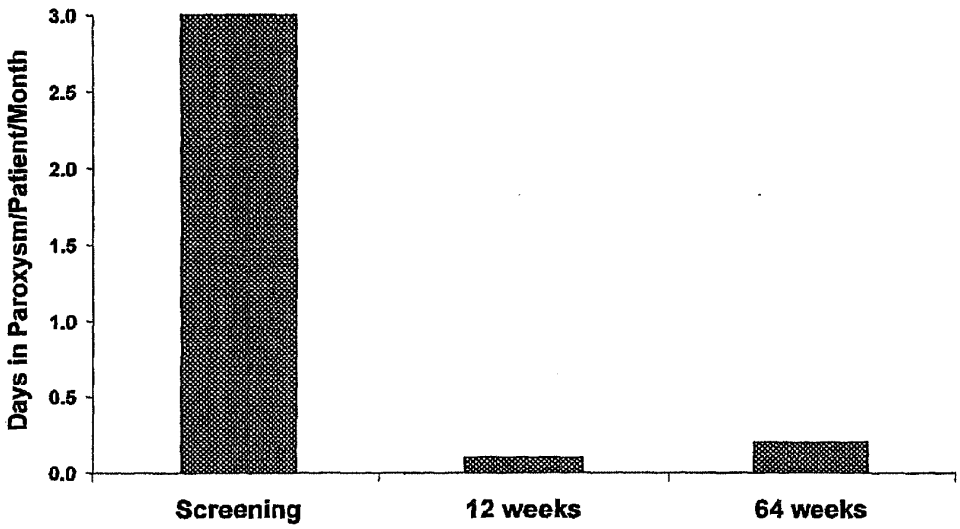


Figure 2

Samsung Bioepis Exhibit No. 1002
Page 1895

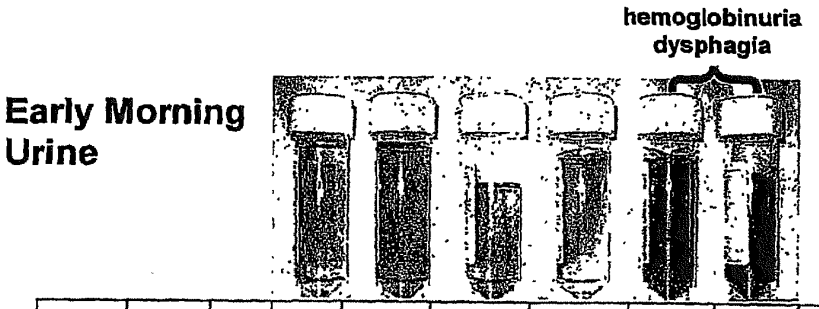
Figure 3.
Effect of Eculizumab on Paroxysm Rate (n=8)



Bars represent the paroxysm rates (number of paroxysms per patient per month) during the screening period (pre-eculizumab treatment), and during the first 12 weeks and the entire 64 weeks of eculizumab treatment. Three patients were not included in the analysis as either pretreatment urine scores were inadvertently not collected (2 patients) or an iron chelating agent that resulted in artificially colored urine was administered during the extension study (1 patient).

Samsung Bioepis Exhibit No. 1002
Page 1896

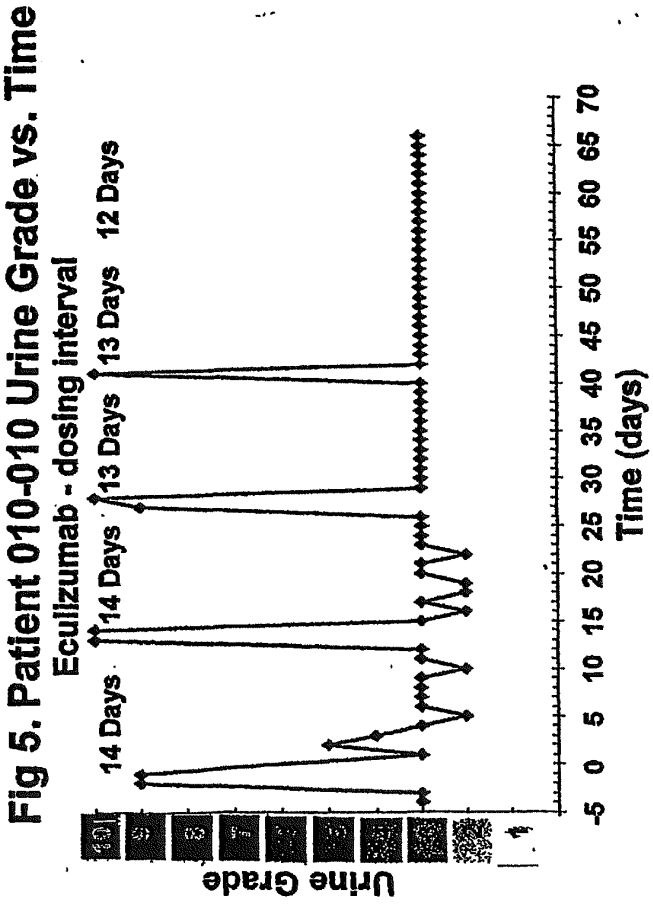
Figure 4.
Analysis of Complement Breakthrough



Visit	4							5			9
Day	0*	1-8	9	10	11	12	13	0*	1-2		0*
Urine	10	2-3	3	3	3	3	9	10	3		2
LDH	2624	–	784	–	–	697	1687	2917	–		495
AST	119	–	38	–	–	31	87	–	–		–
PK	28	–	51	–	–	35	31	23	–		51
PD	72	–	1	–	–	2	56	56	–		2

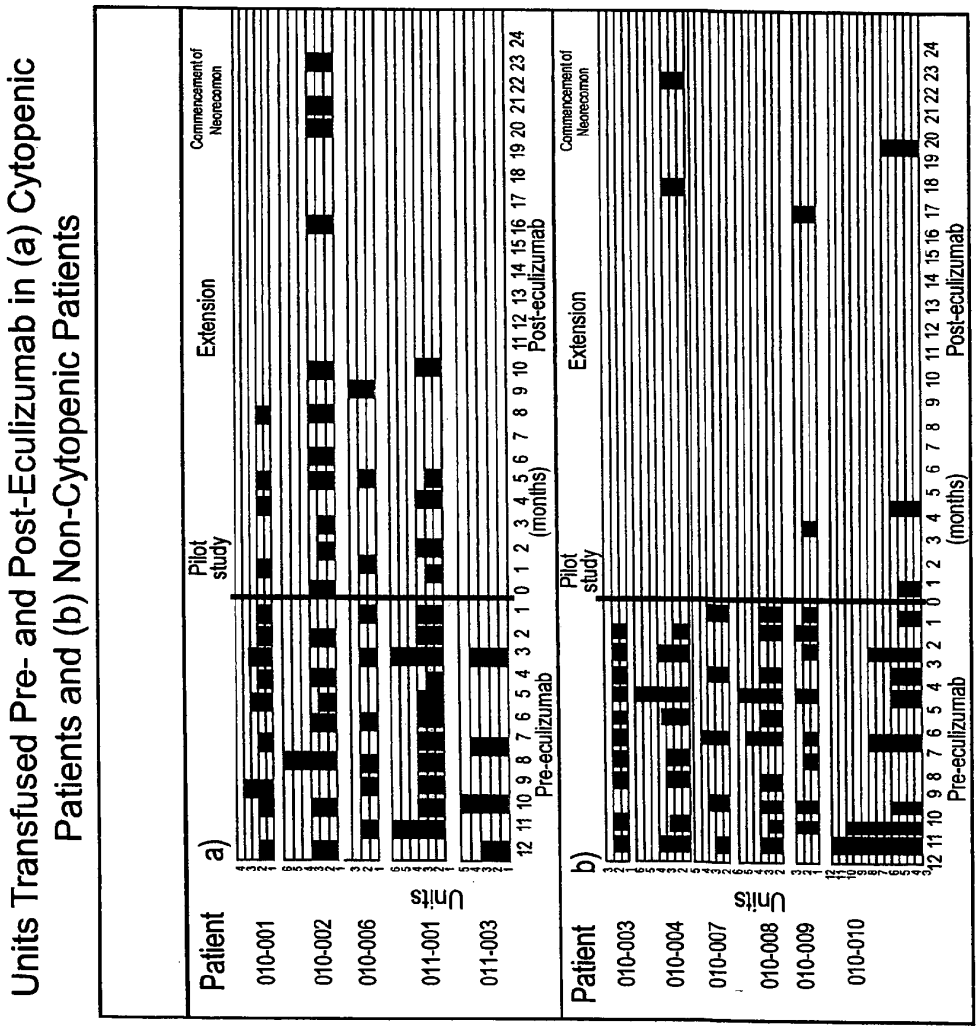
*Dose of eculizumab

Samsung Bioepis Exhibit No. 1002
Page 1897



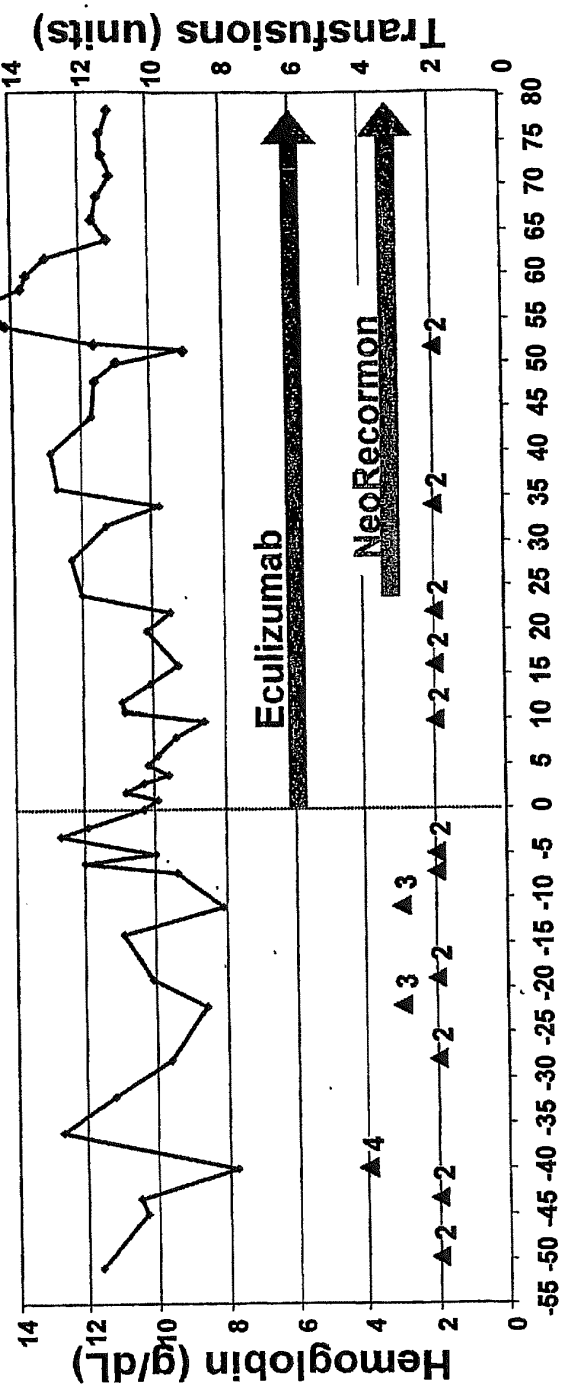
Samsung Bioepis Exhibit No. 1002
Page 1898

Figure 6.



Samsung Bioepis Exhibit No. 1002
Page 1899

Figure 7 Management of thrombocytopenic patient with erythropoietin



Transfusions reduced with eculizumab and transfusion independence with combined eculizumab and erythropoietin (NeoRecormon 18,000U 3x/week)

Samsung Bioepis Exhibit No. 1002
Page 1900

Figure 8.
Eculizumab Pharmacodynamics Pre- and Post-dose

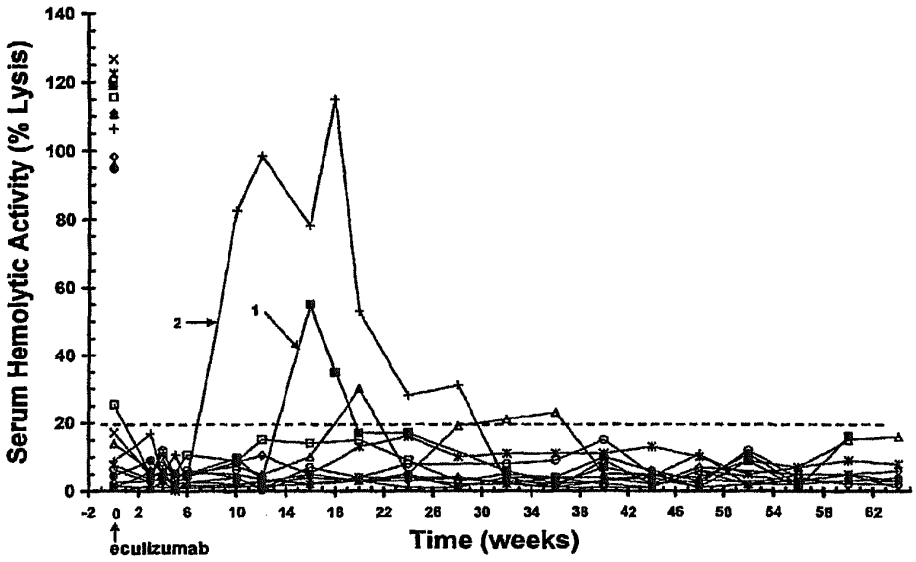


Figure 8 shows serum hemolytic activity (PD) during the 64 week treatment period as determined by the ability of serum to lyse antibody-presensitized chicken erythrocytes. The percentage of hemolytic activity at which complement is considered to be effectively inhibited (≤ 20 %) is indicated by dashed line. Two patients that demonstrated trough serum hemolytic activity values less than 20% are identified (patients 1 and 2).

Samsung Bioepis Exhibit No. 1002
Page 1901

Figure 9.
Eculizumab Pharmacodynamics Pre- and Post-dose

Domain (a)	Mean Base-Line Score (b)	64 Week Change from Base-line (c)	p-value (d)
Global Health Status	56.1	13.8	0.009
Physical Functioning	70.9	14.3	<0.001
Emotional Functioning	70.5	12.5	<0.001
Role Functioning	66.7	14.5	0.003
Cognitive Functioning	77.3	10.3	0.001
Fatigue	47.5	-17.8	<0.001
Dyspnea	39.4	-16.6	0.002
Insomnia	30.3	-8.2	0.031
Pain	21.2	-8.2	0.023
Constipation	3.0	4.1	<0.001

a) the quality of life was assessed using the European Organization for Research and Treatment of Cancer QLQ-C30 instrument
b) numbers represent mean values of linearly transformed scores
c) values represent least-square means; positive change indicates improvement for Global Health Status and Functional Scales while a negative change indicates improvement for Symptom Scales
d) values are from a mixed analysis-of-covariance model with visit as a fixed effect, patient as a random effect, and baseline as a covariate

Samsung Bioepis Exhibit No. 1002
Page 1902

Figure 10.
Symptoms Pre- and 2 Years Post-Eculizumab

Patient	Pre-Eculizumab			Post-Eculizumab		
	Abdominal Pain	Dysphagia	Erectile Dysfunction	Abdominal Pain	Dysphagia	Erectile Dysfunction
010-001	Every 4 – 8 weeks	Every 4 – 8 weeks	Every 4 – 8 weeks	None	None	None
010-002	None	None	--	None	None	--
010-003	Every week	Every week	Every week	None	None	None
010-004	Every 4 – 6 weeks	None	--	None	None	--
010-006	None	None	None	None	None	None
010-007	None	At least every 4 weeks	--	None	None	--
010-008	None	Every 10 weeks	Every 10 weeks	None	None	Intermittent
010-009	None	None	None	None	None	None
010-010	Every 4 weeks	Every 4 weeks	None	None	2 episodes in 2 years*	None
011-001	None	None	--	None	None	--
011-003	None	None	--	None	None	--

*This patient experienced a transient breakthrough in complement blockade with a return of hemolysis and symptoms. Increasing the dosing frequency to 12 days re-established complete complement blockade and prevented further symptoms.

Samsung Bioepis Exhibit No. 1002
Page 1903

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EUROPEAN SEARCH REPORT

Application Number
EP 15 15 0658

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	ROSSE WENDELL F ET AL: "Immune-mediated hemolytic anemia.", HEMATOLOGY / THE EDUCATION PROGRAM OF THE AMERICAN SOCIETY OF HEMATOLOGY. AMERICAN SOCIETY OF HEMATOLOGY. EDUCATION PROGRAM 2004, January 2004 (2004-01), pages 48-62, XP002497825, ISSN: 1520-4391	1,2,8,9	INV. A61K39/395 C07K16/18 A61K45/06 A61K39/00
Y	* the whole document *	1-14	
X	JANE SALODOF MACNEIL: "Eculizumab could be paroxysmal nocturnal hemoglobinuria breakthrough", 19000101 , 13 December 2002 (2002-12-13), pages 1-2, XP009106338, Medscape Medical News Retrieved from the Internet: URL:http://bcbsma.medscape.com/viewarticle/446336 [retrieved on 2008-09-24]	1,2,8,9	TECHNICAL FIELDS SEARCHED (IPC) A61K C07K
Y	* the whole document *	1-14	
A	"Alexion receives FDA, European approval for orphan drug status of new treatment for paroxysmal nocturnal hemoflobinuria", TRANSPLANT NEWS , 15 January 2004 (2004-01-15), XP002497241, Retrieved from the Internet: URL:http://findarticles.com/p/articles/mi_m0YUG/is_1_14/ai_n17208612 [retrieved on 2008-09-25] * the whole document *	1-14	

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	22 June 2015	Chapman, Rob

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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Samsung Bioepis Exhibit No. 1002
Page 1904

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EUROPEAN SEARCH REPORT

Application Number
EP 15 15 0658

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A	HALL CLAIRE ET AL: "Primary prophylaxis with warfarin prevents thrombosis in paroxysmal nocturnal hemoglobinuria (PNH)", BLOOD, AMERICAN SOCIETY OF HEMATOLOGY, US, vol. 102, no. 10, 15 November 2003 (2003-11-15), pages 3587-3591, XP002482083, ISSN: 0006-4971 * The whole document, in particular the abstract *	1-14	
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	22 June 2015	Chapman, Rob

CATEGORY OF CITED DOCUMENTS

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Samsung Bioepis Exhibit No. 1002
Page 1905

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EUROPEAN SEARCH REPORT

Application Number
EP 15 15 0658

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Page 1906

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Samsung Bioepis Exhibit No. 1002
Page 1907

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14177646.8 / 2 815 767
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(71) Applicant: **Alexion Pharmaceuticals, Inc.**
Cheshire, CT 06410 (US)

(72) Inventors:
- **ROTHER, Russell P.**
Oklahoma City, OK 73151 (US)
- **WANG, Hao**
London, Ontario N5X 4H2 (CA)
- **ZHONG, Zhen**
deceased (CA)

(74) Representative: **Soames, Candida Jane**
Maschio & Soames IP Limited
30 Carlton Crescent
Southampton SO15 2EW (GB)

Remarks:
This application was filed on 15-03-2016 as a divisional application to the application mentioned under INID code 62.

(54) PROLONGATION OF SURVIVAL OF AN ALLOGRAFT BY INHIBITING COMPLEMENT ACTIVITY

(57) An antibody specific to C5, which inhibits the cleavage of C5, for use in prolonging survival of an allograft in a recipient mammal, wherein said antibody to C5 is to be administered chronically and said antibody to C5 is administered in conjunction with more than one immunosuppressive drug.

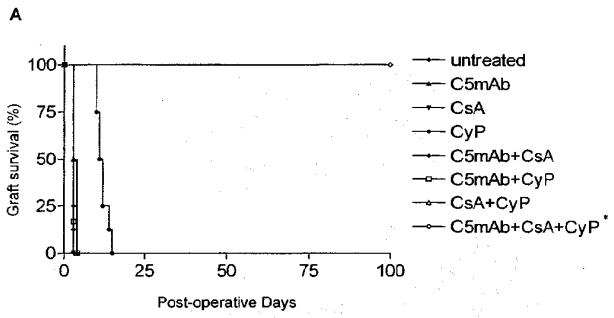


Figure 2A

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EP 3 056 218 A1

Description

RELATED APPLICATIONS

[0001] This application claims the benefit of and priority to U.S. Provisional Patent Application No. 60/571,444, filed May 14, 2004, the disclosure of which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

[0002] The present disclosure relates to methods for prolonging survival of an allograft in a mammal. In particular, the present disclosure relates to prolonging survival of an allograft by administering an inhibitor of complement or terminal complement formation, especially an inhibitor of complement C5 cleavage, in addition to one or more drugs that are immunosuppressant.

BACKGROUND

[0003] Organ transplantation is the preferred treatment for most patients with chronic organ failure. Although transplantation of kidney, liver, lung, and heart offers an excellent opportunity for rehabilitation as recipients return to a more normal lifestyle, it is limited by the medical/surgical suitability of potential recipients, an increasing shortage of donors, and premature failure of transplanted organ function.

[0004] Transplantation of cells, tissues and organs has become very common and is often a life-saving procedure. Organ transplantation is the preferred treatment for most patients with chronic organ failure. Despite great improvement in treatments to inhibit rejection, rejection continues to be the single largest impediment to successful organ transplantation. Rejection includes not only acute rejection but also chronic rejection. One-year survival rates for transplanted kidneys average 88.3% with kidneys from deceased donors and 94.4% with kidneys received from living donors. The corresponding five year survival rates for the transplanted kidneys are 63.3% and 76.5% (OPTN/SRTR Annual Report, 2002). For livers the one year survival rates are 80.2% and 76.5% for livers from deceased and living donors, respectively. The corresponding five year liver graft survival rates are 63.5% and 73.0% (OPTN/SRTR Annual Report, 2002). The use of immunosuppressant drugs, especially cyclosporin A and more recently tacrolimus, has dramatically improved the success rate of organ transplantation especially by preventing acute rejection. But as the numbers above show, there is still a need to improve the success rates, both short-term and especially long-term. As seen from the above numbers for kidney and liver transplants, the five year failure rates for these transplanted organs are on the order of 25-35%. In the year 2001 alone there were more than 23,000 patients who received an organ transplant of which approximately 19,000 were kidney or liver (OPTN/SRTR Annual Report, 2002). For this one year of transplants alone, with present techniques it can be expected that approximately 5,000-6,000 of these transplanted kidneys and livers will fail within 5 years. These numbers do not even include other transplanted organs or transplanted tissues or cells such as bone marrow.

[0005] There are multiple types of transplants. These are described in Abbas et al., 2000. A graft transplanted from one individual to the same individual is called an autologous graft or autograft. A graft transplanted between two genetically identical or syngeneic individual is called a syngeneic graft. A graft transplanted between two genetically different individuals of the same species is called an allogeneic graft or allograft. A graft transplanted between individuals of different species is called a xenogeneic graft or xenograft. The molecules that are recognized as foreign on allografts are called alloantigens and those on xenografts are called xenoantigens. The lymphocytes or antibodies that react with alloantigens or xenoantigens are described as being alloreactive or xenoreactive, respectively.

[0006] Currently more than 40,000 kidney, heart, lung, liver and pancreas transplants are performed in the United States each year (Abbas et al., 2000). Other possible transplants include, but are not limited to, vascular tissue, eye, cornea, lens, skin, bone marrow, muscle, connective tissue, gastrointestinal tissue, nervous tissue, bone, stem cells, islets, cartilage, hepatocytes, and hematopoietic cells. Unfortunately, there are many more candidates for a transplant than there are donors. To overcome this shortage, a major effort is being made to learn how to use xenografts. While progress is being made in this field, the fact is that at present most transplants are allografts. An allogeneic transplant, while presently being more likely to be successful than a xenogeneic transplant, must surmount numerous obstacles to be successful. There are several types of immunological attacks made by the recipient against the donor organ which can lead to rejection of the allograft. These include hyperacute rejection, acute vascular rejection (including accelerated humoral rejection and de novo acute humoral rejection), and chronic rejection. Rejection is normally a result of T-cell mediated or humoral antibody attack, but may include additional secondary factors such as the effects of complement and cytokines.

[0007] An ever growing gap between the number of patients requiring organ transplantation and the number of donor organs available has become a major problem throughout the world. Park et al., 2003. Individuals who have developed

Samsung Bioepis Exhibit No. 1002
Page 1910

anti-HLA antibodies are said to be immunized or sensitized. Gloor, 2005. HLA sensitization is the major barrier to optimal utilization of organs from living donors in clinical transplantation (Warren et al., 2004) due to the development of severe antibody-mediated rejection (ABMR). For example, more than 50% of all individuals awaiting kidney transplantation are presensitized patients (Glotz et al., 2002) who have elevated levels of broadly reactive alloantibodies, resulting from multiple transfusions, prior failed allografts, or pregnancy (Kupiec-Weglinski, 1996). The role of ABMR is currently one of the most dynamic areas of study in transplantation, due to recognition that this type of rejection can lead to either acute or chronic loss of allograft function. Mehra et al., 2003. Numerous cases of ABMR, including hyperacute rejection (HAR) or accelerated humoral rejection (ACHR), have been reported that are characterized by acute allograft injury that is resistant to potent anti-T cell therapy, the detection of circulating donor specific antibodies, and the deposition of complement components in the graft. ABMR with elevated circulating alloantibodies and complement activation that occurs in 20-30% of acute rejection cases has a poorer prognosis than cellular rejection. Mauiyyedi et al., 2002.

[0008] Highly presensitized patients, who exhibit high levels of alloantibodies, usually suffer an immediate and aggressive HAR. In clinical practice, with great efforts and significant advances in technology, HAR is avoided by obtaining a pretransplant lymphocytotoxic crossmatch to identify sensitized patients with antibodies specific for donor HLA antigens. However, circulating antibodies against donor HLA or other non-MHC endothelial antigens may also be responsible for a delayed form of acute humoral rejection, which is associated with an increased incidence of graft loss. Collins et al., 1999. Therefore, development of a novel presensitized animal model to mimic ABMR in clinical settings would be beneficial to studies on the mechanism, and to the much needed progress in the management of allograft rejection in presensitized hosts.

[0009] Some highly presensitized patients can benefit from intervention programs such as immunoadsorption (Palmer et al.,1989; Ross et al., 1993; Kriaa et al., 1995), plasmapheresis and intravenous immunoglobulin (Sonnenday et al., 2002; Rocha et al., 2003), that have been designed and implemented to temporarily eliminate anti-donor antibodies. However, in addition to their benefits, the aforementioned therapies carry with them numerous drawbacks as some individuals are less susceptible to their effects (Kriaa et al., 1995; Hakim et al., 1990; Glotz et al., 1993; Tyan et al., 1994); they are extremely expensive, time-consuming, and risky (Salama et al., 2001). Moreover, the transient and variable effect of these protocols has limited their impact. Glotz et al., 2002; Kupin et al., 1991; Schweitzer et al., 2000. Therefore, developing novel strategies to reduce the risk and cost in prevention of ABMR would be beneficial to presensitized recipients receiving an allograft.

SUMMARY

[0010] Accordingly, methods of prolonging survival of transplanted cells, tissues or organs are provided. In particular, methods of prolonging survival of allotransplanted cells, tissues or organs are provided. These methods are directed to using one or more immunosuppressants in addition to an inhibitor of complement activity. Use of one or more immunosuppressants and an inhibitor of complement activity in the manufacture of one or more medicaments or medicament packages is also provided. Such medicaments or medicament packages are useful in prolonging survival of an allograft in a subject mammal.

[0011] In certain embodiments, the inhibition of complement activity is effected by chronic administration of a drug directed against complement C5. A preferred drug that inhibits complement activity is an antibody specific to one or more components of complement, for example, C5. In certain preferred embodiments, the antibody inhibits the cleavage of C5 and thereby inhibits the formation of both C5a and C5b-9. The antibody may be, e.g., a monoclonal antibody, a chimeric antibody (e.g., a humanized antibody), an antibody fragment (e.g., Fab), a single chain antibody, an Fv, or a domain antibody. The recipient is also treated with one or more immunosuppressive drugs, for example, cyclosporin A.

[0012] In certain embodiments, either an MHC mismatched recipient (i.e., a mammalian recipient of an MHC mismatched allograft), a presensitized recipient or an ABO mismatched recipient (i.e., a mammalian recipient of an AMB mismatched allograft) is treated. In this model, the recipient is again chronically treated with a complement inhibitor, preferably an anti-C5 monoclonal antibody, together with immunosuppressive drugs, preferably a chronic administration of cyclosporin A and a short-term administration of cyclophosphamide. This triple therapy results in extended graft survival in the presensitized allotransplant recipient.

[0013] The present disclosure also provides methods of prolonging survival of an allograft in a mammalian recipient by administering to the recipient agents that modulate the level and/or ratio of subclasses and/or isotypes of anti-donor immunoglobulins (Ig) in the recipient. In certain embodiments, an agent that reduces the level of anti-donor IgG1 in the recipient is preferred. In certain embodiments, an agent that increases the level of anti-donor IgG2a and/or IgG2b in the recipient is preferred. In certain embodiments, an agent that reduces the ratio of anti-donor IgG1/anti-donor IgG2a or IgG2b in the recipient is preferred.

[0014] The present disclosure also provides a method of prolonging survival of an allograft in a second mammalian recipient using an allograft that has been accommodated in a first mammalian recipient (i.e., the allograft has prolonged survival in the first recipient). The present disclosure further provides an allograft that is resistant to anti-donor antibodies

Samsung Bioepis Exhibit No. 1002
Page 1911

in a mammalian recipient, and the allograft is prepared from a first recipient that has accommodated the allograft. In preferred embodiments, the first recipient has accommodated the allograft by receiving a treatment as described herein, such a triple therapy treatment involving administering to the first recipient a drug that inhibits complement activity and two immunosuppressive agents.

[0015] Further provided are pharmaceutical packages. A pharmaceutical package of the present disclosure may comprise a drug that inhibits complement activity and at least one immunosuppressive agent. The pharmaceutical package may further comprise a label for chronic administration. The pharmaceutical package may also comprise a label for self-administration by a patient, for example, a recipient of a transplant graft, or instructions for a caretaker of a recipient of a transplant graft. In certain embodiments, the drug and the agent in the pharmaceutical package are in a formulation or separate formulations that are suitable for chronic administration and/or self-administration.

[0016] The present disclosure also provides lyophilized formulations and formulations suitable for injection. Certain embodiments provide a lyophilized antibody formulation comprising an antibody that inhibits complement activity and a lyoprotectant. In preferred embodiments, the antibody formulation is suitable for chronic administration, for example, the antibody formulation is stable. Alternative embodiments provide an injection system comprising a syringe; the syringe comprises a cartridge containing an antibody that inhibits complement activity and is in a formulation suitable for injection.

[0017] An antibody employed in various embodiments of the present disclosure preferably inhibits the formation of terminal complement or C5a. In certain embodiments, antibody inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment. The whole antibody or antibody fragment may be a human, humanized, chimerized or deimmunized antibody or antibody fragment. In certain embodiments, the whole antibody or antibody fragment may inhibit cleavage of complement C5. In certain embodiments, the antibody fragment is a Fab, an F(ab')2, an Fv, a domain antibody, or a single-chain antibody. In preferred embodiments, the antibody fragment is pexelizumab. In alternative preferred embodiments, the whole antibody is eculizumab.

[0018] In certain embodiments, a drug, such as an antibody, that inhibits complement activity is present in unit dosage form, which can be particularly suitable for self-administration. Similarly, an immunosuppressive agent of the present disclosure may also be present in unit dosage form.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019]

Figures 1A-1D show anti-donor antibody levels in presensitized versus unsensitized recipients under different treatments.

Figures 2A and 2B show comparison between triple therapy using anti-C5 antibody, CsA and CyP in presensitized allograft recipients and combination therapy using only anti-C5 antibody and CsA in presensitized allograft recipients. Figure 2A compares heart-allograft survival in various recipients under different treatments as indicated. Figure 2B shows histology and immunohistology, for example, for lymphocyte infiltration in heart allografts of recipients in different groups.

Figure 3 shows blocked terminal complement activity by anti-C5 antibody as compared to immunosuppressive agents.

Figures 4A-4D compare levels of anti-donor antibodies in presensitized recipients of allografts under monotherapy with anti-C5 antibody alone, double combination therapy with anti-C5 antibody and CsA, and triple combination therapy with anti-C5 antibody, CsA and CyP.

Figures 5A and 5B show change of ratios of IgG isotypes in allograft recipients that were untreated or under different treatments.

Figure 6 shows high-level expression of Bcl-2 and Bcl-xl proteins in long-term surviving heart grafts as compared to heart grafts of untreated animals.

Figure 7 shows improved second transplantation (re-transplantation) of an accommodated graft from a first transplantation recipient.

Figure 8 shows results from re-transplantation experiments.

Figure 9 shows results from re-transplantation experiments.

Samsung Bioepis Exhibit No. 1002
Page 1912

DETAILED DESCRIPTION

Overview: Rejection of Transplants or Grafts

[0020] Hyperacute rejection occurs within minutes to hours after transplant and is due to preformed antibodies to the transplanted tissue antigens. It is characterized by hemorrhage and thrombotic occlusion of the graft vasculature. The binding of antibody to endothelium activates complement, and antibody and complement induce a number of changes in the graft endothelium that promote intravascular thrombosis and lead to vascular occlusion, the result being that the grafted organ suffers irreversible ischemic damage (Abbas et al., 2000). Hyperacute rejection is often mediated by preexisting IgM alloantibodies, e.g., those directed against the ABO blood group antigens expressed on red blood cells. This type of rejection, mediated by natural antibodies, is the main reason for rejection of xenotransplants. Hyperacute rejection due to natural IgM antibodies is no longer a major problem with allografts because allografts are usually selected to match the donor and recipient ABO type. Hyperacute rejection of an ABO matched allograft may still occur, usually mediated by IgG antibodies directed against protein alloantigens, such as foreign MHC molecules, or against less well defined alloantigens expressed on vascular endothelial cells. Such antibodies may arise as a result of prior exposure to alloantigens through blood transfusion, prior transplantation, or multiple pregnancies (this prior exposure being referred to as "presensitization"). Abbas et al., 2000.

[0021] Acute rejection is a process of vascular and parenchymal injury mediated by T cells, macrophages, and antibodies that usually begins after the first week of transplantation. Abbas et al., 2001. T lymphocytes play a central role in acute rejection by responding to alloantigens, including MHC molecules, present on vascular endothelial and parenchymal cells. The activated T cells cause direct lysis of graft cells or produce cytokines that recruit and activate inflammatory cells, which cause necrosis. Both $CD4^+$ and $CD8^+$ cells may contribute to acute rejection. The destruction of allogeneic cells in a graft is highly specific and a hallmark of $CD8^+$ cytotoxic T lymphocyte killing. Abbas et al., 2000. $CD4^+$ T cells may be important in mediating acute graft rejection by secreting cytokines and inducing delayed-type hypersensitivity-like reactions in grafts, with some evidence available that indicates that $CD4^+$ T cells are sufficient to mediate acute rejection. Abbas et al., 2000. Antibodies can also mediate acute rejection after a graft recipient mounts a humoral immune response to vessel wall antigens and the antibodies that are produced bind to the vessel wall and activate complement. Abbas et al., 2000.

[0022] Chronic rejection is characterized by fibrosis with loss of normal organ structures occurring over a prolonged period. The pathogenesis of chronic rejection is less well understood than that of acute rejection. Graft arterial occlusion may occur as a result of the proliferation of intimal smooth muscle cells (Abbas et al., 2000). This process is called accelerated or graft arteriosclerosis and can develop in any vascularized organ transplant within 6 months to a year after transplantation.

[0023] For a transplant to be successful, the several modes of rejection must be overcome. Multiple approaches are utilized in preventing rejection. This may require administration of immunosuppressants, often several types to prevent the various modes of attack, e.g., inhibition of T-cell attack, antibodies, and cytokine and complement effects. Prescreening of donors to match them with recipients is also a major factor in preventing rejection, especially in preventing hyperacute rejection. Immunoadsorption of anti-HLA antibodies prior to grafting may reduce hyperacute rejection. Prior to transplantation the recipient or host may be administered anti-T cell reagents, e.g., the monoclonal antibody OKT3, Anti-Thymocyte Globulin (ATG), cyclosporin A, or tacrolimus (FK 506). Additionally, glucocorticoids and/or azathioprine may be administered to the host prior to transplant. Drugs used to aid in preventing transplant rejection include, but are not limited to, ATG or ALG, OKT3, daclizumab, basiliximab, corticosteroids, 15-deoxyspergualin, cyclosporins, tacrolimus, azathioprine, methotrexate, mycophenolate mofetil, 6-mercaptopurine, bredinin, brequinar, leflunamide, cyclophosphamide, sirolimus, anti-CD4 monoclonal antibodies, CTLA4-Ig, anti-CD154 monoclonal antibodies, anti-LFA1 monoclonal antibodies, anti-LFA-3 monoclonal antibodies, anti-CD2 monoclonal antibodies, and anti-CD45.

[0024] Allografts are rejected in part by the activation of T cells. The transplant recipient mounts a rejection response following $CD4^+$ T cell recognition of foreign antigens in the allograft. These antigens are encoded by the major histocompatibility complex (MHC). There are both Class I and Class II MHC molecules. In humans the class I MHC molecules are HLA-A, HLA-B, and HLA-C. The class II MHC molecules in humans are called HLA-DR, HLA-DQ and HLA-DP. In mice the class I MHC molecules are H-2K, H-2D and H-2L and the class II MHC molecules are I-A and I-E. When $CD4^+$ T cells bind the foreign MHC antigens they are activated and undergo clonal proliferation. The activated T cells secrete cytokines which aid in activating monocytes/macrophages, B cells and cytotoxic $CD8^+$ T cells. The activated monocytes/macrophages release agents which result in tissue damage, the B cells produce alloantibodies which lead to complement mediated tissue destruction, and the $CD8^+$ T cells kill graft cells in an antigen-specific manner through induction of apoptosis and cell lysis.

Samsung Bioepis Exhibit No. 1002
Page 1913

Immunosuppressive Agents

[0025] The numerous drugs utilized to delay graft rejection (i.e., to prolong their survival) work in a variety of ways. Immunosuppressive agents are widely used. See Stepkowski, 2000, for a review of the mechanism of action of several immunosuppressive drugs. Cyclosporin A is one of the most widely used immunosuppressive drugs for inhibiting graft rejection. It is an inhibitor of interleukin-2 or IL-2 (it prevents mRNA transcription of interleukin-2). More directly, cyclosporin inhibits calcineurin activation that normally occurs upon T cell receptor stimulation. Calcineurin dephosphorylates NFAT (nuclear factor of activated T cells) enabling it to enter the nucleus and bind to interleukin-2 promoter. By blocking this process, cyclosporin A inhibits the activation of the $CD4^+$ T cells and the resulting cascade of events which would otherwise occur. Tacrolimus is another immunosuppressant that acts by inhibiting the production of interleukin-2.

[0026] Rapamycin (Sirolimus), SDZ RAD, and interleukin-2 receptor blockers are drugs that inhibit the action of interleukin-2 and therefore prevent the cascade of events described above.

[0027] Inhibitors of purine or pyrimidine biosynthesis are also used to inhibit graft rejection. These prevent DNA synthesis and thereby inhibit cell division including the ability of T cells to divide. The result is the inhibition of T cell activity by preventing the formation of new T cells. Inhibitors of purine synthesis include azathioprine, methotrexate, mycophenolate mofetil (MMF) and mizoribine (bredinin). Inhibitors of pyrimidine synthesis include brequinar sodium and leflunomide. Cyclophosphamide is an inhibitor of both purine and pyrimidine synthesis.

[0028] Yet another method for inhibiting T cell activation is to treat the recipient with antibodies to T cells. OKT3 is a murine monoclonal antibody against CD3 which is part of the T cell receptor. This antibody inhibits the T cell receptor and suppresses T cell activation.

[0029] Numerous other drugs and methods for delaying allotransplant rejection are known to and used by those of skill in the art. One approach has been to deplete T cells, e.g., by irradiation. This has often been used in bone marrow transplants, especially if there is a partial mismatch of major HLA. Administration to the recipient of an inhibitor (blocker) of the CD40 ligand-CD40 interaction and/or a blocker of the CD28-B7 interaction has been used (U.S. Patent 6,280,957). Published PCT patent application WO 01/37860 teaches the administration of an anti-CD3 monoclonal antibody and IL-5 to inhibit the Th1 immune response. Published PCT patent application WO 00/27421 teaches a method for prophylaxis or treatment of corneal transplant rejection by administering a tumor necrosis factor-α antagonist. Glotz et al. (2002) show that administration of intravenous immunoglobulins (IVIg) can induce a profound and sustained decrease in the titers of anti-HLA antibodies thereby allowing a transplant of an HLA-mismatched organ. Similar protocols have included plasma exchanges (Taube et al., 1984) or immunoadsorption techniques coupled to immunosuppressive agents (Hiesse et al., 1992) or a combination of these (Montgomery et al., 2000). Changelian et al. (2003) teach a model in which immunosuppression is caused by an oral inhibitor of Janus kinase 3 (JAK3) which is an enzyme necessary for the proper signaling of cytokine receptors which use the common gamma chain (yc) (Interleukins-2, -4, -7, -9, -15, -21), the result being an inhibition of T cell activation. Antisense nucleic acids against ICAM-1 have been used alone or in combination with a monoclonal antibody specific for leukocyte-function associated antigen 1 (LFA-1) in a study of heart allograft transplantation (Stepkowski, 2000). Similarly, an anti-ICAM-1 antibody has been used in combination with anti-LFA-1 antibody to treat heart allografts (Stepkowski, 2000). Antisense oligonucleotides have additionally been used in conjunction with cyclosporin in rat heart or kidney allograft models, resulting in a synergistic effect to prolong the survival of the grafts (Stepkowski, 2000). Chronic transplant rejection has been treated by administering an antagonist of TGF-β which is a cytokine involved in differentiation, proliferation and apoptosis (U.S. Patent Application Publication US 2003/0180301).

Complement and Transplant/Graft Rejection

[0030] The role of complement in transplant rejection is well known. This is especially true in the case of xenotransplantation, but complement also plays a role in allotransplant rejection. For review, see Platt and Saadi, 1999. One aspect of complement's role is that ischemia-reperfusion injury may occur at the time that an organ graft is reperfused with the blood of the recipient. Complement may also cause some manifestations of allograft rejection.

[0031] The complement system is described in detail in U.S. Patent 6,355,245. The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of cellular and viral pathogens. There are at least 25 complement proteins, which are found as a complex collection of plasma proteins and membrane cofactors. The plasma proteins make up about 10% of the globulins in vertebrate serum. Complement components achieve their immune defensive functions by interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions.

[0032] The complement cascade progresses via the classical pathway or the alternative pathway. These pathways share many components and, while they differ in their initial steps, they converge and share the same "terminal complement" components (C5 through C9) responsible for the activation and destruction of target cells.

Samsung Bioepis Exhibit No. 1002
Page 1914

[0033] The classical complement pathway is typically initiated by antibody recognition of and binding to an antigenic site on a target cell. The alternative pathway is usually antibody independent and can be initiated by certain molecules on pathogen surfaces. Both pathways converge at the point where complement component C3 is cleaved by an active protease (which is different in each pathway) to yield C3a and C3b. Other pathways activating complement attack can act later in the sequence of events leading to various aspects of complement function.

[0034] C3a is an anaphylatoxin. C3b binds to bacterial and other cells, as well as to certain viruses and immune complexes, and tags them for removal from the circulation. C3b in this role is known as opsonin. The opsonic function of C3b is considered to be the most important anti-infective action of the complement system. Patients with genetic lesions that block C3b function are prone to infection by a broad variety of pathogenic organisms, while patients with lesions later in the complement cascade sequence, i.e., patients with lesions that block C5 functions, are found to be more prone only to Neisseria infection, and then only somewhat more prone (Fearon, 1983).

[0035] C3b also forms a complex with other components unique to each pathway to form classical or alternative C5 convertase, which cleaves C5 into C5a and C5b. C3 is thus regarded as the central protein in the complement reaction sequence since it is essential to both the alternative and classical pathways (Wurzner et al., 1991). This property of C3b is regulated by the serum protease Factor I, which acts on C3b to produce iC3b. While still functional as opsonin, iC3b cannot form an active C5 convertase.

[0036] C5 is a 190 kDa beta globulin found in normal serum at approximately 75 μg/mL (0.4 μM). C5 is glycosylated, with about 1.5-3 percent of its mass attributed to carbohydrate. Mature C5 is a heterodimer of a 999 amino acid 115 kDa alpha chain that is disulfide linked to a 656 amino acid 75 kDa beta chain. C5 is synthesized as a single chain precursor protein product of a single copy gene (Haviland et al., 1991). The cDNA sequence of the transcript of this gene predicts a secreted pro-C5 precursor of 1659 amino acids along with an 18 amino acid leader sequence.

[0037] The pro-C5 precursor is cleaved after amino acid 655 and 659, to yield the beta chain as an amino terminal fragment (amino acid residues +1 to 655) and the alpha chain as a carboxyl terminal fragment (amino acid residues 660 to 1658), with four amino acids deleted between the two.

[0038] C5a is cleaved from the alpha chain of C5 by either alternative or classical C5 convertase as an amino terminal fragment comprising the first 74 amino acids of the alpha chain (i.e., amino acid residues 660-733). Approximately 20 percent of the 11 kDa mass of C5a is attributed to carbohydrate. The cleavage site for convertase action is at or immediately adjacent to amino acid residue 733. A compound that would bind at or adjacent to this cleavage site would have the potential to block access of the C5 convertase enzymes to the cleavage site and thereby act as a complement inhibitor.

[0039] C5 can also be activated by means other than C5 convertase activity. Limited trypsin digestion (Minta and Man, 1977; Wetsel and Kolb, 1982) and acid treatment (Yamamoto and Gewurz, 1978; Vogt et al., 1989) can also cleave C5 and produce active C5b.

[0040] C5a is another anaphylatoxin. C5b combines with C6, C7, and C8 to form the C5b-8 complex at the surface of the target cell. Upon binding of several C9 molecules, the membrane attack complex (MAC, C5b-9, terminal complement complex-TCC) is formed. When sufficient numbers of MACs insert into target cell membranes the openings they create (MAC pores) mediate rapid osmotic lysis of the target cells. Lower, non-lytic concentrations of MACs can produce other effects. In particular, membrane insertion of small numbers of the C5b-9 complexes into endothelial cells and platelets can cause deleterious cell activation. In some cases activation may precede cell lysis.

[0041] As mentioned above, C3a and C5a are anaphylatoxins. These activated complement components can trigger mast cell degranulation, which releases histamine and other mediators of inflammation, resulting in smooth muscle contraction, increased vascular permeability, leukocyte activation, and other inflammatory phenomena including cellular proliferation resulting in hypercellularity. C5a also functions as a chemotactic peptide that serves to attract pro-inflammatory granulocytes to the site of complement activation.

[0042] Complement-binding recipient antibodies to donor alloantigens are considered to be the main cause of hyperacute graft rejection. Owing to pretransplant crossmatch testing, this prototype of humoral rejection is now rarely observed (Regele et al., 2001). Data are now showing that humoral immune mechanisms might contribute to other types of allograft rejection (Regele et al., 2001). High levels of panel reactive antibodies indicating humoral presensitization were found to be associated with inferior kidney graft survival (Opelz, 1992), the appearance of alloantibodies during the post-transplant period has been reported to predict poor graft outcome (Jeannet et al., 1970; Halloran et al., 1992), and selective removal of recipient IgG by immunoadsorption reversed some rejection episodes indicating the contribution of humoral immune mechanisms to rejection (Persson et al., 1995; Böhmig et al., 2000). Complement activation within a graft might indicate antibody-mediated graft injury. The complement cleavage product C4d is a marker for activation of the antibody-dependent classical pathway. Capillary C4d deposits in kidney allograft biopsies were associated with poor graft outcome.

[0043] Recently increasing evidence indicates that complement activation significantly contributes to the sensitization of allograft recipients and the development of tissue injury in allografts (Platt et al., 1999). Antibodies are the most thoroughly investigated mediators of activating the classical complement pathway. Clinically, alloantibodies are known

Samsung Bioepis Exhibit No. 1002
Page 1915

to activate complement (Baldwin et al., 2001). Halloran and Collins indicate that C4d deposition in peritubular capillaries of renal allografts is a sensitive and diagnostic marker of acute humoral rejection that correlates strongly with the presence of circulating donor-specific antibodies (Collins et al., 1999; Halloran, 2003). Further supporting evidence is seen in animals with complement inhibition (Pratt et al., 1996; Pruitt et al., 1991; Forbes et al., 1978) or deficiency (Pratt et al., 2000; Baurer et al., 1995) which exhibit significantly reduced inflammatory injury and lowered anti-donor immune responses. In ABMR, complement is suggested to be activated by the classical pathway and to play a key role in the pathogenesis (Collard et al., 1997). Although the role of complement in HAR or acute vascular rejection (AVR) following xenotransplantation has been well documented (Platt et al., 1999), precise mechanisms of complement in the pathogenesis of ABMR following allotransplantation has not yet been elucidated.

[0044] The C5 component of complement is cleaved to form products with multiple proinflammatory effects and thus represents an attractive target for complement inhibition within the immune-mediated inflammatory response. As described above, C5a is a powerful anaphylatoxin and chemotactic factor. Cellular activation by C5a induces the release of multiple additional inflammatory mediators (Jose et al., 1983). The complement activation pathways (classical, alternative, or mannan-binding lectin pathway) ultimately lead to the formation of the cytolytic membrane attack complex C5b-9 (Kirschfunk, 2001), which can mediate both direct tissue injury by cell lysis, and proinflammatory cell activation at sublytic doses (Saadi et al., 1995; Papadimitriou et al., 1991). Therefore, blocking both C5a and C5b-9 generation may be required for the optimal inhibition of complement-mediated inflammatory response following transplantation. At the same time, inhibition of the complement cascade at C5 does not impair the generation of C3b, preserving C3b-mediated opsonization of pathogenic microorganisms as well as solubilization and clearance of immune complexes (Liszewski, 1993).

[0045] The beneficial effect of anti-C5 mAb has previously been reported in several experimental models including myocardial reperfusion (Vakeva et al., 1998), systemic lupus erythematosus (Wang et al., 1996) and rheumatoid arthritis (Wang et al., 1995); as well as in human clinical trials (Kirschfink, 2001) of autoimmune disease, cardiopulmonary bypass and acute myocardial infarction. In addition, complement inactivation by a functionally blocking anti-C5 monoclonal Ab (mAb) prevented HAR in xenotransplantation models (Kroshus et al., 1995; Wang et al., 1999).

[0046] Methods of delaying allotransplant rejection by administration of complement inhibitors have been tested. Published PCT patent application WO 92/10205 discloses the use of a combination of cyclosporin and a soluble complement receptor (sCR1) to inhibit rejection of a cardiac allotransplant in a presensitized rat model. Complement receptor 1 binds complements C3b and C4b. Soluble forms of complement receptor 1 occur naturally or can be generated via recombinant DNA procedures. These soluble complement receptors have inhibited in vitro the consequences of complement activation (U.S. Patent 6,057,131). In WO 92/10205, rats, which had been presensitized to the cardiac allograft they were receiving, were administered cyclosporin A intramuscularly at 10 mg/kg/day beginning two days prior to transplant and continued until the time of graft rejection. Additionally, soluble complement receptor 1 (sCR1) was administered as a single intravenous bolus at 15 mg/kg immediately prior to reperfusion of the graft. Control animals with no drug treatment had the graft rejected at an average of 3.8 days. Those administered cyclosporin A alone rejected the grafts at an average of 57 days (this was quite variable with two rats rejecting quickly at 2 and 4 days and a third rat rejecting at 166 days). Rats administered sCR1 alone rejected the grafts at an average of 44 days. Those rats administered the combination of cyclosporin A and sCR1 rejected the grafts at an average of 147 days. The combination of chronic cyclosporin A and single bolus sCR1 was seen to result in a synergistic effect greatly prolonging the time until graft rejection. Earlier studies by Pruitt and Bollinger (1991) used a similar model of a presensitized rat allograft to show that administration of sCR1 alone to inactivate complement resulted in increased time before graft rejection.

[0047] Sims et al. (U.S. Patent 5,135,916) suggest using inhibitors of complement, e.g., CD59 or antibodies against C7 or C9 to block the formation of the C5b-9 complex, to treat the vascular endothelium of organs and tissues to be transplanted. This would prevent the C5b-9 initiated cell necrosis. The C5b-9 inactivators would be added to the perfusate or storage medium to protect the vascular lining cells from ongoing complement activation during in vitro storage. Additionally the organ or tissue would be protected from the cytolytic and thrombotic effects arising from complement activation initiated upon transplantation, thereby circumventing complement mediated acute rejection. Sims et al. (U.S. Patent 5,573,940 and U.S. Patent 6,100,443) also teach a method of expressing CD59 in the transplanted tissue or organ to protect the transplanted organ from rejection. This can be accomplished by transfecting the cells being transplanted.

[0048] Although the several drugs developed to date in combination with methods of prescreening donors and recipients to match the donor allograft to the recipient have over time increased the average length of time of survival of allografts, many allografts are nonetheless rejected during the life-time of the recipient. In general, the prior art advances have mainly been directed to overcoming acute graft rejection. Further, the role of activated terminal complement components in antibody-mediated allograft rejection has not been examined using inhibitors that specifically target the complement cascade at the C5 protein level. The methods described herein and as exemplified in the Examples advance the allotransplant art by inhibiting chronic rejection of allografts, in particular, allografts in a presensitized recipient. New methods are presented for further prolonging allograft survival by using a proper combination of immunosuppressive

Samsung Bioepis Exhibit No. 1002
Page 1916

drugs in combination with a chronic administration of a complement inhibitor.

Methods and Uses

[0049] The methods disclosed herein are used to prolong allograft survival. The methods generally include administering an inhibitor of complement activity in combination with one or more immunosuppressants.

[0050] Suitable complement inhibitors are known to those of skill in the art. Antibodies can be made to individual components of activated complement, e.g., antibodies to C5a, C7, C9, etc. (see, e.g., U.S. Patent 6,534,058; published U.S. patent application US 2003/0129187; and U.S. Patent 5,660,825). Proteins are known which inhibit complement-mediated lysis, including CD59, CD55, CD46 and other inhibitors of C8 and C9 (see, e.g., U.S. Patent 6,100,443). U.S. Patent 6,355,245 teaches an antibody which binds to C5 and prevents it from being cleaved into C5a and C5b thereby preventing the formation not only of C5a but also the C5b-9 complex. Proteins known as complement receptors and which bind complement are also known (see, Published PCT Patent Application WO 92/10205 and U.S. Patent 6,057,131). Use of soluble forms of complement receptors, e.g., soluble CR1, can inhibit the consequences of complement activation such as neutrophil oxidative burst, complement mediated hemolysis, and C3a and C5a production. Those of skill in the art recognize the above as some, but not all, of the known methods of inhibiting complement and its activation.

[0051] Suitable immunosuppressants include, but are not limited to, ATG or ALG, OKT3, daclizumab, basiliximab, corticosteroids, 15-deoxyspergualin, cyclosporins, tacrolimus, azathioprine, methotrexate, mycophenolate mofetil, 6-mercaptopurine, bredinin, brequinar, leflunamide, cyclophosphamide, sirolimus, anti-CD4 monoclonal antibodies, CTLA4-Ig, anti-CD154 monoclonal antibodies, anti-LFA1 monoclonal antibodies, anti-LFA-3 monoclonal antibodies, anti-CD2 monoclonal antibodies, and anti-CD45.

[0052] An allograft can include a transplanted organ, part of an organ, tissue or cell. These include, but are not limited to, heart, kidney, lung, pancreas, liver, vascular tissue, eye, cornea, lens, skin, bone marrow, muscle, connective tissue, gastrointestinal tissue, nervous tissue, bone, stem cells, islets, cartilage, hepatocytes, and hematopoietic cells.

[0053] At least part of the reason for the failure of allografts is that one response by the recipient of an allograft is the activation of complement. This results in the formation of C5a and C5b-9 which are potent proinflammatory molecules which aid in causing graft failure. Without wishing to be bound by any proposed theory, Applicants theorized that inhibiting the formation of C5a and C5b-9 or inhibiting C5a and C5b-9 which was present would aid in preventing graft failure. Furthermore, it was theorized that so long as the allograft is present, the recipient will continue to attempt to mount an immune response against the graft, and this response will include attempts to produce C5a and C5b-9. If not prevented, this complement response will lead to acute vascular rejection in the short term and could contribute to chronic graft rejection in the long term. Prior art methods of using inhibitors of complement activity were limited to administering these inhibitors only at the time of transplant. This was helpful in preventing acute rejection, but as the results disclosed herein illustrate, improved results are obtained by administration of such inhibitors for a longer term. This long-term administration aids in preventing a chronic rejection of the allograft as opposed to only aiding in preventing an acute rejection. The result is a longer term survival of the allograft as compared to either not administering an inhibitor of complement activity or administering such an inhibitor only at the time of transplant of the allograft. Although very commonly it is desirable that the allograft will survive for the remaining lifetime of the recipient, there are times when the allograft is needed only for a shorter length of time, e.g., a bridge organ to bridge the time until the recipient's own organ can recover on its own, at which time the allograft will no longer be needed. The length of time such a graft will be needed will vary, but will usually be longer than the time at which acute rejection would occur and may be long enough for chronic rejection to occur. This period of desired survival for a bridge graft may be several months, e.g., six months.

[0054] To prove that long-term inhibition of complement activity will prolong allograft survival, experiments were performed in which complement activation was inhibited in a chronic fashion and not merely at the time of transplant. Chronic treatment means treatment during an extended period up to the lifetime of the allograft. This can be daily treatment but is not limited to daily treatment. Chronic treatment will maintain an effective amount of the drug in the allograft recipient. For example, a preferred method is to include the anti-C5 monoclonal antibody eculizumab in the treatment. In studies of persons suffering from paroxysmal nocturnal hemoglobinuria (PNH), eculizumab has been administered at a dose of 900 mg/patient once every 12-14 days. This dosing has been found to completely and consistently block terminal complement activity and has greatly inhibited the symptoms of PNH (Hillmen et al., 2004). The administered dose is able to block the effects of complement for approximately two weeks before the eculizumab is inactivated or removed from the body. Therefore, a chronic treatment of eculizumab may be, e.g., the administration of 900 mg to the allograft recipient once every two weeks for the remaining life-time of the patient. Similarly, other drugs can be delivered chronically as needed, whether this is on a daily basis or another schedule is required to maintain an effective amount of the drug in the allograft recipient. Because it is well known that graft rejection can be caused by more than just complement activation, e.g., by T cell activity, the experiments included immunosuppressants such as cyclosporin to further aid in preventing graft rejection.

[0055] A preferred method of inhibiting complement activity is to use a monoclonal antibody which binds to complement

Samsung Bioepis Exhibit No. 1002
Page 1917

C5 and prevents C5 from being cleaved. This prevents the formation of both C5a and C5b-9 while at the same time allowing the formation of C3a and C3b which are beneficial to the recipient. Such antibodies that are specific to human complement are known (U.S. Patent 6,355,245). These antibodies disclosed in U.S. Patent 6,355,245 include both a whole or full-length antibody (now named eculizumab) and a single-chain antibody (now named pexelizumab). A similar antibody against mouse C5 is called BB5.1 (Frei et al., 1987). BB5.1 was utilized in the experiments set forth below. Antibodies to inhibit complement activity need not be monoclonal antibodies. They can be, e.g., polyclonal antibodies. They may additionally be antibody fragments. An antibody fragment includes, but is not limited to, an Fab, F(ab'), F(ab')2, a single-chain antibody, a domain antibody and an Fv. Furthermore, it is well known by those of skill in the art that antibodies can be humanized (Jones et al., 1986), chimerized, or deimmunized. An antibody may also comprise a mutated Fc portion, such that the mutant Fc does not activate complement. The antibodies to be used in the present disclosure may be any of these. It is preferable to use humanized antibodies when the recipient of the allograft is a human.

Administration and Formulations

[0056] Administration of the inhibitor of complement activity is performed according to methods known to those of skill in the art. These inhibitors are administered preferably before the time of allograft transplantation or at the time of transplantation with administration continuing in a chronic fashion. These inhibitors can additionally be administered during a rejection episode in the event such an episode does occur.

[0057] The present disclosure also provides uses of a drug that inhibits complement activity and an immunosuppressive agent in the manufacture of a medicament or medicament package. Such medicament or medicament package is useful in prolonging allograft survival in a recipient, in particular, chronic survival of the allograft. In preferred embodiments, the medicament or medicament package is formulated and prepared such that it is suitable for chronic administration to the recipient of the allograft, for example, stable formulations are employed. In certain embodiments, the medicament or medicament package is formulated and prepared such that it is suitable for concurrent administration of the drug that inhibits complement activity and the immunosuppressive drug to the recipient of the allograft. In certain embodiments, the medicament or medicament package is formulated and prepared such that it is suitable for sequential (in either order) administration of the drug that inhibits complement activity and the immunosuppressive drug to the recipient of the allograft.

[0058] A pharmaceutical package of the present disclosure may comprise a drug that inhibits complement activity and at least one immunosuppressive agent. The pharmaceutical package may further comprise a label for chronic administration. The pharmaceutical package may also comprise a label for self-administration by a patient, for example, a recipient of a transplant graft, or instructions for a caretaker of a recipient of a transplant graft. In certain embodiments, the drug and the agent in the pharmaceutical package are in a formulation or separate formulations that are suitable for chronic administration and/or self-administration.

[0059] The present disclosure also provides lyophilized formulations and formulations suitable for injection. Certain embodiments provide a lyophilized antibody formulation comprising an antibody that inhibits complement activity and a lyoprotectant. In preferred embodiments, the antibody formulation is suitable for chronic administration, for example, the antibody formulation stable. Alternative embodiments provide an injection system comprising a syringe; the syringe comprises a cartridge containing an antibody that inhibits complement activity and is in a formulation suitable for injection.

[0060] An antibody employed in various embodiments of the present disclosure preferably inhibits the formation of terminal complement or C5a. In certain embodiments, antibody inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment. The whole antibody or antibody fragment may be a human, humanized, chimerized or deimmunized antibody or antibody fragment. In certain embodiments, the whole antibody or antibody fragment may inhibit cleavage of complement C5. In certain embodiments, the antibody fragment is a Fab, an F(ab')2, an Fv, a domain antibody, or a single-chain antibody. In preferred embodiments, the antibody fragment is pexelizumab. In alternative preferred embodiments, the whole antibody is eculizumab.

[0061] In certain embodiments, a drug, such as an antibody, that inhibits complement activity is present in unit dosage form, which can be particularly suitable for self-administration. Similarly, an immunosuppressive agent of the present disclosure may also be present in unit dosage form. A formulated product of the present disclosure can be included within a container, typically, for example, a vial, cartridge, prefilled syringe or disposable pen. A doser such as the doser device described in U.S. Patent 6,302,855 may also be used, for example, with an injection system of the present disclosure.

[0062] A "stable" formulation is one in which the drug (e.g., an antibody) or agent therein essentially retains its physical and chemical stability and integrity upon storage. Various analytical techniques for measuring protein stability are available in the art and are reviewed in Peptide and Protein Drug Delivery, 247-301, Vincent Lee Ed., Marcel Dekker, Inc., New York, N.Y., Pubs. (1991) and Jones, A. Adv. Drug Delivery Rev. 10: 29-90 (1993). Stability can be measured at a selected temperature for a selected time period. For example, the extent of aggregation following lyophilization and storage can be used as an indicator of protein stability. For example, a "stable" formulation may be one wherein less

Samsung Bioepis Exhibit No. 1002
Page 1918

than about 10% and preferably less than about 5% of the protein is present as an aggregate in the formulation. In other embodiments, any increase in aggregate formation following lyophilization and storage of the lyophilized formulation can be determined. For example, a "stable" lyophilized formulation may be one wherein the increase in aggregate in the lyophilized formulation is less than about 5% and preferably less than about 3%, when the lyophilized formulation is stored at 2-8 °C for at least one year. In other embodiments, stability of the protein formulation may be measured using a biological activity assay.

[0063] A "reconstituted" formulation is one which has been prepared by dissolving a lyophilized protein formulation in a diluent such that the protein is dispersed in the reconstituted formulation. The reconstituted formulation in suitable for administration (e.g. parenteral administration) to a patient to be treated with the protein of interest and, in certain embodiments of the invention, may be one which is suitable for subcutaneous administration.

[0064] An isotonic reconstituted formulation is preferable in certain embodiments. By "isotonic" is meant that the formulation of interest has essentially the same osmotic pressure as human blood. Isotonic formulations will generally have an osmotic pressure from about 250 to 350 mOsm. Isotonicity can be measured using a vapor pressure or ice-freezing type osmometer, for example.

[0065] A "lyoprotectant" is a molecule which, when combined with a drug (e.g., antibody) of interest, significantly prevents or reduces chemical and/or physical instability of the drug (e.g., antibody) upon lyophilization and subsequent storage. Exemplary lyoprotectants include sugars such as sucrose or trehalose; an amino acid such as monosodium glutamate or histidine; a methylamine such as betaine; a lyotropic salt such as magnesium sulfate; a polyol such as trihydric or higher sugar alcohols, e.g. glycerin, erythritol, glycerol, arabitol, xylitol, sorbitol, and mannitol; propylene glycol; polyethylene glycol; Pluronics; and combinations thereof. The preferred lyoprotectant is a non-reducing sugar, such as trehalose or sucrose.

[0066] The lyoprotectant is added to the pre-lyophilized formulation in a "lyoprotecting amount" which means that, following lyophilization of the drug (e.g., antibody) in the presence of the lyoprotecting amount of the lyoprotectant, the drug (e.g., antibody) essentially retains its physical and chemical stability and integrity upon lyophilization and storage.

[0067] The "diluent" of interest herein is one which is pharmaceutically acceptable (safe and non-toxic for administration to a human) and is useful for the preparation of a reconstituted formulation. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (e.g. phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution.

[0068] A "preservative" is a compound which can be added to the diluent to essentially reduce bacterial action in the reconstituted formulation, thus facilitating the production of a multi-use reconstituted formulation, for example. Examples of potential preservatives include octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride (a mixture of alkylbenzyldimethylammonium chlorides in which the alkyl groups are long-chain compounds), and benzethonium chloride. Other types of preservatives include aromatic alcohols such as phenol, butyl and benzyl alcohol, alkyl parabens such as methyl or propyl parahen, catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol.

[0069] A "bulking agent" is a compound which adds mass to the lyophilized mixture and contributes to the physical structure of the lyophilized cake (e.g. facilitates the production of an essentially uniform lyophilized cake which maintains an open pore structure). Exemplary bulking agents include mannitol, glycine, polyethylene glycol and xorbitol.

[0070] Accordingly, a stable lyophilized antibody formulation can be prepared using a lyoprotectant (preferably a sugar such as sucrose or trehalose), which lyophilized formulation can be reconstituted to generate a stable reconstituted formulation having an antibody concentration which is significantly higher (e.g. from about 2-40 times higher, preferably 3-10 times higher and most preferably 3-6 times higher) than the antibody concentration in the pre-lyophilized formulation. Such high protein concentrations in the reconstituted formulation are considered to be particularly useful where the formulation is intended for subcutaneous administration. Despite the very high protein concentration in the reconstituted formulation, the reconstituted formulation can be stable (i.e. fails to display significant or unacceptable levels of chemical or physical instability of the protein) at 2-8 °C. for at least about 30 days. See U.S. Patent 6,821,515. In certain embodiments, the reconstituted formulation is isotonic.

[0071] When reconstituted with a diluent comprising a preservative (such as bacteriostatic water for injection, BWFI), the reconstituted formulation may be used as a multi-use formulation. Such a formulation is useful, for example, where a subject patient requires frequent administrations of the drug or antibody and/or agent to treat a chronic medical condition. The advantage of a multi-use formulation is that it facilitates ease of use for the patient, reduces waste by allowing complete use of vial contents, and results in a significant cost savings for the manufacturer since several doses are packaged in a single vial (lower filling and shipping costs).

[0072] The present disclosure also provides a method for preparing a formulation comprising the steps of: (a) lyophilizing a mixture of an antibody and a lyoprotectant; and (b) reconstituting the lyophilized mixture of step (a) in a diluent such that the reconstituted formulation is isotonic and stable.

[0073] An article of manufacture is also provided herein which comprises: (a) a container which holds a lyophilized mixture of an antibody and a lyoprotectant; and (b) instructions for reconstituting the lyophilized mixture with a diluent to a desirable antibody concentration in the reconstituted formulation. The article of manufacture may further comprise

Samsung Bioepis Exhibit No. 1002
Page 1919

a second container which holds a diluent (e.g. bacteriostatic water for injection (BWFI) comprising an aromatic alcohol).

[0074] An injection system of the present disclosure may employ a medication delivery pen as described in U.S. Patent 5,308,341. Medication delivery pens have been developed to facilitate the self-administration of medication. A medication of the present disclosure can be a drug that inhibits complement activity, for example an antibody specific to complement C5, and/or an immunosuppressive agent. One medication delivery pen includes a vial holder into which a vial of insulin or other medication may be received. The vial holder is an elongate generally tubular structure with proximal and distal ends. The distal end of the vial holder includes mounting means for engaging a double-ended needle cannula. The proximal end also includes mounting means for engaging a pen body which includes a driver and dose setting apparatus. A disposable medication containing vial for use with the prior art vial holder includes a distal end having a pierceable elastomeric septum that can be pierced by one end of a double-ended needle cannula. The proximal end of this vial includes a stopper slidably disposed in fluid tight engagement with the cylindrical wall of the vial. This medication delivery pen is used by inserting the vial of medication into the vial holder. A pen body then is connected to the proximal end of the vial holder. The pen body includes a dose setting apparatus for designating a dose of medication to be delivery by the pen and a driving apparatus for urging the stopper of the vial distally for a distance corresponding to the selected dose.

[0075] The user of the pen mounts a double-ended needle cannula to the distal end of the vial holder such that the proximal point of the needle cannula pierces the septum on the vial. The patient then selects a dose and operates the pen to urge the stopper distally to deliver the selected dose. The dose selecting apparatus returns to zero upon injection of the selected dose. The patient then removes and discards the needle cannula, and keeps the prior art medication delivery pen in a convenient location for the next required medication administration. The medication in the vial will become exhausted after several such administrations of medication. The patient then separates the vial holder from the pen body. The empty vial may then be removed and discarded. A new vial can be inserted into the vial holder, and the vial holder and pen body can be reassembled and used as explained above.

[0076] Accordingly, a medication delivery pen generally has a drive mechanism for accurate dosing and ease of use. A dosage mechanism such as a rotatable knob allows the user to accurately adjust the amount of medication that will be injected by the pen from a prepackaged vial of medication. To inject the dose of medication, the user inserts the needle under the skin and depresses the knob once as far as it will depress. The pen may be an entirely mechanical device or it may be combined with electronic circuitry to accurately set and/or indicate the dosage of medication that is injected into the user. See U.S. Patent 6,192,891.

[0077] The present disclosure also presents controlled-release or extended-release formulations suitable for chronic and/or self-administration of a medication.

[0078] The various formulations can be administered to a patient in need of treatment (e.g., a recipient of an allograft) with the medication (e.g., an antibody of the present disclosure and at least one immunosuppressive agent) by intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes.

[0079] In certain embodiments, a formulation is administered to the patient by subcutaneous (i.e. beneath the skin) administration. For such purposes, the formulation may be injected using a syringe. However, other devices for administration of the formulation are available such as injection devices (e.g. the Inject-ease® and Genject® devices); injector pens (such as the GenPen®); needleless devices (e.g. MediJector® and BioJector®); and subcutaneous patch delivery systems.

[0080] The present methods and uses are described with reference to the following Examples, which are offered by way of illustration and are not intended to limit the disclosure in any manner. Standard techniques well known in the art or the techniques specifically described below are utilized. The following abbreviations are used herein: ABMR, antibody-mediated rejection; ACHR, accelerated humoral rejection; ACR, acute cellular rejection; AVR, acute vascular rejection; CsA, cyclosporin; CyP, cyclophosphamide; HAR, hyperacute rejection; MCP-1, monocyte chemotactic protein 1; MST, mean survival time; POD, postoperative day.

Example 1

Methods

[0081] *Animals and Immunosuppressive Drugs* Male adult C3H (H-2^k) mice and BALB/c (H-2^d) mice (Jackson Labs, Bar Harbor, Maine) weighing 25-30 g were chosen as donors and recipients, respectively. In the groups receiving immunosuppression, the recipients were injected with CsA (15 mg/kg/day, s.c., daily from day 0 to endpoint rejection or until day 100), or with CyP (40 mg/kg/day, i.v., on day 0 and 1), or with anti-C5 mAb (clone BB5.1, Alexion Pharmaceuticals Inc., 40 mg/kg/day, i.p., day 0-2, followed by twice a week, day 0-60). Animals were housed under conventional conditions at the Animal Care Facility, University of Western Ontario, and were cared for in accordance with the guidelines established by the Canadian Council on Animal Care. Olfert et al., 1993.

[0082] *Skin Presensitization* Full-thickness skin grafts taken from C3H donors were cut into square pieces of 1×1 cm^2

Samsung Bioepis Exhibit No. 1002
Page 1920

and transplanted onto the back of the BALB/c recipients' thorax one week prior to heart transplantation from the same donors. Rejection was defined as complete necrosis of the skin grafts.

[0083] *Abdominal and Cervical Cardiac Transplantation* Seven days after skin presensitization, C3H mouse hearts were transplanted into the abdomen of presensitized BALB/c recipients by anastomosing the donor aorta and recipient aorta, and the donor pulmonary artery and recipient inferior vena cava. In the groups with re-transplantation, second heart grafts harvested from either naive C3H mice or long-term surviving presensitized BALB/c recipients were transplanted into the cervical area of the recipients carrying a long-term surviving first abdominal heart graft by anastomosing the donor aorta and recipient carotid artery, and the donor pulmonary artery and recipient external jugular vein (end-to-side). The heart grafts were monitored daily until rejection unless otherwise indicated and rejection was defined as complete cessation of pulsation.

[0084] *Experimental Groups* Presensitized recipients were randomly assigned to eight groups, each consisting of eight animals: Group 1, mice with no treatment; Group 2, mice treated with CsA; Group 3, mice treated with CyP; Group 4, mice treated with CsA plus CyP; Group 5, mice treated with anti-C5 mAb; Group 6, mice treated with anti-C5 mAb plus CsA; Group 7, mice treated with anti-C5 mAb plus CyP; Group 8, mice treated with anti-C5 mAb in combination of CsA and CyP. When cardiac impulses were no longer palpable or at POD100, the grafts were removed for routine histology, immunohistochemistry and western blot analysis, serum samples were collected for flow cytometric analysis and complement hemolytic assay. Five additional animals were placed and sacrificed in groups 6 and 8 on POD3 (MST for groups 1-5, 7) to allow for comparisons at a uniform time point. Serum samples were also collected on POD 11, 21, 28 and 60 in Group 8 for detecting the sequential changes of anti-donor antibody levels and complement activity. In addition, when triple therapy treated presensitized recipients carried a first heart graft for 100 days, they were re-transplanted with a second heart. A naive C3H heart or a 100-day surviving C3H heart from another presensitized BALB/c recipient was used as the second heart. Eight animals were included in each re-transplant group.

[0085] *Graft Histology* Tissue samples were fixed in 10% buffered formaldehyde. Specimens were then embedded in paraffin, and sectioned for H&E staining. The microscopic sections were examined in a blinded fashion for severity of rejection by a pathologist. Criteria for graft rejection included the presence of vasculitis, thrombosis, hemorrhage and lymphocyte infiltration. These changes were scored as: 0, no change; 1, minimum change; 2, mild change; 3, moderate change; or 4, marked change.

[0086] *Immunohistochemistry* Four micrometer sections were cut from tissue samples embedded in Tissue-Tek O.C.T gel (Optimum Cutting Temperature, Skura Finetek, Torrance, CA) mounted on gelatin-coated glass microscope slides and stained by a standard indirect avidin-biotin immunoperoxidase staining method using an Elite Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA). Specimens were stained for $CD4^+$ and $CD8^+$ cells with biotin-conjugated rat anti-mouse CD4 mAb (clone YTS 191.1.2, Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) and biotin-conjugated rat anti-mouse CD8 mAb (clone 53-6.7, Pharmingen, Franklin Lakes, NJ), respectively. Intragraft monocyte/macrophage infiltration was detected by staining with biotin-conjugated rat anti-mouse Mac-1 mAb (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada). Mouse IgG and IgM deposition in grafts was detected using biotin-conjugated goat anti-mouse-IgG and goat anti-mouse-IgM (Cedarlane). For identification of complement deposition, sections were serially incubated with goat anti-C3 or anti-C5 polyclonal Abs (Quidel, San Diego, CA), biotinylated rabbit anti-goat IgG (Vector Laboratories), and HRP-conjugated-streptavidin (Zymed Laboratories, South San Francisco, CA). Slides were washed with phosphate-buffered saline between steps, and examined under light microscopy. Negative controls were performed by omitting the primary antibodies. The immunostaining was scored in five high-power fields of each section, and five independent experiments were performed. The sections of immunoperoxidase staining were graded from 0 to 4+ according to the staining intensity: 0, negative; 1+, equivocal; 2+, weak staining; 3+, moderate staining; and 4+, very intensive staining.

[0087] *Flow Cytometry* The circulating anti-donor specific IgG and IgM antibodies were evaluated in the recipient serum by FACScan flow cytometry (Becton Dickinson, Mountain View, CA). Glotz et al., (1993); Tyan et al. (1994). Briefly, C3H mouse splenocytes were isolated and incubated at 37°C for 30 minutes with serum from naive control and experimental groups. To stain for total IgG, IgG1, IgG2a, IgG2b and IgM, the cells were washed and incubated with FITC-conjugated goat antibody specific for the Fc portion of mouse IgG or with phycoerythrin-conjugated goat antibody specific for the μ-chain of mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA), or with FITC-conjugated goat anti-mouse IgG1 (CALTAG Laboratories, Burlingame, CA), or with FITC-conjugated goat anti-mouse IgG2a (CALTAG), or with FITC-conjugated goat anti-mouse IgG2b (CALTAG). After 1 hour of staining at 4°C, the cells were washed with PBS, resuspended at 5×10^6/mL, and analyzed by flow cytometry for mean channel fluorescence intensity, which represents the antibody-binding reactivity.

[0088] *Complement Hemolytic Assay* The purified anti-C5 mAb was serially diluted twofold (175-0.1 μg/ml) in GVB^{2+} buffer (gelatin Veronal-buffered saline: 0.1% gelatin, 141 mM NaCl, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, and 1.8 mM sodium barbital) and added in triplicate (50 μl/well) to a 96-well plate. BALB/c mouse serum was diluted to 40% v/v with GVB^{2+} buffer and added (50 μl/ml) to the rows of the same 96-well plate such that the final concentration of BALB/c mouse serum in each well was 20%. The plate was then incubated at room temperature for approximately 30 min while chicken

Samsung Bioepis Exhibit No. 1002
Page 1921

erythrocytes were prepared. Chicken erythrocytes were washed 5×1 ml with GVB^{2+} buffer and resuspended to a final concentration of 5×10^7/ml in GVB^{2+}. Four milliliters of the chicken erythrocytes were sensitized by adding anti-chicken RBC polyclonal antibody (Intercell Technologies, Hopewell, NJ, 0.1% v/v) and the cells were incubated at 4 °C for 15 min with frequent vortexing. The cells were then washed 2×1 ml with GVB^{2+} and resuspended to a final volume of 2.4 ml in GVB^{2+}. The chicken erythrocytes (30 μl/well, 2.5×10^6 cells) were added to the plate containing serum and anti-C5 mAb as described above, mixed well, and incubated at 37 °C for 30 min. The plate was then centrifuged at 1000×g for 2 min, and 85 μl of the supernatant was transferred to a new 96-well microtiter plate. The plate was read at OD 415 nm using a microplate reader and the percentage of hemolysis was determined using this formula:

$$\%\ \text{hemolysis} = 100 \times \frac{(\text{OD sample}) - (\text{OD } GVB^{2+} \text{ control})}{(\text{OD 100\% lysed control}) - (\text{OD } GVB^{2+} \text{ control})}$$

with 100% lysed control obtained by the addition of 100 μl GVB^{2+} containing 0.1% NP-40 to the 30 μg/ml of chicken erythrocytes as prepared above.

[0089] *Western Blot Analysis* Sonication of frozen heart samples was performed in RIPA lysis buffer (Santa Cruz Biotechnology, Inc.) at 4°C for 1 minute at 10-second intervals, followed by microcentrifugation at 13,000 rpm for 10 minutes at 4°C. Clarified supernatants were immediately quantitated in triplicate for protein content using Detergent-compatible protein assay kit (BIO-RAD). Heart lysates (10μg protein/well) were separated on NuPAGE, 4-12% gradient Bis-Tris gels and MES buffer system (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membrane (0.45 μm pore size; Invitrogen) using a semi-dry transfer apparatus (BIO-RAD). Membranes were cut appropriately at the correct molecular weights to allow the development of the blots with two different primary antibodies per blot such that each blot was exposed to a test antibody and an internal control antibody to insure equal sample loading. The test primary antibodies including anti-Bcl-2 (N-19) rabbit polyclonal sera (Santa Cruz Biotechnology, Inc.) and anti-Bcl-XS/L (M-125) rabbit polyclonal sera (Santa Cruz Biotechnology, Inc.) were used to detect intragraft expression of Bcl-2 and Bcl-xl proteins. Anti-calsequestrin rabbit polyclonal sera (Calbiochem) were used as internal control primary antibody (Kobayashi et al., 1999). Detection of primary antibody binding was performed as previously described (Arp et al., 1996) by exposing washed incubated blots to a polyclonal goat anti-rabbit IgG fraction conjugated to horseradish peroxidase (HRP; Roche Laboratories) and then appropriately developed by exposure to enhance chemiluminescence for HRP-conjugated antibodies (Roche Laboratories).

[0090] *Statistical Analysis* The data were reported as the mean ± SD. Allograft survival among experimental groups was compared using the rank-log test. Histological and immunohistological findings were analyzed using the Mann-Whitney U test. Flow cytometric data and western blot data were analyzed using one-way ANOVA. Differences with p values less than 0.05 were considered significant.

Example 2

Presensitization with C3H donor skin graft induces antibody-mediated ACHR in heart allografts of BALB/c recipients.

[0091] To develop a suitable small animal model that mimics presensitized patients in the clinic and to study ABMR, a novel, fully MHC-mismatched mouse ABMR model has been developed through presensitization of mouse recipients. In this model, BALB/c recipients were presensitized with C3H donor skin grafts one week prior to heart transplantation from the same donor. Seven days after donor skin presensitization, serum level of anti-donor IgG, but not IgM antibody was markedly elevated and reached to a peak level in the presensitized BALB/c recipients (Figure 1A). Heart transplantation from same donor was then performed in these highly sensitized recipients. Without immunosuppression, C3H heart grafts were rapidly rejected in 3.1 ± 0.4 days by ACHR, characterized by severe thrombosis, hemorrhage and infarction (Figure 1B-a). In contrast, same heart grafts in unsensitized BALB/c recipients (with mean survival time, MST of 8.2 ± 0.8 days) show the normal histology on post-operative day (POD) 3 (Figure 1B-b). When compared to unsensitized BALB/c recipients at the same day, heart grafts in presensitized animals revealed massive IgG antibody and complement (C3 and C5) deposition, but minimal $CD4^+$ and $CD8^+$ cell infiltration (Table 1). Furthermore, circulating anti-donor IgG levels in presensitized recipients were significantly higher than those of unsensitized same recipients receiving a heart graft on POD3 ($P< 0.01$, Figure 1C). However, anti-donor IgM remained at very low levels both in circulation (Figure 1C) and in heart grafts (Table 1) and it showed no significant difference between unsensitized and presensitized recipients. In addition, normal levels of complement hemolytic activity were shown in both presensitized and unsensitized

Samsung Bioepis Exhibit No. 1002
Page 1922

heart recipients without treatment (Figure 1D). These data indicate that this is an ideal transplant model to study ABMR in presensitized recipients in which complement plays an important role in the pathogenesis.

Table 1.
Comparison of immunohistological changes of C3H heart allografts in unsensitized and presensitized BALB/c recipients on POD3

Groups	Unsensitized	Presensitized
IgG	1+	4+
IgM	1+	1+
C3	2+	3+
C5	2+	3+
CD4	0	1+
CD8	0	1+

[0092] Grades for immunoperoxidase staining: 0, negative; 1+, equivocal; 2+, weak; 3+, moderate; 4+, intense.

Anti-C5 mAb in combination with CsA and CyP prevents ABMR and achieves indefinite heart allograft survival in presensitized mouse recipients.

[0093] Complement has been shown to play an important role in ABMR. However, the inhibitory effect of functionally blocking terminal complement cascade at the C5 level in highly sensitized recipients is unknown. In the study presented herein, the presensitized model was used to study the efficacy of anti-C5 mAb either alone or combined with CsA and /or CyP in prevention of ABMR. As presented in Figure 2A, treatment with either CsA or CyP or the two drugs in combination did not prevent ABMR and grafts were rejected in 3.0 $\pm$ 0.0 days, 3.3 $\pm$ 0.5 days and 3.5 $\pm$ 0.6 days, respectively with typical pathological features of ACHR including intravascular thrombosis and interstitial hemorrhage (Figure 2B-b, c, d), which were indistinguishable from heart grafts in untreated presensitized BALB/c recipients (Figure 2B-a). Anti-C5 monotherapy or combined with CyP was not able to improve graft survival and heart grafts were rejected by ACHR (Figure 2B-e, f) in 3.5 $\pm$ 0.6 days and 3.2 $\pm$ 0.4 days, respectively (Figure 2A). Although the combination therapy of anti-C5 mAb and CsA, the protocol capable of inducing long-term heart allograft survival in unsensitized animals, marginally prolonged graft survival in this presensitized model, heart grafts were also rejected by severe humoral rejection with vasculitis, thrombosis, hemorrhage and minimal cell infiltration (Figure 2B-g) in 11.9 $\pm$ 1.8 days (Figure 2A). In contrast, triple therapy of anti-C5 mAb in combination of CsA and CyP achieved indefinite heart graft survival over 100 days (Figure 2A) in presensitized animals ($P<0.01$ vs. the animals without treatment or treated with either monotherapy or two drugs in combination) with no evidence of rejection (Figure 2B-h). In this presensitized mouse model, as shown in Table 2, only minor intragraft $CD4^+$ and $CD8^+$ cell infiltration was observed in the recipients that rejected their heart grafts within 3 days. However, the number of these T cells was slightly increased if heart grafts survived longer in anti-C5 mAb plus CsA-treated recipients at the time of rejection (POD11) and in triple therapy-treated recipients at early stages of graft survival (e.g. POD11). Furthermore, with continuous treatment of CsA in the triple therapy group, $CD4^+$ and $CD8^+$ cell infiltration was inhibited in long-term surviving heart grafts on POD60 and 100. In addition, moderate intragraft Mac-1^+ cell infiltration, including monocytes and macrophages, was found in untreated and CsA-, CyP- or CsA plus CyP-treated animals, while the infiltration of these cells was significantly reduced in anti-C5 mAb treated animals (Table 2). These results indicate that functionally blocking anti-C5 mAb enables the use and efficacy of conventional immunosuppressive agents, thereby preventing ABMR and achieving indefinite heart graft survival in presensitized recipients.

Table 2.
Grades for immunoperoxidase staining of heart allografts in presensitized mouse recipients at necropsy

Groups	Date for sample collection (POD)	C3	C5	CD4	CD8	Mac-1	IgG	IgM
Untreated	3	3+	3+	1+	1+	3+	4+	1+
CsA	3	3+	3+	1+	1+	3+	4+	1+
CyP	3	3+	3+	1+	1+	3+	3+	1+
CsA+CyP	3	3+	3+	1+	1+	3+	3+	1+
Anti-C5mAb	3	3+	0	1+	1+	2+	4+	1+

Samsung Bioepis Exhibit No. 1002
Page 1923

(continued)

Grades for immunoperoxidase staining of heart allografts in presensitized mouse recipients at necropsy

Groups	Date for sample collection (POD)	C3	C5	CD4	CD8	Mac-1	IgG	IgM
Anti-C5mAb+CsA	11	3+	0	2+	2+	2+	4+	1+
Anti-C5mAb+CyP	3	3+	0	1+	1+	2+	3+	1+
Anti-C5mAb+CsA+CyP	3	3+	0	1+	1+	2+	3+	1+
Anti-C5mAb+CsA+CyP	11	3+	0	2+	2+	1+	3+	1+
Anti-C5mAb+CsA+CyP	60	3+	0	1+	1+	0	2+	1+
Anti-C5mAb+CsA+CyP	100	3+	2+	0	0	0	2+	1+

Grades: 0, negative; 1+, equivocal; 2+, weak; 3+, moderate; 4+, intense.

Anti-C5 mAb completely inhibits total complement hemolytic activity and local C5 deposition in presensitized recipients receiving a heart allograft.

[0094] Anti-C5 mAb was previously shown to block the cleavage of complement protein C5 into the proinflammatory molecules C5a and C5b-9 (Kroshus et al., 1995), and to completely and consistently block terminal complement activity in mice (Wang et al., 1999). In the current study, terminal complement activity was measured by assessing the ability of recipient mouse sera to lyse antibody presensitized chicken erythrocytes and was compared at the same time-point (POD3). Treatment of mice with either CsA or CyP or the two drugs in combination had no effect on terminal complement activity, while treatment with anti-C5 mAb either alone or combined with CsA or/and CyP completely inhibited this activity (Figure 3; $P<0.01$, vs. naive and untreated animals, as well as CsA-, CyP-, or CsA plus CyP-treated animals). In addition, sera obtained from anti-C5 mAb treated animals at several earlier time points showed similarly diminished hemolytic activity, suggesting that serum terminal complement was inhibited throughout the treatment period. Furthermore, local C5 deposition in heart grafts was completely prevented in the anti-C5 mAb treated presensitized recipients, but not in untreated, or CsA-, CyP- and CsA plus CyP-treated presensitized animals (Table 2). As predicted, treatment with anti-C5 mAb did not prevent C3 deposition in the grafts (Table 2). These results suggest anti-C5 therapy completely blocks total complement activity after cardiac allografting in highly sensitized recipients.

Long-term surviving heart grafts in presensitized animals are resistant to humoral injury in the presence of low level of anti-donor antibodies and complement- a situation of accommodation.

[0095] To further investigate the role of anti-C5 mAb in humoral rejection, anti-donor alloantibody levels were measured in recipient sera by flow cytometry and intragraft antibody deposition by using immunostaining techniques in different groups. Figure 4A shows that on POD3 untreated presensitized BALB/c recipients had high levels of circulating anti-donor IgG antibodies. When presensitized recipients receiving either monotherapy or two drugs in combination, CsA and/or CyP partially down-regulated circulating anti-donor IgG levels, while treatment with anti-C5 mAb either alone or combined with CsA or CyP did not further affect anti-donor antibody levels at the same day. In contrast, with triple therapy of anti-C5 mAb, CsA and CyP, a high level of circulating anti-donor IgG was gradually downregulated and reached a low level on POD60, thereafter remaining at this level until day 100 (Figure 4B). Similar to levels of circulating antibodies in the different treatment groups, Table 2 shows that strong deposition of anti-mouse IgG was present in the rapidly rejected heart grafts of presensitized animals with no treatment or treated with monotherapy or two drugs in combination therapy. Interestingly, with triple therapy, IgG antibody deposition was gradually attenuated to a mild level in the long-term surviving heart grafts on POD 100 (Figure 4C-a, Table 2). In this model, IgM remained at very low levels in either circulation (Figure 4A. B) or transplanted heart grafts (Figure 4C-b, Table 2) in presensitized recipients with or without treatment. In addition, treatment with anti-C5 mAb eliminated complement activity to an undetectable level until day 60, followed by a progressive recovery to predepletion levels on POD 100 after discontinuation of anti-C5 therapy in presensitized mouse recipients receiving triple therapy (Figure 4D). Furthermore, intragraft C5 deposition was also detected in 100-day surviving presensitized animals (Table 2). These data demonstrate that ongoing transplant accommodation occurs in triple therapy treated presensitized recipients despite the presence of anti-graft antibodies and complement activation.

Samsung Bioepis Exhibit No. 1002
Page 1924

Anti-C5 mAb in combination with CsA and CyP reduces the IgG1/IgG2a ratio and leads to a shift in IgG subclass to IgG2b in recipients with accommodated grafts.

[0096] To determine whether anti-C5 mAb-based triple therapy would induce a shift in IgG subclass, which may be associated with accommodation, serum levels of anti-donor IgG subclasses of IgG1, IgG2a and IgG2b were compared between untreated recipients and the recipients with accommodated heart graft. Sera from untreated recipients contained predominant IgG1 isotype, indicated by a high ratio of IgG1/IgG2a (Figure 5A). In contrast, a significant reduction in the ratio of IgG1/IgG2a was observed in the recipients carrying accommodated grafts (Figure 5A, $P<0.01$). Furthermore, presensitized recipients with the accommodated heart grafts displayed an increased level of anti-donor IgG2b as compared to the same recipients with rejected grafts (Figure 5B, $P<0.01$). In addition, the pattern of IgG isotypes in the recipients treated with either monotherapy or two drugs in combination is indistinguishable from that of untreated animals. These data indicate that anti-donor IgG1 isotype may be associated with graft rejection, while production of anti-donor IgG2b subclass may function as a protective antibody and plays an important role in the induction of accommodation.

Anti-C5 mAb in combination with CsA and CyP induces intragraft Bcl-2 and Bel-xl expression in highly sensitized mouse recipients.

[0097] To determine whether a causal relationship exists between intragraft expression of protective proteins and graft resistance to humoral injury in this model, western blot analysis was employed to detect proteins of interest in heart graft tissues from highly sensitized mouse recipients. Long-term surviving heart grafts were found to express high levels of Bcl-2 and Bcl-xl proteins on POD100, and these proteins were detected as early as 12 days after heart transplantation in highly sensitized recipients receiving anti-C5 mAb-based triple therapy (Figure 6). In contrast, there were no Bcl-2 and Bcl-xl proteins expressed on heart grafts of untreated animals (Figure 6) or animals treated with either monotherapy or two drugs in combination therapy. This result suggests that graft resistance to humoral injury in indefinite surviving animals is associated with the protection provided by Bcl-2 and Bcl-xl proteins in this presensitized model.

Presensitized recipients with an accommodating first heart graft accept a second accommodated heart graft but reject a second naive heart graft from the same donors.

[0098] The ability of accommodated grafts to resist rejection has not been tested directly under pathophysiological conditions where naive grafts undergo rejection following allotransplantation. In this model, to determine whether presensitized recipients with an accommodating first heart graft will accept a second accommodated graft but reject a second naive graft, we performed re-transplantation scenarios. After the accommodated C3H heart grafts have survived to the 100-day point; the time at which low levels of alloantibodies were detected (Figure 4B) and complement activity has returned to pretreatment levels (Figure 4D), in presensitized BALB/c recipient treated with anti-C5 mAb-based triple therapy, these recipients received a second heart graft. Specifically, either a naive (Figure 7A) or a 100-day accommodated C3H heart (Figure 7B) from another presensitized BALB/c recipient was transplanted into the neck of the presensitized recipients carrying an accommodating first C3H heart. These recipients rejected a second naive heart at 6.6 ± 1.1 days (Figure 8A) with severe AVR (Figure 8B-a) while the first heart continued to survive. In contrast, when the accommodated hearts that had been already surviving for 100 days in different presensitized mice were used as second grafts, these grafts were accepted by the presensitized recipients carrying an accommodating first heart graft (Figure 8A). There was no sign of rejection in those accommodated second heart grafts 90 days after second transplantation (Figure 8B-b). These data indicate that accommodated grafts become resistant to the effects of anti-donor antibodies and complement that normally mediate allograft rejection in these presensitized recipients. Furthermore, the fact that the host of the accommodated graft rejected a new graft suggests that accommodation involves changes to the graft.

Presensitized recipients being treated with CsA reject accommodated heart grafts.

[0099] Another re-transplantation was performed to determine whether accommodation in this presensitized model would be caused by the changes in the grafts and/or the recipients. Specifically, after C3H heart grafts have been accommodated in presensitized BALB/c mice for 100 days, the accommodated heart graft will then be re-transplanted into a second presensitized BALB/c recipient being treated with CsA alone (Figure 7C), a therapy that can prevent cellular rejection but cannot prevent accelerated humoral rejection of a fresh C3H heart in presensitized recipients. The accommodated C3H heart grafts were rapidly rejected in CsA treated presensitized BALB/c recipients. After re-transplantation, the pathology in accommodated heart grafts was changed from normal (Figure 9A) to severe ACHR with massive interstitial hemorrhage but few cell infiltrates (Figure 9B). In addition, high levels of anti-donor IgG and normal levels of complement hemolytic activity in these recipients receiving an accommodated C3H heart were similar to those of CsA treated presensitized recipients receiving a naive C3H heart. This result further indicates that accommodation

Samsung Bioepis Exhibit No. 1002
Page 1925

induced by anti-C5 mAb-based triple therapy can originate from mechanisms involving changes not only to the graft, but also to the recipient.

Example 3

Acute Vascular Rejection in a Heart Transplantation Model

[0100] Experiments were performed to determine whether inclusion of an inhibitor of formation of terminal complement would attenuate acute vascular rejection and whether the use of such an inhibitor in conjunction with an immunosuppressant would achieve long-term allograft survival. In this set of experiments an anti-C5 monoclonal antibody was used in conjunction with cyclosporin. The model used was an allograft heterotopic heart transplant from C3H mice into BALB/c mice. This model is a stringent acute vascular rejection model with the C3H and BALB/c mice being strongly MHC mismatched. The transplantations and other methods were performed as described in Wang et al. (2003).

Heterotopic Cardiac Transplantation

[0101] Intra-abdominal heterotopic cardiac transplantation was performed as previously described by Wang et al. (2003). Briefly, a median sternotomy was performed on the donor, and the heart graft was slowly perfused in situ with 1.0 ml of cold heparinized Ringer's lactate solution through the inferior vena cava and aorta before the superior vena cava and pulmonary veins were ligated and divided. The ascending aorta and pulmonary artery were transected, and the graft was removed from the donor. The graft was then revascularized with end-to-side anastomoses between the donor's pulmonary artery and the recipient's inferior vena cava as well as the donor's aorta and the recipient's abdominal aorta using 11-0 nylon suture. The beating of the grafted heart was monitored daily by direct abdominal palpation. The degree of pulsation was scored as: A, beating strongly; B, noticeable decline in the intensity of pulsation; or C, complete cessation of cardiac impulses. When cardiac impulses were no longer palpable, the graft was removed for routine histology. In certain instances, mice in which the graft was still functioning were sacrificed to perform histology.

Results

[0102] Mice (male 8-12 week old mice weighing 25 - 30 g) were split into six experimental groups with six to eight mice per group. Transplant occurred on day 0. Histological changes were checked at the endpoint (the endpoint being graft failure) or in some cases a mouse was sacrificed prior to graft failure. The dosage of BB5.1 which was administered (40 mg/kg body weight three times per week) was known from prior studies to completely inhibit terminal complement activity.

Group 1' (control) - mice were administered 0.75 mL of saline intraperitoneally on days -1, 0, 1 and 2. Subsequently these mice were treated with 0.75 mL of saline intraperitoneally three times per week (Monday, Wednesday, Friday) until the endpoint.

Group 2' (cyclosporin A alone) - mice were administered 15 mg/kg body weight of cyclosporin A subcutaneously on a daily basis beginning at day 0 (day of transplant) until the endpoint.

Group 3' (anti-complement antibody alone) - mice were administered the anti-mouse C5 antibody BB5.1 (Frei et al., 1987) at 40 mg/kg body weight intraperitoneally on days -1, 0, 1 and 2 followed by 40 mg/kg body weight administered three times per week (Monday, Wednesday, Friday) until the endpoint.

Group 4' (anti-complement antibody until day 14 post-transplant plus cyclosporin A) - mice were administered the anti-mouse C5 antibody BB5.1 at 40 mg/kg body weight intravenously on days -1 through day 14 and were also administered cyclosporin A at 15 mg/kg of body weight on a daily basis beginning at day 0 until the endpoint. Note that this differs from the other groups in that the BB5.1 was administered intravenously and on a daily basis.

Group 5' (anti-complement antibody until day 28 post-transplant plus cyclosporin A) - mice were administered the anti-mouse C5 antibody BB5.1 at 40 mg/kg body weight intraperitoneally on days -1,0,1 and 2 followed by 40 mg/kg body weight administered three times per week (Monday, Wednesday, Friday) until day 28 and were also administered cyclosporin A at 15 mg/kg of body weight on a daily basis beginning at day 0 until the endpoint.

Group 6' (anti-complement antibody chronically until 100 days plus cyclosporin) - mice were administered the anti-mouse C5 antibody BB5.1 at 40 mg/kg body weight intraperitoneally on days -1, 0, 1 and 2 followed by 40 mg/kg

Samsung Bioepis Exhibit No. 1002
Page 1926

body weight administered three times per week (Monday, Wednesday, Friday) until 100 days and were also administered cyclosporin A at 15 mg/kg of body weight on a daily basis beginning at day 0 until 100 days.

[0103] The results of this experiment are shown in Tables 3 and 4. Table 3 shows the survival time for the grafts. Table 4 sets forth the histological scores.

Table 3

Allograft Survival		
Group (Treatment)	Individual Survival (days)	Mean Survival Time (days)
1'. Saline	8, 8, 8, 8, 8, 9	8.3 ± 0.5
2'. Cyclosporin A	14, 15, 15, 16, 16, 16, 17	15.5 ± 1.1
3'. BB5.1	7, 8, 8, 8, 8, 9	8.0 ± 0.6
4'. BB5.1 until day 14 + cyclosporin A	35, 38, 43, 45, 46, 47	42.3 ± 4.8
5'. BB5.1 until day 28 + cyclosporin A	77, 80, 80, 81, 82	80 ± 1.9
6'. BB5.1 until day 100 + cyclosporin A	>100 days (7 mice; one sacrificed for histology)	> 100 days

Table 4

Median Scores of Histological Changes of Heart Allografts at Necropsy							
Groups	Vasc*	Infarc	Lymph	Throm	Hemo	Fibrin	PMN
1'. Saline (endpoint)	3.0	3.0	1.0	4.0	3.0	3.0	3.0
2'. Cyclosporin A (endpoint)	2.0	1.0	2.0	3.0	2.0	2.0	3.0
3'. BB5.1 (endpoint)	2.0	1.0	2.0	2.0	1.0	0.0	0.0
4'. BB5.1 until day 14 + cyclosporin A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
5'. BB5.1 28 days + Cyclosporin A (post-operative day 8)	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5'. BB5.1 28 days + Cyclosporin A (endpoint)	0.0	0.0	1.0	1.0	2.0	1.0	0.0
6'. BB5.1 until day 100 + Cyclosporin A (post-operative day 100)	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Median scores: 0 - normal; 1 o- minimum change; 2 o - mild change; 3 - moderate change; 4 - marked change. N/A - not available. *Vasc - vasculitis; Infar - infarction; Lymph - lymphocyte infiltration; Throm - thrombosis; Hemo - hemorrhage; Fibrin - fibrin deposition; PMN - polymorphonuclear cell infiltrate							

[0104] The results indicate the synergistic effects of using a complement inhibiting drug in addition to an immunosuppressant. In untreated mice the grafts were rejected in about 8 davs. Use of the immunosuppressant cyclosporin A alone on a daily, chronic basis resulted in an increase in graft survival until approximately 15 days post-transplant. The use of the anti-C5 antibody BB5.1 to inhibit formation of terminal complement had no effect on its own, graft rejection occurring at 8 days post-transplant as in the control group (Group 1'). The combination of BB5.1 through day 28 post-transplant plus cyclosporin A showed a synergistic effect with graft survival being extended until approximately day 80. A more surprising result is that of Group 5' in which BB5.1 and cyclosporin A were each administered chronically post-transplant. In this case the graft survival was for more than 100 days (as much data as presently available). Additionally, the histological results shown in Table 4 indicate that the administration of both BB5.1 and cyclosporin A protected the graft from changes much better than either BB5.1 or cyclosporin A alone, and that the chronic administration of BB5.1 and cyclosporin A protected the graft to such an extent that even at 100 days post-transplant there were no histological changes seen in the engrafted hearts. A survival time of 100 days in these models is considered to be the gold standard.

Samsung Bioepis Exhibit No. 1002
Page 1927

A survival of 100 days in the model is believed to indicate that there will be an indefinite survival of the allograft. When BB5.1 administration was stopped after 28 days, the grafts were protected but they did begin to show some minimal to mild histological changes by about day 80 which was the time at which graft failure occurred.

[0105] The Group 4' mice were treated differently in that they were administered BB5.1 on a daily basis by an intravenous administration. These animals became ill, showing weight loss and urine retention and were sacrificed at a time at which the grafted hearts were still beating although their function had declined. This was the first group of mice studied and it is unknown why these ill effects were seen. These ill effects were not seen when the BB5.1 was administered intraperitoneally with a schedule of three times per week. As seen below in Example 4, daily administration of BB5.1 via an intraperitoneal route did not cause ill effects. Also, intravenous administration was not necessarily the cause of the illness in these animals. Intravenous administration of eculizumab (a human equivalent antibody to BB5.1 in that it binds to human C5) has been successfully administered intravenously without ill effects to humans in a study of PNH (Hillmen et al., 2004). Complement inhibitors may be administered by other routes in addition to intravenous and intraperitoneal, with all such routes being well known by those skilled in the art.

Example 4

Accelerated Rejection in a Presensitized Heart Transplantation Model

[0106] A second set of experiments similar to those of Example 3 was performed but the recipient mouse was presensitized to the donor organ. In these experiments, the presensitization was brought about by prior transplantation of a skin graft. In general, presensitization can occur not only as a result of having received an earlier allograft, but can also be caused by having received multiple blood transfusions or in women who have been pregnant. Besides such presensitization methods, allografts with an ABO mismatch will be rapidly attacked and rejected because of preformed antibodies to the ABO antigens unless steps are taken to prevent such an attack.

[0107] Some mice in these studies were administered cyclophosphamide in addition to BB5.1 and/or cyclosporin A. For these experiments BALB/c recipient mice were presensitized with C3H skin grafts one week prior to heart transplantation from the same donor (using the method of Pruitt and Bollinger, 1991). This model is designed to mimic presensitized transplantation in humans, especially in relation to accelerated humoral rejection. Recipient mice were split into eight groups of six to eight mice each. The treatments were as follow.

Group 1" (control) - mice (male 8-12 week old mice weighing 25 - 30 g) were administered 0.75 mL saline intraperitoneally on a daily basis beginning at day -1 and continuing until the endpoint (graft rejection).

Group 2" (cyclosporin A alone) - mice were administered cyclosporin A subcutaneously at a dose of 15 mg/kg body weight beginning on day 0 (day of transplant) until the endpoint.

Group 3" (BB5.1 alone) - mice were administered the anti-mouse complement monoclonal antibody BB5.1 at a dose of 40 mg/kg body weight delivered intraperitoneally on a daily basis beginning at day -1 and continuing until the endpoint.

Group 4" (cyclophosphamide alone) - mice were administered cyclophosphamide intravenously at a dose of 40 mg/kg body weight on each of days 0 and 1.

Group 5" (BB5.1 plus cyclosporin A) - mice were administered BB5.1 intraperitoneally at a dose of 40 mg/kg body weight on a daily basis beginning at day -1 and continuing until the endpoint. These mice were additionally administered cyclosporin A subcutaneously at a dose of 15 mg/kg body weight on a daily basis from day 0 until the endpoint.

Group 6" (BB5.1 plus cyclophosphamide) - mice were administered BB5.1 intraperitoneally at a dose of 40 mg/kg body weight on a daily basis beginning at day -1 and continuing until the endpoint. These mice were additionally administered cyclophosphamide intravenously at a dose of 40 mg/kg body weight on each of days 0 and 1.

Group 7" (cyclosporin A plus cyclophosphamide) - mice were administered cyclosporin A subcutaneously at a dose of 15 mg/kg body weight on a daily basis from day 0 until the endpoint. These mice were additionally administered cyclophosphamide intravenously at a dose of 40 mg/kg body weight on each of days 0 and 1.

Group 8" (BB5.1 plus cyclosporin A plus cyclophosphamide) - mice were administered BB5.1 intraperitoneally at a dose of 40 mg/kg body weight on a daily basis beginning at day -1 and continuing until 100 days. These mice were also administered cyclosporin A subcutaneously at a dose of 15 mg/kg body weight on a daily basis from day 0 until

Samsung Bioepis Exhibit No. 1002
Page 1928

100 days. These mice were additionally administered cyclophosphamide intravenously at a dose of 40 mg/kg body weight on each of days 0 and 1. Two mice in this group were sacrificed at day 60 for histological studies (no rejection had yet occurred) and the four remaining mice still had not rejected their grafts by day 100.

[0108] Additionally a control group of mice which was not presensitized and received only the saline treatment as for Group 1" was tested.

[0109] The results of these experiments are shown in Tables 5 and 6. Table 5 lists survival times for the grafts and Table 6 summarizes the histological results.

Table 5

Allograft Survival		
Groups (Treatment)	Individual survival (days)	Mean survival time (days)
No presensitization	8, 8, 8, 8, 8,9	8.3 ± 0.5*
1". One skin presensitization	3, 3, 3, 3, 3, 3, 3, 4	3.1 ± 0.4
2". Cyclosporin A	3, 3, 3, 3	3.0 ± 0.0
3". BB5.1	3, 3, 4, 4	3.5 ± 0.6
4". Cyclophosphamide	3, 3, 3, 4	3.3 ± 0.5
5". BB5.1 + Cyclosporin A	10, 10, 11, 11, 12, 12, 14, 15	11.9 ± 1.8**
6". BB5.1 + Cyclophosphamide	3, 3, 3, 3, 3, 4	3.2 ± 0.4
7". Cyclosporin A + Cyclophosphamide	3, 3, 3, 4, 4, 4	3.5 ± 0.6
8". BB5.1 + Cyclosporin A + Cyclophosphamide	> 100 days (4 mice)	> 100***
*P < 0.01 group 1" vs. no presensitization **P < 0.01 group 5" vs. groups 1"-4" and 6"-7". ***P<0.01 group 8" vs. groups 1"-7".		

Table 6

Median Scores of Histological Changes of Heart Allografts at Necropsy							
Groups	Vasc*	Infar	Lymph	Throm	Hemo	Fibrin	PMN
No presensitization (endpoint)	3.0	3.0	1.0	4.0	3.0	3.0	3.0
1". One skin presensitization (endpoint)	0.0	4.0	1.0	4.0	3.0	0.0	0.0
2". Cyclosporin A (endpoint)	0.0	4.0	0.0	3.0	3.0	N/A	N/A
3". BB5.1 (endpoint)	2.0	2.0	2.0	2.0	3.0	N/A	N/A
4". Cyclophosphamide (endpoint)	2.0	4.0	0.0	3.0	2.0	N/A	N/A
5". BB5.1 + Cyclosporin A (post-operative day 3)	0.0	1.0	1.0	0.0	0.0	0.0	0.0
5". BB5.1 + Cyclosporin A (endpoint)	2.0	2.0	2.0	2.0	2.0	0.0	0.0
6". BB5.1 + Cyclophosphamide (endpoint)	1.0	3.0	0.0	3.0	2.0	N/A	N/A
7". Cyclosporin A + Cyclophosphamide (endpoint)	0.0	1.0	1.0	2.0	3.0	N/A	N/A
8". BB5.1 + Cyclosporin A + Cyclophosphamide (post-operative day 3)	0.0	0.0	1.0	0.0	0.0	N/A	N/A
8. BB5.1 + Cyclosporin A + Cyclophosphamide (post-operative day 12)	0.0	0.0	1.0	0.0	0.0	N/A	N/A
8". BB5.1 + Cyclosporin A + Cyclophosphamide (post-operative day 60)	0.0	0.0	1.0	0.0	0.0	N/A	N/A

Samsung Bioepis Exhibit No. 1002
Page 1929

(continued)

Median Scores of Histological Changes of Heart Allografts at Necropsy							
Groups	Vasc*	Infar	Lymph	Throm	Hemo	Fibrin	PMN
8". BB5.1 + Cyclosporin A + Cyclophosphamide (post-operative day 100)	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Median scores: 0 - normal; 1 - minimum change; 2 - mild change; 3 - moderate change; 4 - marked change. N/A - not available. *Vasc - vasculitis; Infar - infarction; Lymph - lymphocyte infiltration; Throm - thrombosis; Hemo - hemorrhage; Fibrin - fibrin deposition; PMN - polymorphonuclear cell infiltrate							

[0110] The results shown in Table 5 indicate a difference between the presensitized mouse model and the nonpresensitized mouse model as used in Example 3. The results indicate that in the absence of presensitization, grafts are rejected in approximately 8 days in the absence of treatment with any drugs. Presensitizing the animals causes a more rapid rejection, the rejection of the graft in the presensitized animals being in approximately 3 days in the absence of any drug treatment. Treatment with either BB5.1, cyclosporin A or cyclophosphamide had no effect upon graft survival, with the grafts being rejected in approximately 3-4 days in each of these groups of animals. The combination of BB5.1 and cyclosporin A showed some effect with rejection occurring about day 12. The combination of BB5.1 and cyclophosphamide had no protective effect with rejection occurring about day 3. Similarly the combination of cyclosporin A and cyclophosphamide had essentially no protective effect with rejection occurring at 3-4 days. Very surprisingly, the combination of all three drugs (chronic administration of BB5.1 and cyclosporin plus administration of cyclophosphamide at the time of transplant) showed a highly synergistic effect with all of the mice surviving for more than 100 days. Again, a survival of 100 days in this model is considered to be the gold standard and assumes an indefinite survival.

[0111] These results as well as the histological results as shown in Table 6 indicate that the combination of chronic treatment with a complement inhibitor and an immunosuppressant such as cyclosporin A in treating a presensitized mouse results in some attenuation of accelerated rejection. Treatment of these animals additionally with cyclophosphamide at the time of transplant and on the first day after transplant results in a much greater time of survival, no rejection having been seen by at least day 100.

[0112] It will be appreciated that the methods and compositions of the instant disclosure can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent to the artisan that other embodiments exist and do not depart from the spirit of the disclosure. Thus, the described embodiments are illustrative and should not be construed as restrictive.

LIST OF REFERENCES

[0113] The publications and other materials used herein to illuminate the background of the disclosure, and in particular, cases to provide additional details respecting the practice, are incorporated herein by reference in their entirety, and for convenience, are referenced by author and date in the text and respectively grouped in the following List of References.

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U.S. Patent 6,821,515

The contents of all references cited herein are incorporated by reference in their entirety.

[0114] The invention furthermore comprises the following items:

1. A method to prolong survival of an MHC mismatched allograft in a recipient mammal, said method comprising administering to said mammal a) a drug which inhibits complement activity and b) at least one immunosuppressive drug, wherein said drug which inhibits complement activity is administered chronically.

2. The method of item 1 wherein said mammal is a human.

3. The method of item 2 wherein said MHC mismatched allograft is an HLA mismatched allograft.

4. The method of item 1 wherein said drug which inhibits complement activity inhibits the formation of terminal complement or C5a.

5. The method of item 4 wherein said drug which inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment.

6. The method of item 5 wherein said whole antibody or antibody fragment is a human, humanized, chimerized or deimmunized antibody or antibody fragment.

Samsung Bioepis Exhibit No. 1002
Page 1933

7. The method of item 5 wherein said whole antibody or antibody fragment inhibits cleavage of complement C5.

8. The method of item 5 wherein said antibody fragment is selected from the group consisting of an Fab, an $F(ab')_2$, an Fv, a domain antibody, and a single-chain antibody.

9. The method of item 5 wherein said antibody fragment is pexelizumab.

10. The method of item 5 wherein said whole antibody is eculizumab.

11. The method item 10 wherein said eculizumab is administered once every 2 weeks.

12. The method of item 1 wherein said inhibitor of complement activity is selected from the group consisting of a i) soluble complement receptor, ii) CD59, iii) CD55, iv) CD46, and v) an antibody to C5, C6, C7, C8, or C9.

13. The method of item 1 wherein said immunosuppressive drug inhibits T-cell activity or B-cell activity.

14. The method of item 1 wherein said immunosuppressive drug inhibits T-cell activity and B-cell activity.

15. The method of item 1 wherein said immunosuppressive drug is selected from the group consisting of cyclosporin A, tacrolimus, sirolimus, OKT3, a corticosteroid, daclizumab, basiliximab, azathioprene, mycophenolate mofetil, methotrexate, 6-mercaptopurine, anti-T cell antibodies, cyclophosphamide, leflunamide, brequinar, ATG, ALG, 15-deoxyspergualin, and bredinin.

16. The method of item 1 wherein more than one immunosuppressive drug is administered.

17. The method of item 1 wherein said method comprises administering i) a drug which inhibits complement activity and ii) cyclosporin A.

18. The method of item 17 wherein said drug which inhibits complement activity is an antibody which inhibits cleavage of complement C5.

19. The method of item 1 wherein said allograft is selected from the group consisting of i) heart, ii) kidney, iii) lung, iv) pancreas, v) liver, vi) vascular tissue, vii) eye, viii) cornea, ix) lens, x) skin, xi) bone marrow, xii) muscle, xiii) connective tissue, xiv) gastrointestinal tissue, xv) nervous tissue, xvi) bone, xvii) stem cells, xviii) islets, xix) cartilage, xx) hepatocytes, and xxi) hematopoietic cells.

20. The method of item 1 wherein said allograft survives for a time at least 20% longer than would occur if said method were to be performed without said drug which inhibits complement activity.

21. The method of item 1 wherein said allograft survives for a time at least 40% longer than would occur if said method were to be performed without said drug which inhibits complement activity.

22. The method of item 1 wherein said allograft survives for the remaining life-time of said mammal.

23. The method of item 2 wherein said allograft survives for at least six months.

24. The method of item 2 wherein said allograft survives for at least one year.

25. The method of item 2 wherein said allograft survives for at least five years.

26. The method of item 2 wherein said allograft survives for the remaining life-time of said human.

27. The method of item 1 wherein said drug which inhibits complement activity is administered chronically for at least 14 days.

28. The method of item 1 wherein said drug which inhibits complement activity is administered chronically for at least 28 days.

Samsung Bioepis Exhibit No. 1002
Page 1934

29. The method of item 1 wherein said drug which inhibits complement activity is administered chronically for at least 3 months.

30. The method of item 1 wherein said drug which inhibits complement activity is administered chronically for at least 6 months.

31. The method of item 1 wherein said drug which inhibits complement activity is administered chronically for at least 1 year.

32. The method of item 1 wherein said drug which inhibits complement activity is administered chronically for at least 5 years.

33. The method of item 1 wherein said drug which inhibits complement activity is administered chronically for the remaining life-time of said mammal.

34. The method of item 1 wherein at least one immunosuppressive drug is administered chronically for the remaining life-time of said mammal.

35. The method of item 34 wherein at least cyclosporin A is administered chronically for the remaining life-time of said mammal.

36. A method to prolong survival of an allograft in a recipient mammal presensitized to said allograft, said method comprising administering to said mammal a) a drug which inhibits complement activity and b) at least one immunosuppressive drug, wherein said drug which inhibits complement activity is administered chronically.

37. The method of item 36 wherein said mammal is a human.

38. The method of item 36 wherein said drug which inhibits complement activity inhibits the formation of terminal complement or C5a.

39. The method of item 38 wherein said drug that inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment.

40. The method of item 39 wherein said whole antibody or antibody fragment is a human, humanized, chimerized or deimmunized antibody or antibody fragment.

41. The method of item 39 wherein said whole antibody or antibody fragment inhibits cleavage of complement C5.

42. The method of item 39 wherein said antibody fragment is selected from the group consisting of an Fab, an $F(ab')_2$, an Fv, a domain antibody, and a single-chain antibody.

43. The method of item 39 wherein said antibody fragment is pexelizumab.

44. The method of item 39 wherein said whole antibody is eculizumab.

45. The method item 44 wherein said eculizumab is administered once every 2 weeks.

46. The method of item 36 wherein said inhibitor of complement activity is selected from the group consisting of a i) soluble complement receptor, ii) CD59, iii) CD55, iv) CD46, and v) an antibody to C5, C6, C7, C8, or C9.

47. The method of item 36 wherein said immunosuppressive drug inhibits T-cell activity or B-cell activity.

48. The method of item 36 wherein said immunosuppressive drug inhibits T-cell activity and B-cell activity.

49. The method of item 36 wherein said immunosuppressive drug is selected from the group consisting of cyclosporin A, tacrolimus, sirolimus, OKT3, a corticosteroid, daclizumab, basiliximab, azathioprene, mycophenolate mofetil, methotrexate, 6-mercaptopurine, anti-T cell antibodies, cyclophosphamide, leflunamide, brequinar, ATG, ALG, 15-deoxyspergualin, and bredinin.

Samsung Bioepis Exhibit No. 1002
Page 1935

50. The method of item 36 wherein more than one immunosuppressive drug is administered.

51. The method of item 36 wherein said method comprises administering i) a drug which inhibits complement activity and ii) cyclosporin A.

52. The method of item 51 wherein said method comprises administering i) a drug which inhibits complement activity, ii) cyclosporin A, and iii) cyclophosphamide.

53. The method of item 51 wherein said drug which inhibits complement activity is an antibody which inhibits cleavage of complement C5.

54. The method of item 36 wherein said allograft is selected from the group consisting of i) heart, ii) kidney, iii) lung, iv) pancreas, v) liver, vi) vascular tissue, vii) eye, viii) cornea, ix) lens, x) skin, xi) bone marrow, xii) muscle, xiii) connective tissue, xiv) gastrointestinal tissue, xv) nervous tissue, xvi) bone, xvii) stem cells, xviii) islets, xix) cartilage, xx) hepatocytes, and xxi) hematopoietic cells.

55. The method of item 36 wherein said allograft survives for a time at least 20% longer than would occur if said method were to be performed without said drug which inhibits complement activity.

56. The method of item 36 wherein said allograft survives for a time at least 40% longer than would occur if said method were to be performed without said drug which inhibits complement activity.

57. The method of item 36 wherein said allograft survives for the remaining life-time of said mammal.

58. The method of item 37 wherein said allograft survives for at least six months.

59. The method of item 37 wherein said allograft survives for at least one year.

60. The method of item 37 wherein said allograft survives for at least five years.

61. The method of item 37 wherein said allograft survives for the remaining life-time of said human.

62. The method of item 36 wherein said drug which inhibits complement activity is administered chronically for at least 14 days.

63. The method of item 36 wherein said drug which inhibits complement activity is administered chronically for at least 28 days.

64. The method of item 36 wherein said drug which inhibits complement activity is administered chronically for at least 3 months.

65. The method of item 36 wherein said drug which inhibits complement activity is administered chronically for at least 6 months.

66. The method of item 36 wherein said drug which inhibits complement activity is administered chronically for at least 1 year.

67. The method of item 36 wherein said drug which inhibits complement activity is administered chronically for at least 5 years.

68. The method of item 36 wherein said drug which inhibits complement activity is administered chronically for the remaining life-time of said mammal.

69. The method of item 36 wherein at least one immunosuppressive drug is administered chronically for the remaining life-time of said mammal.

70. The method of item 69 wherein at least cyclosporin A is administered chronically for the remaining life-time of said mammal.

Samsung Bioepis Exhibit No. 1002
Page 1936

71. A method to prolong survival of an allograft in a recipient mammal which is an ABO mismatch to said allograft, said method comprising administering to said mammal a) a drug which inhibits complement activity and b) at least one immunosuppressive drug, wherein said drug which inhibits complement activity is administered chronically.

72. The method of item 71 wherein said mammal is a human.

73. The method of item 71 wherein said drug which inhibits complement activity inhibits the formation of terminal complement or C5a.

74. The method of item 73 wherein said drug which inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment.

75. The method of item 74 wherein said whole antibody or antibody fragment is a human, humanized, chimerized or deimmunized antibody or antibody fragment.

76. The method of item 74 wherein said whole antibody or antibody fragment inhibits cleavage of complement C5.

77. The method of item 74 wherein said antibody fragment is selected from the group consisting of an Fab, an $F(ab')_2$, an Fv, a domain antibody, and a single-chain antibody.

78. The method of item 74 wherein said antibody fragment is pexelizumab.

79. The method of item 74 wherein said whole antibody is eculizumab.

80. The method item 79 wherein said eculizumab is administered once every 2 weeks.

81. The method of item 71 wherein said inhibitor of complement activity is selected from the group consisting of a i) soluble complement receptor, ii) CD59, iii) CD55, iv) CD46, and v) an antibody to C5, C6, C7, C8, or C9.

82. The method of item 71 wherein said immunosuppressive drug inhibits T-cell activity or B-cell activity.

83. The method of item 71 wherein said immunosuppressive drug inhibits T-cell activity and B-cell activity.

84. The method of item 71 wherein said immunosuppressive drug is selected from the group consisting of cyclosporin A, tacrolimus, sirolimus, OKT3, a corticosteroid, daclizumab, basiliximab, azathioprene, mycophenolate mofetil, methotrexate, 6-mercaptopurine, anti-T cell antibodies, cyclophosphamide, leflunamide, brequinar, ATG, ALG, 15-deoxyspergualin, and bredinin.

85. The method of item 71 wherein more than one immunosuppressive drug is administered.

86. The method of item 71 wherein said method comprises administering i) a drug which inhibits complement activity and ii) cyclosporin A.

87. The method of item 86 wherein said method comprises administering i) a drug which inhibits complement activity, ii) cyclosporin A, and iii) cyclophosphamide.

88. The method of item 86 wherein said drug that inhibits complement activity is an antibody that inhibits cleavage of complement C5.

89. The method of item 71 wherein said allograft is selected from the group consisting of i) heart, ii) kidney, iii) lung, iv) pancreas, v) liver, vi) vascular tissue, vii) eye, viii) cornea, ix) lens, x) skin, xi) bone marrow, xii) muscle, xiii) connective tissue, xiv) gastrointestinal tissue, xv) nervous tissue, xvi) bone, xvii) stem cells, xviii) islets, xix) cartilage, xx) hepatocytes, and xxi) hematopoietic cells.

90. The method of item 71 wherein said allograft survives for a time at least 20% longer than would occur if said method were to be performed without said drug that inhibits complement activity.

91. The method of item 71 wherein said allograft survives for a time at least 40% longer than would occur if said

Samsung Bioepis Exhibit No. 1002
Page 1937

method were to be performed without said drug that inhibits complement activity.

92. The method of item 71 wherein said allograft survives for the remaining life-time of said mammal.

93. The method of item 72 wherein said allograft survives for at least six months.

94. The method of item 72 wherein said allograft survives for at least one year.

95. The method of item 72 wherein said allograft survives for at least five years.

96. The method of item 72 wherein said allograft survives for the remaining life-time of said human.

97. The method of item 71 wherein said drug which inhibits complement activity is administered chronically for at least 14 days.

98. The method of item 71 wherein said drug which inhibits complement activity is administered chronically for at least 28 days.

99. The method of item 71 wherein said drug which inhibits complement activity is administered chronically for at least 3 months.

100. The method of item 71 wherein said drug which inhibits complement activity is administered chronically for at least 6 months.

101. The method of item 71 wherein said drug which inhibits complement activity is administered chronically for at least 1 year.

102. The method of item 71 wherein said drug which inhibits complement activity is administered chronically for at least 5 years.

103. The method of item 71 wherein said drug which inhibits complement activity is administered chronically for the remaining life-time of said mammal.

104. The method of item 71 wherein at least one immunosuppressive drug is administered chronically for the remaining life-time of said mammal.

105. The method of item 104 wherein at least cyclosporin A is administered chronically for the remaining life-time of said mammal.

106. Use of a drug that inhibits complement activity and an immunosuppressive drug in the manufacture of a medicament or medicament package for prolong survival of an allograft in a mammal.

107. The use of item 106, wherein more than one immunosuppressive drug is included in said medicament or medicament package.

108. The use of item 106, wherein said drug that inhibits complement activity and said immunosuppressive drug are in a formulation suitable for concurrent administration to said mammal.

109. The use of item 106, wherein said drug that inhibits complement activity and said immunosuppressive drug are in a formulation or formulations suitable for sequential administration to said mammal.

110. The use of item 106, wherein said drug that inhibits complement activity is in a formulation suitable for chronic administration to said mammal.

111. The use of item 106, wherein said immunosuppressive drug is in a formulation suitable for chronic administration to said mammal.

112. A method to prolong survival of allograft in a recipient mammal, comprising preparing an allograft from a first

Samsung Bioepis Exhibit No. 1002
Page 1938

allograft accommodated by a first recipient mammal, wherein said first recipient mammal, after receiving said first allograft, has been treated with a drug that inhibits complement activity and at least one immunosuppressive agent.

113. An allograft that has prolonged survival in a recipient mammal, wherein said allograft is prepared from a first recipient mammal and was transplanted to the first recipient mammal that has been treated with a drug that inhibits complement activity and at least one immunosuppressive agent.

114. A pharmaceutical package comprising a drug that inhibits complement activity and at least one immunosuppressive agent, wherein said drug and said agent are formulated for chronic administration.

115. The pharmaceutical package of item 114, wherein said drug is an antibody in a lyophilized formulation comprising the antibody and a lyoprotectant.

116. The pharmaceutical package of item 114, wherein said agent is in a lyophilized formulation comprising the agent and a lyoprotectant.

117. The pharmaceutical package of item 114, wherein said drug and said agent are in the same lyophilized formulation comprising said drug, said agent, and a lyoprotectant.

118. The pharmaceutical package of item 114, wherein said drug is in an injection system comprising a syringe that comprises a cartridge, wherein said cartridge contains said drug in a formulation suitable for injection.

119. The pharmaceutical package of item 114, wherein said agent is in an injection system comprising a syringe that comprises a cartridge, wherein said cartridge contains said agent in a formulation suitable for injection.

120. The pharmaceutical package of item 114, wherein said drug and said agent are in an injection system comprising a syringe that comprises a cartridge, wherein said cartridge contains said drug and said agent in a formulation suitable for injection.

121. The pharmaceutical package of item 114, wherein said drug is present in unit dosage form.

122. The pharmaceutical package of item 114, wherein said agent is present in unit dosage form.

123. A lyophilized antibody formulation comprising an antibody that inhibits complement activity and a lyoprotectant, wherein said antibody formulation is suitable for chronic administration.

124. The lyophilized antibody formulation of item 123, wherein the antibody inhibits the formation of terminal complement or C5a.

125. The lyophilized antibody formulation of item 123, wherein the antibody is a whole antibody or an antibody fragment.

126. The lyophilized antibody formulation of item 125, wherein the antibody wherein said whole antibody or antibody fragment is a human, humanized, chimerized or deimmunized antibody or antibody fragment.

127. The lyophilized antibody formulation of item 125, wherein said whole antibody or antibody fragment inhibits cleavage of complement C5.

128. The lyophilized antibody formulation of item 125, wherein said antibody fragment is selected from the group consisting of an Fab, an $F(ab')_2$, an Fv, a domain antibody, and a single-chain antibody.

129. The lyophilized antibody formulation of item 125, wherein said antibody fragment is pexelizumab.

130. The lyophilized antibody formulation of item 125, wherein said whole antibody is eculizumab.

131. An injection system comprising a syringe, wherein said syringe comprises a cartridge containing an antibody that inhibits complement activity and is formulated suitable for injection.

Samsung Bioepis Exhibit No. 1002
Page 1939

Claims

1. An antibody specific to C5, which inhibits the cleavage of C5, for use in prolonging survival of an allograft in a recipient mammal, wherein said antibody to C5 is to be administered chronically and said antibody to C5 is administered in conjunction with more than one immunosuppressive drug.

2. An antibody specific to C5, which inhibits the cleavage of C5, for use in prolonging survival of an allograft in a recipient mammal according to claim 1 wherein said recipient mammal is pre-sensitised.

3. An antibody specific to C5 which inhibits the cleavage of C5 for use in prolonging survival of an allograft in a pre-sensitised recipient mammal according to claim 1 or claim 2 wherein said antibody to C5 is to be administered chronically and said antibody to C5 is administered in conjunction with more than one immunosuppressive drug and wherein at least one immunosuppressive drug is administered chronically.

4. The antibody to C5 for use in prolonging survival of an allograft in a recipient mammal according to any preceding claim wherein said mammal is a human.

5. The antibody according to any preceding claim wherein said antibody to C5 which inhibits the cleavage of C5, inhibits the formation of terminal complement or C5a.

6. The antibody to C5 according to claim 5 wherein said antibody to C5 which inhibits formation of terminal complement or C5a is a whole antibody or an antibody fragment.

7. The antibody to C5 and more than one immunosuppressive drug for use in prolonging survival of an allograft in a recipient mammal according to any preceding claim, wherein said whole antibody or antibody fragment is a humanized, chimerized or deimmunized antibody or antibody fragment.

8. The antibody according to any one of claims 6 or 7, wherein said antibody fragment is selected from the group consisting of an Fab, an $F(ab')_2$, Fv, and a single-chain antibody.

9. The antibody according to claim 8, wherein said antibody fragment is pexelizumab.

10. The antibody to C5 according to claim 9 wherein said whole antibody is eculizumab.

11. The antibody to C5 according to claim 12 wherein said immunosuppressive drug is selected from the group consisting of cyclosporine A, tacrolimus, sirolimus, OKT3, a corticosteroid, daclizumab, basiliximab, azathioprene, mycophenolate mofetil, methotrexate, 6-mercaptopurine, anti-T- cell antibodies, cyclophosphamide, leflunamide, brequinar, ATG, ALG, 15-deoxyspergualin, and bredinin.

12. The antibody to C5 according to any preceding claim wherein said antibody to C5 is to be administered in conjunction with (cyclosporin A.

13. The antibody to C5 according to any preceding claim wherein said antibody to C5 is to be administered in conjunction with cyclosporin A and cyclophosphamide.

14. The antibody to C5 for use in prolonging survival of an allograft according to any preceding claim wherein said allograft is selected from the group consisting of i) heart, ii) kidney, iii) lung, iv) pancreas, v) liver, vi) vascular tissue, vii) eye, viii) cornea,.ix) lens, x) skin, xi) bone marrow, xii) muscle, xiii) connective tissue, xiv) gastrointestinal tissue, xv) nervous tissue, xvi) bone, xvii) stem cells, xviii) islets, xix) cartilage, xx) hepatocytes, and xxi) hematopoietic cells.

15. The antibody to C5 for use in prolonging survival of an allograft according to any preceding claim wherein said allograft survives for at least 6 months.

16. The antibody to C5 according to any preceding claim wherein said antibody to C5 is to be administered chronically for at least 28 days.

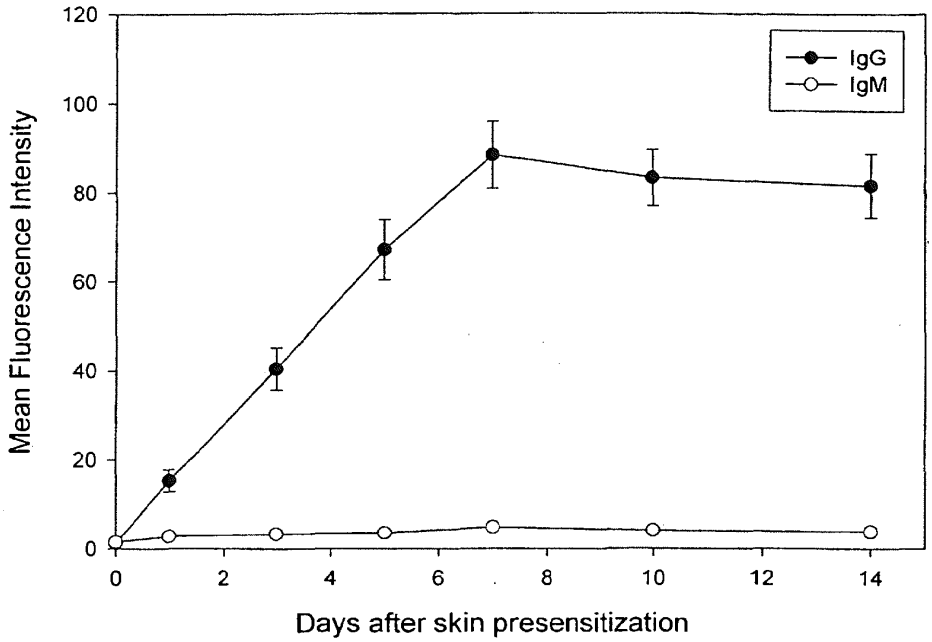
17. Use of an antibody specific to C5, which inhibits the cleavage of C5 according to any preceding claim, for the manufacture of a medicament for prolonging the survival of an allograft in a recipient mammal wherein said medi-

Samsung Bioepis Exhibit No. 1002
Page 1940

cament is to be administered chronically and said medicament is administered in conjunction with more than one immunosuppressive drug; preferably wherein said recipient mammal is pre-sensitised.

Samsung Bioepis Exhibit No. 1002
Page 1941

A



B

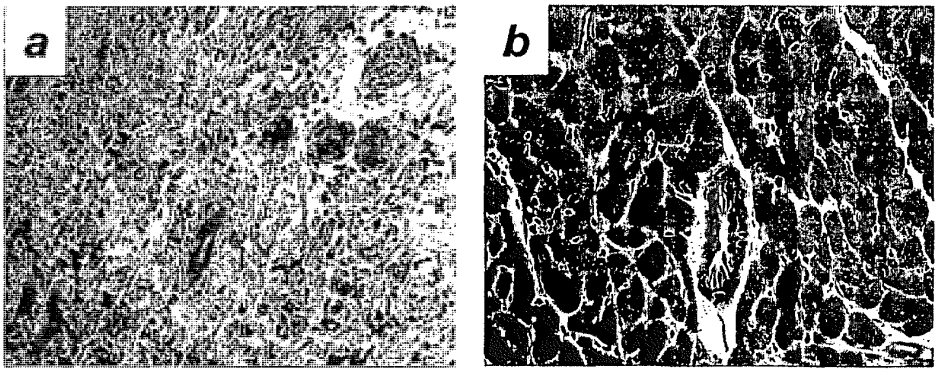


Figure 1A, B

Samsung Bioepis Exhibit No. 1002
Page 1942

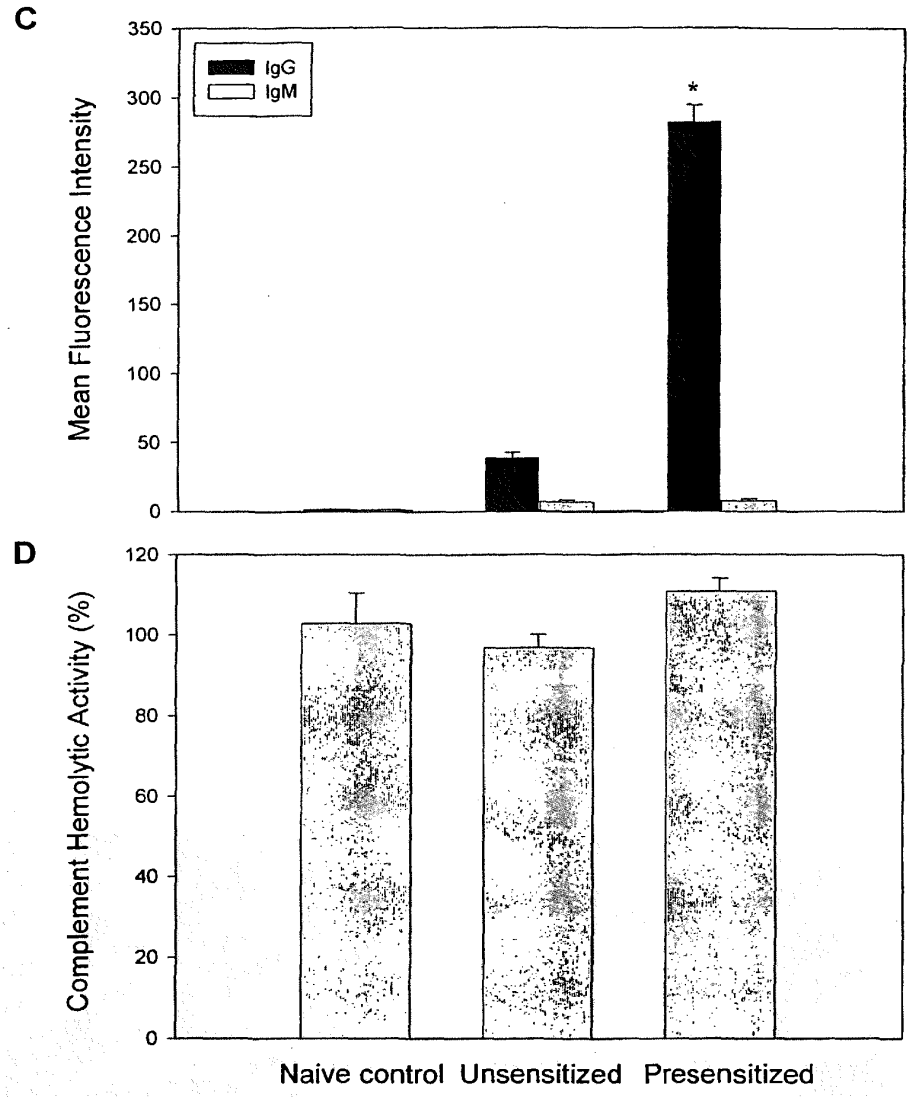


Figure 1C, D

Samsung Bioepis Exhibit No. 1002
Page 1943

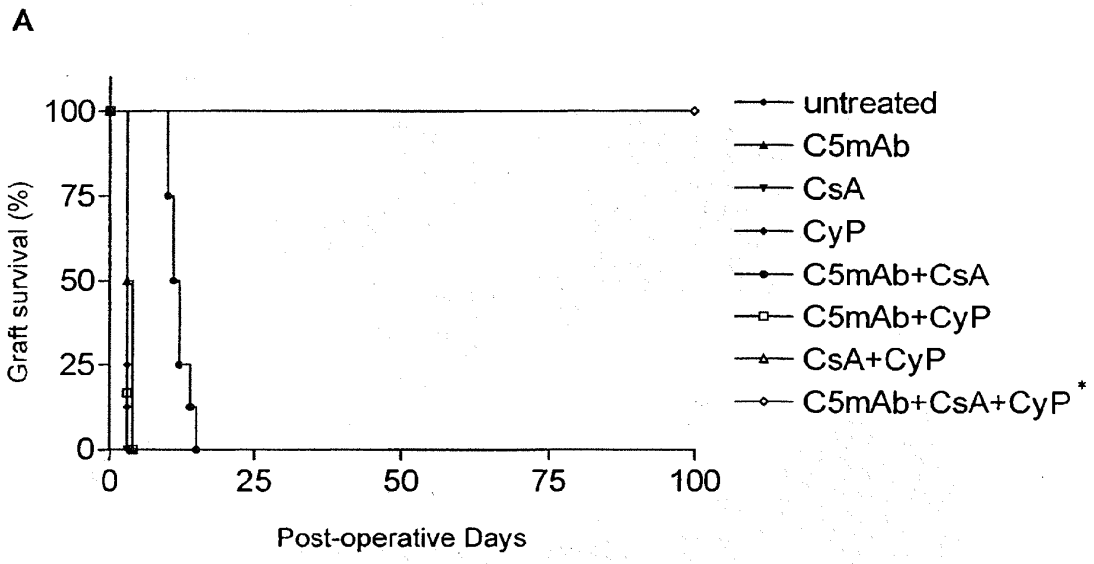


Figure 2A

Samsung Bioepis Exhibit No. 1002
Page 1944

B

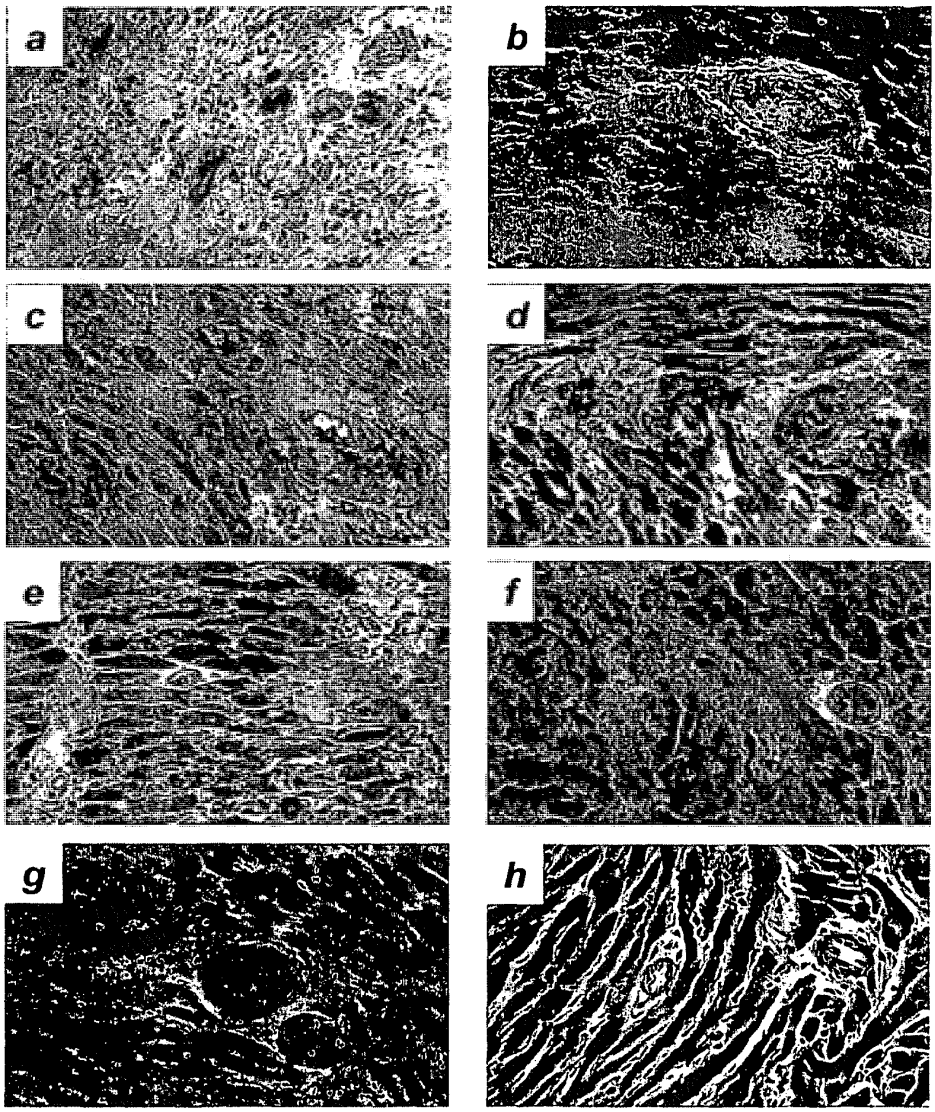


Figure 2B

Samsung Bioepis Exhibit No. 1002
Page 1945

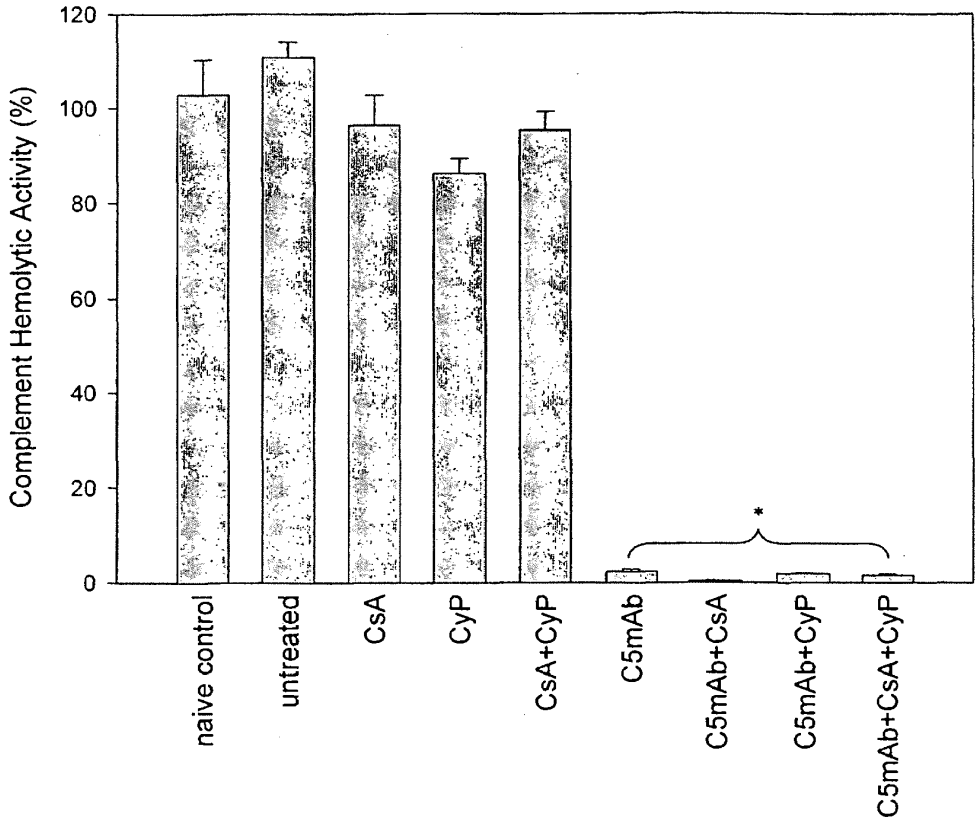


Figure 3

Samsung Bioepis Exhibit No. 1002
Page 1946

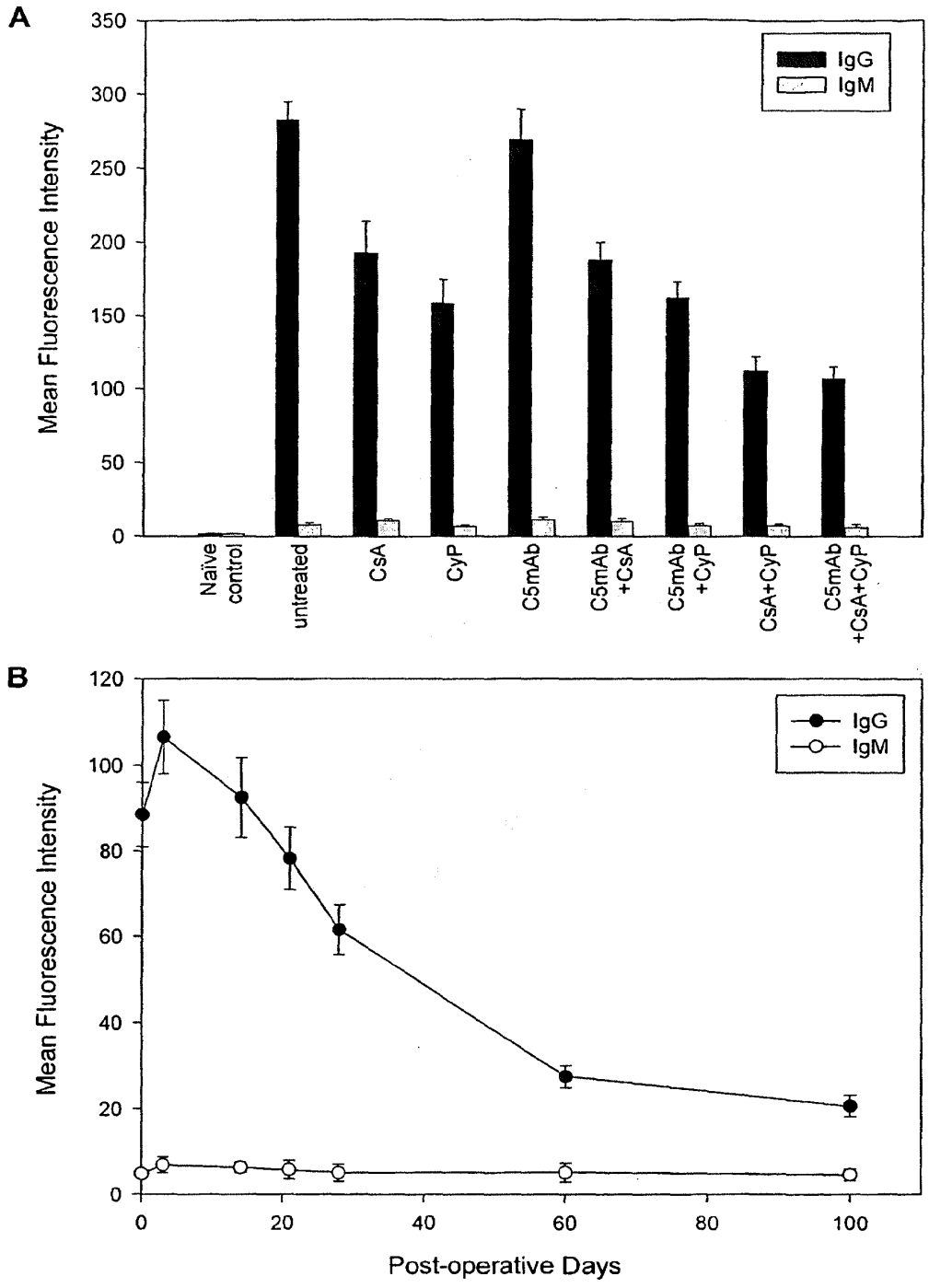
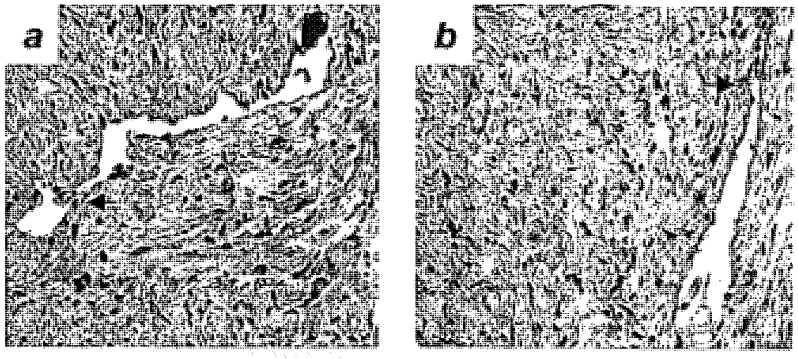


Figure 4A, B

Samsung Bioepis Exhibit No. 1002
Page 1947

C



D

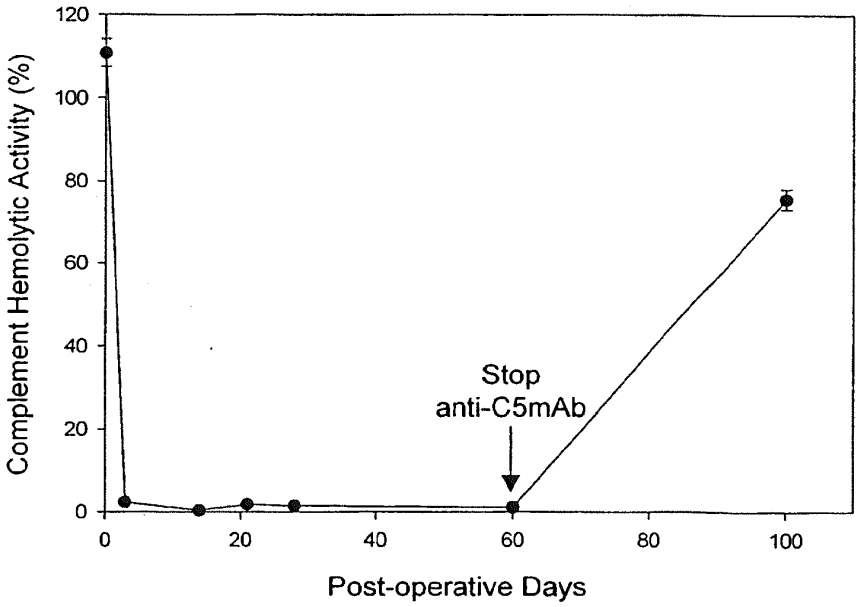


Figure 4C, D

Samsung Bioepis Exhibit No. 1002
Page 1948

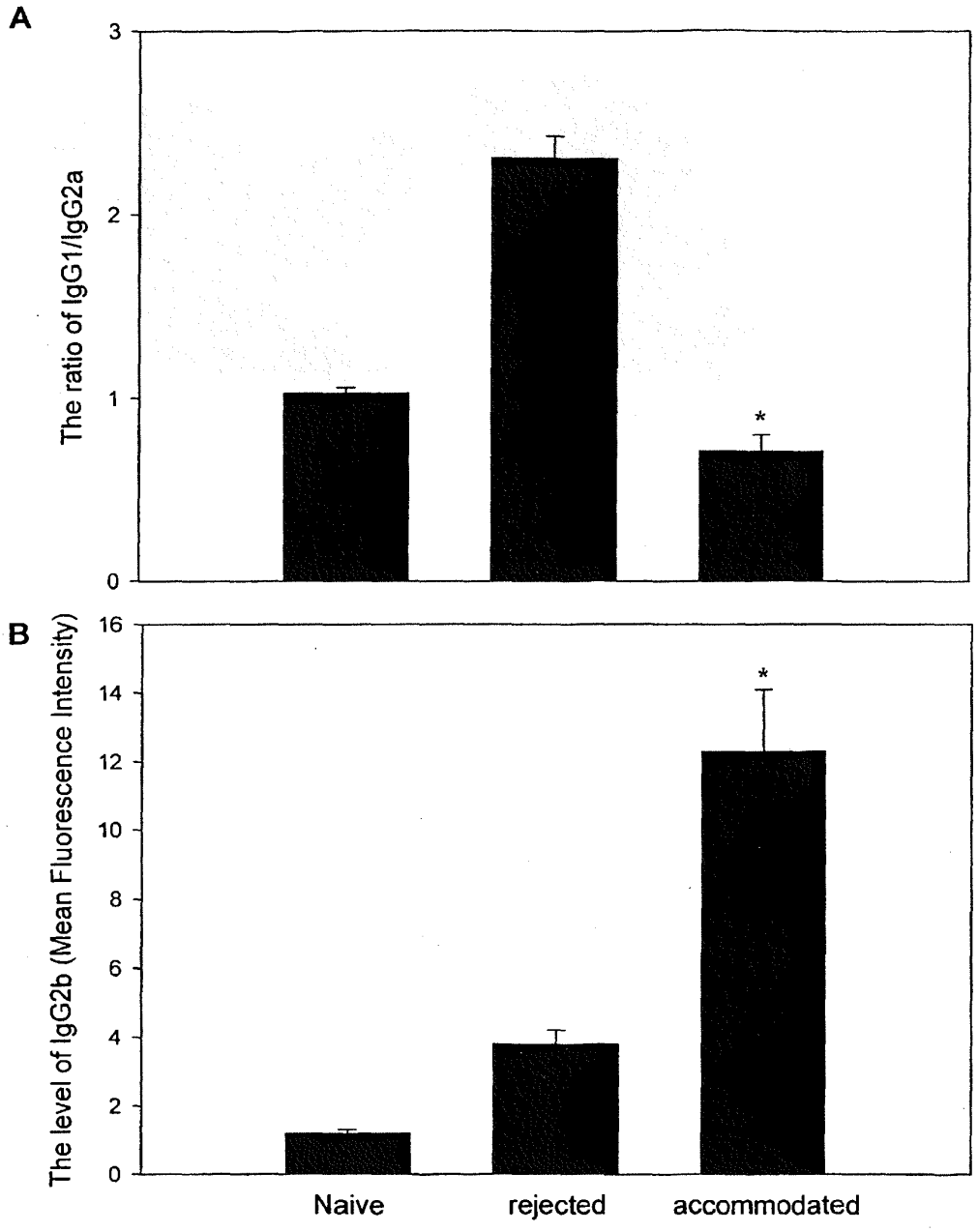


Figure 5A, B

Samsung Bioepis Exhibit No. 1002
Page 1949

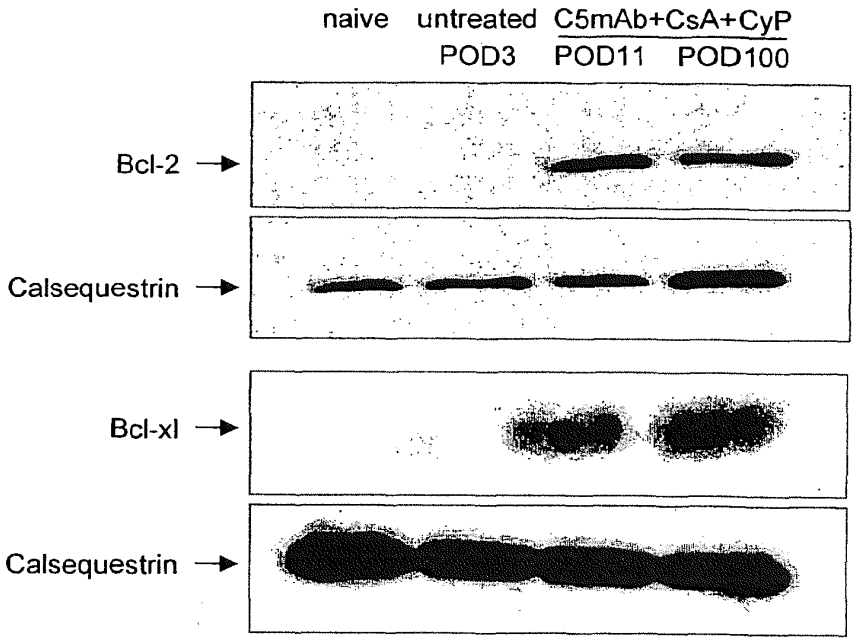


Figure 6

Samsung Bioepis Exhibit No. 1002
Page 1950

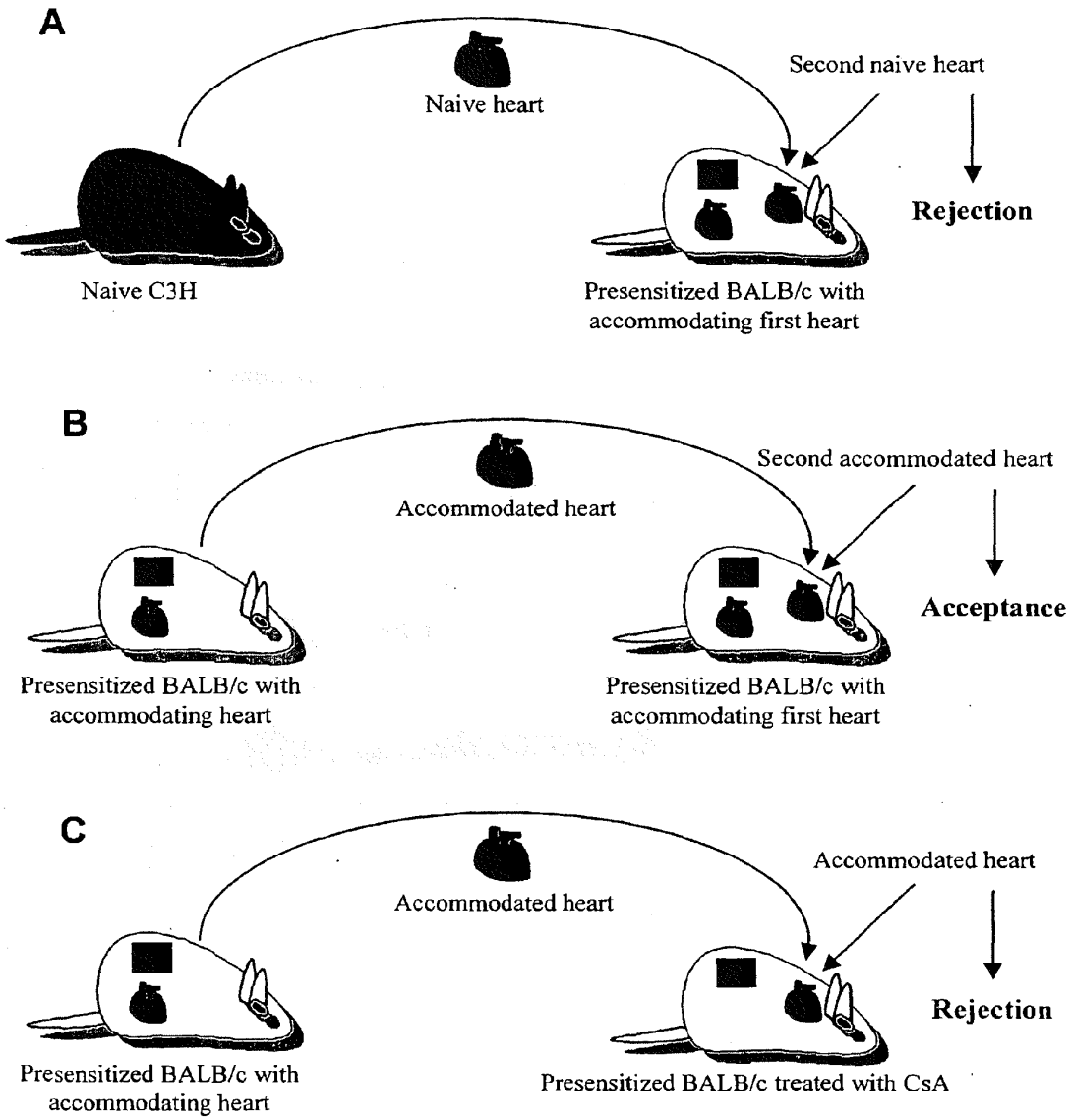


Figure 7

Samsung Bioepis Exhibit No. 1002
Page 1951

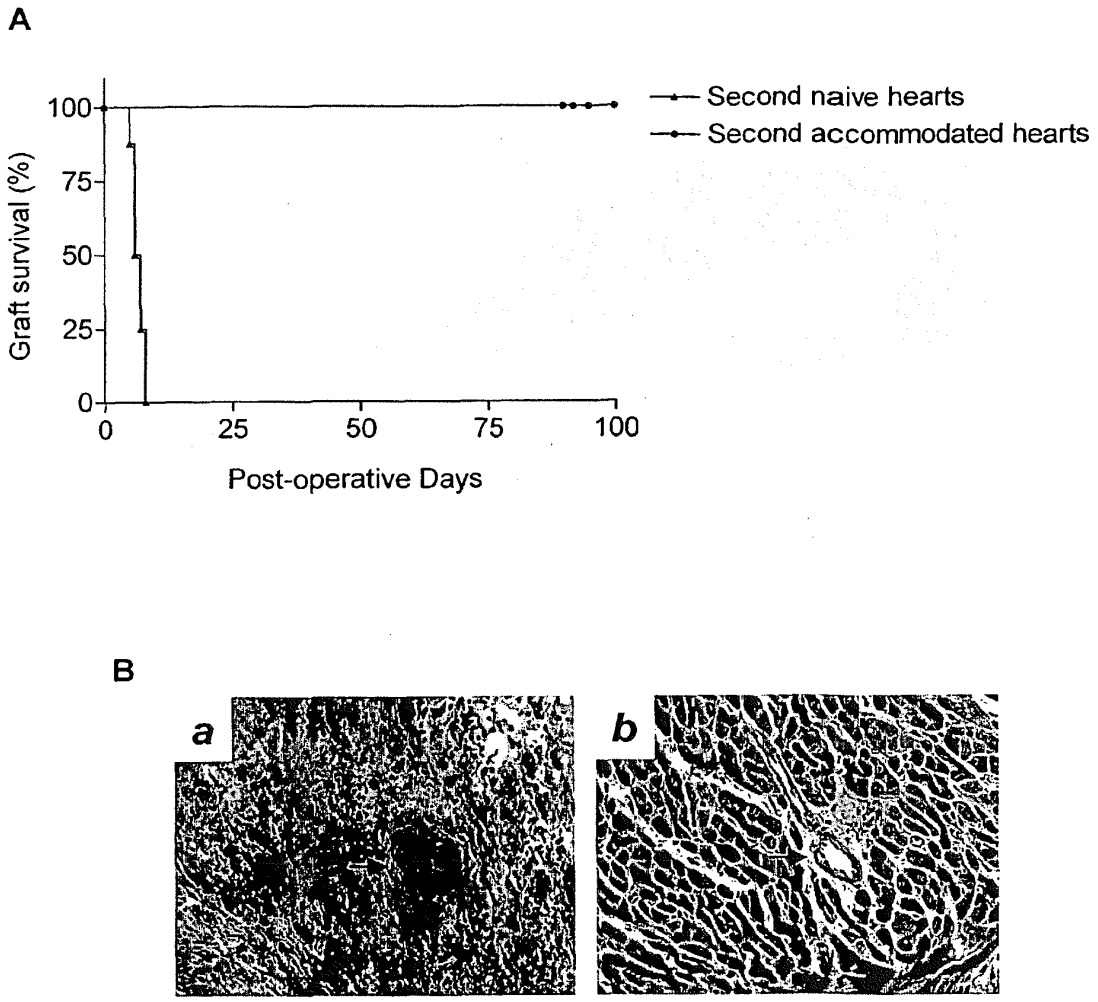


Figure 8

Samsung Bioepis Exhibit No. 1002
Page 1952

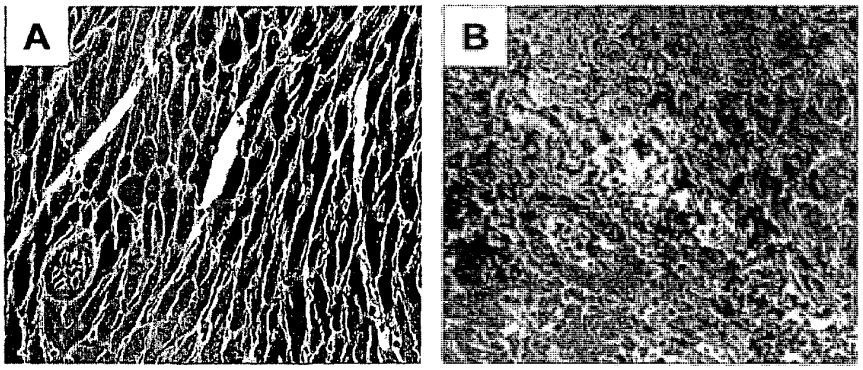


Figure 9

Samsung Bioepis Exhibit No. 1002
Page 1953

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European Patent Office
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EUROPEAN SEARCH REPORT

Application Number
EP 16 16 0321

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A,D	KROSHUS TIMOTHY J ET AL: "Complement inhibition with an anti-C5 monoclonal antibody prevents acute cardiac tissue injury in an ex vivo model of pig-to-human xenotransplantation", TRANSPLANTATION (BALTIMORE), vol. 60, no. 11, 1995, pages 1194-1202, XP002356728, ISSN: 0041-1337 * the whole document *	1-17	INV. A61K39/395 C07K16/18
A,D	WANG H ET AL: "COMPLEMENT INHIBITION WITH AN ANTI-C5 MONOCLONAL ANTIBODY PREVENTS HYPERACUTE REJECTION IN A XENOGRAFT HEART TRANSPLANTATION MODEL", TRANSPLANTATION, WILLIAMS AND WILKINS, BALTIMORE, MD, US, vol. 68, no. 11, 15 December 1999 (1999-12-15), pages 1643-1651, XP001095658, ISSN: 0041-1337 * the whole document *	1-17	
A,D	US 6 355 245 B1 (EVANS MARK J ET AL) 12 March 2002 (2002-03-12) * the whole document *	1-17	TECHNICAL FIELDS SEARCHED (IPC) C07K
A	US 2002/041875 A1 (FUNG MICHAEL S.C ET AL) 11 April 2002 (2002-04-11) * the whole document *	1-17	

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	14 June 2016	Rojo Romeo, Elena

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
O : non-written disclosure
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Samsung Bioepis Exhibit No. 1002
Page 1954

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EUROPEAN SEARCH REPORT

Application Number
EP 16 16 0321

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A,D	VAKEVA ANTTI P ET AL: "Myocardial infarction and apoptosis after myocardial ischemia and reperfusion: Role of the terminal complement components and inhibition by anti-C5 therapy", CIRCULATION, vol. 97, no. 22, 9 June 1998 (1998-06-09), pages 2259-2267, XP002356729, ISSN: 0009-7322 * the whole document *	1-17	
T	WANG HAO ET AL: "Prevention of acute vascular rejection by a functionally blocking anti-C5 monoclonal antibody combined with cyclosporine", TRANSPLANTATION (HAGERSTOWN), vol. 79, no. 9, May 2005 (2005-05), pages 1121-1127, XP002356730, ISSN: 0041-1337 * the whole document *	1-17	

TECHNICAL FIELDS SEARCHED (IPC)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	14 June 2016	Rojo Romeo, Elena

CATEGORY OF CITED DOCUMENTS

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D : document cited in the application
L : document cited for other reasons

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Samsung Bioepis Exhibit No. 1002
Page 1955

Europäisches Patentamt
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Application Number
EP 16 16 0321

CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing claims for which payment was due.

☐ Only part of the claims have been paid within the prescribed time limit. The present European search report has been drawn up for those claims for which no payment was due and for those claims for which claims fees have been paid, namely claim(s):

☒ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for those claims for which no payment was due.

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

☐ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.

☐ As all searchable claims could be searched without effort justifying an additional fee, the Search Division did not invite payment of any additional fee.

☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid, namely claims:

☐ None of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims, namely claims:

☐ The present supplementary European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims (Rule 164 (1) EPC).

Samsung Bioepis Exhibit No. 1002
Page 1956

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 16 16 0321

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

14-06-2016

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Samsung Bioepis Exhibit No. 1002
Page 1957

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(54) **Methods and compositions for the treatment of antibody mediated neuropathies**

Verfahren und Zusammensetzungen zur Behandlung von antikörpervermittelten Neuropathien

Procédés et compositions pour le traitement de neuropathies à médiation par anticorps

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(73) Proprietor: **ALEXION PHARMACEUTICALS, INC.**
Cheshire, CT 06410 (US)

(72) Inventors:
- **Halstead, Susan**
  Shawlands, Glasgow G41 3BX (GB)
- **Willison, Hugh**
  Glasgow G61 4AJ (GB)
- **Rother, Russell, P.**
  Oklahoma City
  OK 73151 (US)

(74) Representative: **Vossius & Partner**
Patentanwälte Rechtsanwälte mbB
Siebertstrasse 3
81675 München (DE)

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EP 2 380 907 B1

Description

FIELD OF THE INVENTION

[0001] This application provides compositions for the treatment of antibody mediated neuropathies that activate complement to induce nerve injury. In specific embodiments, the application relates to the use of complement cascade inhibitors as therapeutic agents to treat antibody mediated neuropathies.

BACKGROUND OF THE INVENTION

[0002] Neuropathy is a generic term used to describe diseases of the peripheral nervous system. There are about 200 known different causes of peripheral neuropathies. Although most neuropathies affect all three major classes of nerve fibers to varying degrees, some diseases involve only one or two, and are thus said to be purely or predominantly motor, sensory, or autonomic neuropathies.

[0003] Guillain-Barré syndrome (GBS) is an acute illness involving the peripheral nervous system that usually occurs two to three weeks after a flu-like disease or other infection, including *Campylobacter* enteritis. It is mostly a motor neuropathy, meaning that its symptoms are largely related to the involvement of the motor nerves. Despite the primarily motor nature of the disease, the earliest symptoms may be numbness and tingling felt in the lower extremities followed shortly by weakness of the distal muscles of the lower extremities. The danger occurs when the weakness involves the muscles of respiration. GBS is associated in a proportion of cases with antiganglioside autoantibodies (AGAs) to a wide range of specific glycolipid structures, and also with antibodies to other nerve components including myelin proteins and glycosaminoglycans in some cases, and in other GBS cases, antibodies are presumed to be present but have yet to be formally identified.

[0004] Miller Fisher syndrome (MFS) is a variant of Guillain-Barré syndrome, accounting for 5-10% of cases. In one survey, the annual incidence has been estimated at 0.09 per 100,000 population. MFS is characterized by the acute onset of ophthalmoplegia, ataxia and areflexia. Anti-GQ1b ganglioside antibodies are the serological hallmark of MFS. The antibodies may arise in some cases through molecular mimicry with *Campylobacter* lipopolysaccharides. It is now widely accepted that well over 90% of patients with MFS have anti-GQ1b IgG antibodies during the acute phase of the illness. Equally significant is the complete absence of anti-GQ1b IgG antibodies from normal and other disease control groups, indicative of a high level of specificity for this disease association. The antibody titers peak at clinical presentation, decaying rapidly with the course of clinical recovery. Anti-GQ1b IgG antibody titers are also elevated in the acute phase sera of some patients with Guillain-Barré syndrome with ophthalmoplegia. The anti-GQ1b IgG antibody marker also identifies a cluster of closely related syndromes, often *considered formes frustes*. of MFS, that have in common the presence of external ophthalmoplegia or cerebellar-like ataxia.

[0005] The current treatment for GBS, including AGA-mediated cases is intravenous immunoglobulin (IVIg), plasma exchange, or a combination of both. Although some neuropathies like GBS are usually self-limiting illnesses, intensive therapeutic intervention is often needed. Some individuals are left with residual deficits. Other neuropathies are chronic and only symptoms such as pain are treated. There is a great need for additional treatments to improve symptoms and decrease recovery time of antibody mediated neuropathies.

[0006] Halstead et al. 2005 (Annals of Neurology (58), 203 - 210) describes that APT070 abrogates nerve terminal injury in rodent models of Miller Fisher syndrome, by reduction of intermediate C3 convertase as well as by inhibition of C5 convertase. APT070 is a soluble form of complement receptor 1 (sCR1) protein that binds to C3b and C4b and thereby inhibits, among other things, opsonisation by host phagocytes (e.g., macrophages) of objects to which C3b and C4b are bound.

[0007] US 2005/004031 describes small molecule inhibitors of one or more of C5a receptor, C1r, C1q, C1s, and MASP-2 and the use of these small molecule inhibitors in treating neurodegenerative diseases.

[0008] WO 2004/007553 describes the generation and characterization of an antibody designated TS-A12/22 that binds to complement component C5. TS-A12/22 inhibits hemolysis and MAC formation *in vifro* and inhibits bovine serum albumin-mediated influx of polymorphonuclear (PMN) cells into the joints of rats.

[0009] WO 95/29697 describes anti-C5 antibodies for use in treating glomerulonephritis (GN).

SUMMARY OF THE INVENTION

[0010] Compositions for treating patients suffering from antibody-mediated neuropathies are presented as characterised in the appended claims.

[0011] In a number of neuropathies antibodies to specific components of nerves have been identified. Some of these diseases are mediated by anti-ganglioside and anti-glycolipid autoantibodies such as the Miller Fisher variant of Guillain-Barré syndrome. Antibodies to a wide range of glycolipids including GM1, GM1(NeuGc),GM1b, GalNAc-GM1b, GDIa, GalNAc-GD1a, GD1b, 9-0-acetyl GD1b, GD3, GT1a, GT1b, GQ1b, GQ1ba, LM1, galactocerebroside and SGPG have been reported in papers on inflammatory neuropathies, as case reports and in larger series.

[0012] Antibodies to other nerve components including proteins and glycoproteins have also been reported to be causative in neuropathies. For example, monoclonal IgM against myelin-associated glycoprotein causes anti-MAG IgM paraproteinemic neuropathy (Willison and Yuki, Brain, 2002, 125, 2591-2625). In a proportion of neu-

Samsung Bioepis Exhibit No. 1002
Page 1961

ropathy cases, antibodies are presumed to be present owing to similar pathological patterns and treatment responses as seen in antibody-associated cases, but the specificity of the presumed antibody has yet to be formally identified. In accordance with the present invention, the antibody mediated neuropathy may be one or more of the following: acute motor axonal neuropathy, acute inflammatory demyelinating polyneuropathy, Bickerstaff's brainstem encephalitis, acute ophthalmoparesis, ataxic Guillain-Barré syndrome, pharyngeal-cervical-brachial weakness, chronic neuropathy syndromes with anti-glycolipid antibodies, anti-MAG IgM paraproteinemic neuropathy, chronic sensory ataxic neuropathy with anti-disialosyl antibodies, IgM, IgG and IgA paraproteinemic neuropathy, motor neuropathy with anti-GM1 and anti-GM2 antibodies, chronic inflammatory demyelinating neuropathy (CIDP), multifocal motor neuropathy (MMN), and multifocal acquired demyelinating sensory and motor neuropathy (MADSAM).

[0013] In some neuropathies the activation of complement may be antibody-independent, for example, in hereditary amyloid neuropathy. In this disease, early classical pathway activation markers, Cl q and C4, have been detected in amyloid deposits in the absence of detectable antibody (Hafer-Macko et al., J Peripher Nerv Syst. 2000 Sep;5(3):131-9; incorporated herein by reference). This suggests that antibody-independent activation of the classical pathway can occur and that complement cascade inhibitors may be effective in treating antibody-independent, complement mediated neuropathies. An antibody of the application inhibits activation of the classical pathway, the alternative pathway or the lectin complement pathway. In certain embodiments, said antibody is an antibody against any member of the group comprising the complement components C5 or C5b. In certain embodiments, said antibody is an antibody against C5. In certain embodiments, said antibody is an inhibitor of C5 cleavage.

[0014] Also described herein is a complement cascade inhibitor selected from: a polypeptide, a polypeptide analog, a peptidomimetic, an antibody, a nucleic acid, an RNAi construct, a nucleic acid analog, and a small molecule.

[0015] In certain embodiments, an antibody of the application is a whole antibody or an antibody fragment. In certain embodiments, said whole antibody or antibody fragment is selected from the group consisting of: a polyclonal antibody, a monoclonal antibody or antibody fragment, a diabody, a chimerized or chimeric antibody or antibody fragment, a humanized antibody or antibody fragment, a deimmunized human antibody or antibody fragment, a fully human antibody or antibody fragment, a single chain antibody, an Fv, an Fab, an Fab', or an $F(ab')_2$.

[0016] In certain embodiments, an antibody of the application inhibits activation of the classical pathway, the alternative pathway or the lectin complement pathway. In certain embodiments, said antibody is an antibody against any member of the group comprising the complement components C5 and C5b. In certain embodiments, said antibody is an antibody against C5. In certain embodiments, said antibody is an inhibitor of C5 cleavage.

[0017] In certain embodiments, an antibody of the application is eculizumab. In certain embodiments, said antibody is pexelizumab. In certain embodiments, said treatment with eculizumab is chronic. In certain embodiments, said treatment with pexelizumab comprises treating an acute episode.

[0018] In certain embodiments, a mammal of the application is to be treated by administering said antibody intravenously. In certain embodiments, said antibody is to be administered systemically to said mammal. In certain embodiments, said agent is to be administered locally to said mammal.

[0019] In certain embodiments, treatments of the application result in decreased neural injury in a mammal of the application.

[0020] In certain embodiments, a mammal of the application is to be treated to inhibit or decrease the amount and/or extent of an undesirable physiological condition resulting from excess nerve injury due to antibody dependent neuropathy. In certain embodiments, said undesirable physiological condition is selected from the group consisting of ophthalmoplegia, ataxia, areflexia, abnormal muscle coordination, paralysis of the eye muscles, aching pain in the muscles, absence of the tendon reflexes, numbness and tingling felt in the lower extremities, weakness of the distal muscles of the lower extremities, a footdrop, weakness involving the entire lower extremities, weakness involving the upper extremities, weakness in the muscles of respiration, and death.

[0021] In certain embodiments, the antibody for use according to the invention results in decreased membrane attack complex (MAC) formation at presynaptic motor axons.

[0022] In certain embodiments, the antibody for use according to the invention results in restoration of a normal frequency of miniature endplate, potentials.

[0023] In certain embodiments, the antibody for use according to the invention results in restoration of synaptic transmission at the neuromuscular junctions.

[0024] In certain embodiments, the antibody for use according to the invention in decreased loss of terminal integrity at the neuromuscular junctions.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025]

FIGURES 1A-1F show an immunohistological demonstration of the protective effect of eculizumab against MAC deposition and terminal motor axonopathy at NMJs in the in *vitro* MFS model. Mouse hemidiaphragm preparations werepre-incubated with anti-GQ1b ganglioside mAb CGM3 (50μg/ml) and sub-

Samsung Bioepis Exhibit No. 1002
Page 1962

sequently treated with 40% normal human serum (NHS) with either added eculizumab (anti-human C5 neutralizing mAb; 100μg/ml) or non-specific isotype-matched control mAb (100 μg/ml), and compared with untreated tissue. FIG. 1A shows tissues treated with NHS/eculizumab showed intense C3c staining at neuromuscular junctions (NMJs), similar to that of NHS/control mAb-treated tissues (p=0.12). FIG. 1B shows that membrane attack complex (MAC) staining was not observed in the presence of eculizumab (intensity comparable to baseline, p=0.92), indicating complete inhibition of terminal complement. FIG. 1C shows that axonal integrity was preserved in the presence of eculizumab, as assessed by the presence of neurofilament (NF) signal, comparable to baseline in untreated controls (p=0.80). FIGs. 4D, 4E and 4F are illustrative immunofluorescent images ofNMJs in whole-mount muscle demonstrating the protective effect of eculizumab against MAC deposition (FIG. 4D), perisynaptic Schwann cell (pSC) damage (FIG. 4E) and terminal motor axonal damage (FIG. 4F), as compared to NHS/control mAb treatment. Postsynaptic nicotinic acetylcholine receptor (nAChR; purple) staining was used to delineate the NMJ. Intermediate complement protein C3c (FIG. 4D) remains abundantly deposited at NMJs. *p<0.01, different from untreated; #p<0.01, different from control mAb-treated; scale bar = 20 μm.

FIGURES 2A-2G show eculizumab protects against electrophysiological and functional defects induced at NMJs in *the in vitro* MFS model. Mouse hemi-diaphragm preparations were pre-incubated with anti-GQ1b ganglioside mAb CGM3 (50 μg/ml) and subsequently treated with 40% normal human serum (NHS) with either added 100 μg/ml eculizumab (anti-human C5 neutralizing mAb) or non-specific isotype-matched control mAb. FIG. 2A shows spontaneous uniquantal acetylcholine release, measured as MEPP frequency, at the NMJ. Eculizumab prevented the induction of a high MEPP frequency (p<0.001, n=5 muscles). FIG. 2B shows representative 1 s duration traces obtained during incubation with NHS, either with added eculizumab (upper trace) or control mAb (lower trace). FIG. 2C shows asynchronous twitching of muscle fibers induced by NHS was largely prevented by eculizumab (p<0.01, n= 5 muscles). FIG. 2D shows that eculizumab completely prevented the occurrence of'silent' NMJs (i.e. without detectable synaptic electrophysiological signals). For FIGs. 2E, 2F and 2G hemi-diaphragm muscle-nerve preparations were pre-incubated with 200 μg/ml CGM3 and the protective effect of several concentrations of eculizumab (0-100 μg/ml, n=2-5 muscles per concentration) added to the NHS was observed in muscle contraction force recording experiments. FIG. 2E shows examples of the contraction profiles observed at 0, 30, 60 and 90 min after the start of the NHS incubation with either no eculizumab added or 6 or 100 μg/ml eculizumab added. Each contraction was elicited by supramaximal electric stimulation of the phrenic nerve for 3 s at 40 Hz. FIG. 2F shows development of the contraction loss during NHS incubation with the various concentrations of eculizumab added. FIG. 2G shows the concentration-effect relationship of eculizumab and the protection against loss of contraction after 90 min after the start of the NHS incubation. A Boltzmann sigmoidal curve is fitted through the data points, yielding an EC_{50} of 7.1 μg/ml. Error bars in FIGs. 2A, 2D, 2F and 2G represent S.E.M.

FIGURES 3A-3B show eculizumab dose response *curve in vivo.* FIG. 3A shows that an increasing dose of eculizumab results in a dose-dependent reduction in MAC deposition at the NMJ. FIG. 3B shows the neurofilament signal and demonstrates preservation of axonal integrity at all doses of eculizumab investigated when compared to PBS treated baseline control.

FIGURES 4A-4H show that respiratory paralysis in the *in vivo* MFS model is prevented by eculizumab due to inhibition of presynaptic block of neuromuscular transmission in the diaphragm. Mice (n=6) were intraperitoneally injected with 1.5 mg anti-GQ1b ganglioside mAb CGM3 and, 16 h later, with 0.5 ml 100% normal human serum (NHS) as complement source. A dose of 200 μg eculizumab or control mAb was injected in the tail vein shortly before the NHS injection. FIG. 4A shows grip-strength analysis at 2h post-NHS injection. Eculizumab prevented the loss of pulling force observed in the control mAb group (p<0.01). FIG. 4B shows average tidal volume and FIG. 4C shows breathing rate measured with whole-body plethysmography before NHS injection and during the 2nd, 4th and 6th hour post-NHS injection. The development of breathing difficulty was prevented by eculizumab. FIG. 4D shows examples of 1 s traces of plethysmography signals obtained in eculizumab- and control mAb-treated mice. FIG. 4E shows twitch force and FIG. 4F shows tetanic force elicited in the dissected diaphragm muscle by single and 40 Hz electrical nerve stimulation, respectively. Eculizumab completely prevented the severe paralysis observed in muscles from control mAb-treated mice (p<0.01). FIG. 4G shows that eculizumab almost completely prevented the occurrence of'silent' NMJs (i.e. without detectable synaptic electrophysiological signals) in these mice (p<0.001). FIG. 4H shows thirty superimposed representative traces of 1 s duration obtained in NMJs of muscle from eculizumab-treated (upper traces) or control mAb-treated (lower traces) mice. Error bars in the bar graphs of these figures represent S.E.M.

Samsung Bioepis Exhibit No. 1002
Page 1963

FIGURES 5A-5F show a morphological analysis of NMJs of diaphragm tissue from *in vivo* MFS mice. Mice were passively immunized with 1.5 mg anti-GQ1b ganglioside mAb, CGM3, followed 16 h later with concomitant injections of 0.5 ml 100% normal human serum (intraperitoneal) and eculizumab or control mAb (intravenous; 200μg). FIGs. 5A and 5B show that C3c and membrane attack complex (MAC) deposition is reduced at neuromuscular junctions (NMJs) of both eculizumab- and control mAb-treated mice ($p< 0.01$). FIG. 5C shows that neurofilament (NF) signal was significantly greater at NMJs of muscles from eculizumab-treated mice, compared with control ($p<0.01$). FIGs. 5D and 5E show illustrative immunofluorescent images of NMJs in whole mount muscle. Postsynaptic nicotinic acetylcholine receptors (nAChR; purple) staining was used to delineate NMJs. FIG. 5D further shows C3c and MAC deposition. FIG. 5E further shows NF integrity and MAC deposition. FIG. 5F is an electron micrograph of eculizumab-protected nerve terminals with tightly packed synaptic vesicle and electron dense mitochondria with parallel cristae. At NMJs from control mAb-treated mice, nerve terminals are electron lucent with sparse synaptic vesicles and swollen mitochondria. #$p<0.01$, different from control mAb-treated. Scale bars in FIGs. 5D and 5E = 20 μm; and in FIG. 5F = 1 μm.

DETAILED DESCRIPTION OF THE INVENTION

Overview

[0026] It is here proposed that treatment of an antibody mediated neuropathy patient with a complement inhibitor will attenuate neural injury. For example, the antibody mediated neuropathy may be an AGA mediated neuropathy. Inhibitors of members of the complement cascade include, for example, antibodies to components such as C5, C5b, C6, C7, C8, and C9. In particular embodiments, this application contemplates the use of the anti-C5 antibody eculizumab (a whole antibody) which is known to inhibit cleavage of C5 into C5a and C5b. Treatment of patients with such inhibitors thereby decreases complement mediated neural injury. Eculizumab has been used in clinical studies and found to be well tolerated with minimal side effects. See U.S. Patent 6,355,245 and Hillmen et al., New Engl. J. Med. 350:552-559 (2004).

The Complement System

[0027] The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of cellular and viral pathogens. There are at least 25 complement proteins, which are found as a complex collection of plasma proteins and membrane cofactors. The plasma proteins (which are also found in most other body fluids, such as lymph, bone marrow, synovial fluid, and cerebrospinal fluid) make up about 10% of the globulins in vertebrate serum. Complement components achieve their immune defensive functions by interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions.

[0028] The complement cascade progresses via the classical pathway or the alternative pathway. These pathways share many components and, while they differ in their early steps, both converge and share the same terminal complement components responsible for the destruction of target cells and viruses.

[0029] The classical complement pathway is typically initiated by antibody recognition of and binding to an antigenic site on a target cell. This surface bound antibody subsequently reacts with the first component of complement, C1. The C1 thus bound undergoes a set of autocatalytic reactions that result in, inter alia, the induction of C1 proteolytic activity acting on complement components C2 and C4.

[0030] This activated C1 cleaves C2 and C4 into C2a, C2b, C4a, and C4b. The function of C2b is poorly understood. C2a and C4b combine to form the C4b,2a complex, which is an active protease known as classical C3 convertase. C4b,2a acts to cleave C3 into C3a and C3b. C3a and C4a are both relatively weak anaphylatoxins that may induce degranulation of mast cells, resulting in the release of histamine and other mediators of inflammation.

[0031] C3b has multiple functions. As opsonin, it binds to bacteria, viruses and other cells and particles and tags them for removal from the circulation. C3b can also form a complex with C4b,C2a to produce C4b,2a,3b, or classical C5 convertase, which cleaves C5 into C5a (another anaphylatoxin) and C5b. Alternative C5 convertase is C3b,Bb,C3b and performs the same function. C5b combines with C6 yielding C5b,6, and this complex combines with C7 to form the ternary complex C5b,6,7. The C5b,6,7 complex binds C8 at the surface of a cell membrane. Upon binding of C9, the complete membrane attack complex (MAC) is formed (C5b-9) which mediates the lysis of foreign cells, microorganisms, and viruses.

[0032] Further discussions of the classical complement pathway, as well as a detailed description of the alternative pathway of complement activation, can be found in numerous publications including, for example, Muller-Eberhard, Annu Rev Biochem. 1988;57:321-47.

Inhibitors of the Complement Cascade

[0033] In certain embodiments, a complement inhibitor may be a small molecule (up to 6,000 Da in molecular weight), a nucleic acid or nucleic acid analog, a peptidomimetic, or a macromolecule that is not a nucleic acid or a protein. These agents include, but are not limited to, small organic molecules, RNA aptamers, L-RNA aptamers, Spiegelmers, antisense compounds, double strand-

Samsung Bioepis Exhibit No. 1002
Page 1964

ed RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

[0034] In certain embodiments, a complement inhibitor may be a protein or protein fragment. Proteins are known which inhibit the complement cascade, including CD59, CD55, CD46 and other inhibitors of C8 and C9 (see, e.g., U.S. Patent 6,100,443). Proteins known as complement receptors and which bind complement are also known (see, Published PCT Patent Application WO 92/10205 and U.S. Patent 6,057,131). Use of soluble forms of complement receptors, e.g., soluble CR1, can inhibit the consequences of complement activation such as neutrophil oxidative burst, complement mediated neural injury, and C3a and C5a production. Those of skill in the art recognize the above as some, but not all, of the known methods of inhibiting complement and its activation.

[0035] In certain embodiments, a complement inhibitor may be an antibody capable of inhibiting complement, such as an antibody that can block the formation of MAC. For example, an antibody complement inhibitor may include an anti-C5 antibody. Such anti-C5 antibodies may directly interact with C5 and/or C5b, so as to inhibit the formation of and/or physiologic function of C5b.

[0036] Suitable anti-C5 antibodies are known to those of skill in the art. Antibodies can be made to individual components of activated complement, e.g., antibodies to C7, C9, etc. (see, e.g., U.S. Patent 6,534,058; published U.S. patent application US 2003/0129187; and U.S. Patent 5,660,825). U.S. Patent 6,355,245 teaches an antibody which binds to C5 and inhibits cleavage into C5a and C5b thereby decreasing the formation not only of C5a but also the downstream complement components.

[0037] The concentration and/or physiologic activity of C5a and C5b in a body fluid can be measured by methods well known in the art. For C5a such methods include chemotaxis assays, RIAs, or ELISAs (see, for example, Ward and Zvaifler, J Clin Invest. 1971 Mar,50(3):606-16; Wurzner, et al., Complement Inflamm. 8:328-340, 1991). For C5b, hemolytic assays or assays for soluble C5b-9 as discussed herein can be used. Other assays known in the art can also be used. Using assays of these or other suitable types, candidate antibodies capable of inhibiting complement such as anti-C5 antibodies, now known or subsequently identified, can be screened in order to 1) identify compounds that are useful in the practice of the application and 2) determine the appropriate dosage levels of such compounds.

[0038] An antibody capable of inhibiting complement such as an anti-C5 antibody affecting C5b is preferably used at concentrations providing substantial reduction (i.e., reduction by at least about 25% as compared to that in the absence of the anti-C5 antibody) in the C5b levels present in at least one blood-derived fluid of the patient following activation of complement within the fluid. Such concentrations can be conveniently determined by measuring the cell-lysing ability (e.g., hemolytic activity) of complement present in the fluid or the levels of soluble C5b-9 present in the fluid. Accordingly, a specific concentration for an antibody that affects C5b is one that results in a substantial reduction (i.e., a reduction by at least about 25%) in the cell-lysing ability of the complement present in at least one of the patient's blood-derived fluids. Reductions of the cell-lysing ability of complement present in the patient's body fluids can be measured by methods well known in the art such as, for example, by a conventional hemolytic assay such as the hemolysis assay described by Kabat and Mayer (eds.), "Experimental Immunochemistry, 2d Edition", 135-240, Springfield, IL, CC Thomas (1961), pages 135-139, or a conventional variation of that assay such as the chicken erythrocyte hemolysis method described below.

[0039] Specific antibodies capable of inhibiting complement, such as an anti-C5 antibody, are relatively specific and do not block the functions of early complement components. In particular, such specific agents will not substantially impair the opsonization functions associated with complement component C3b, which functions provide a means for clearance of foreign particles and substances from the body.

[0040] C3b is generated by the cleavage of C3, which is carried out by classical and/or alternative C3 convertases and results in the generation of both C3a and C3b. Therefore, in order not to impair the opsonization functions associated with C3b, specific antibodies capable of inhibiting complement downstream of C3, such as an anti-C5 antibody, do not substantially interfere with the cleavage of complement component C3 in a body fluid of the patient (e.g., serum) into C3a and C3b. Such interference with the cleavage of C3 can be detected by measuring body fluid levels of C3a and/or C3b, which are produced in equimolar ratios by the actions of the C3 convertases. Such measurements are informative because C3a and C3b levels will be reduced (compared to a matched sample without the antibody capable of inhibiting complement such as an anti-C5 antibody) if cleavage is interfered with by an antibody capable of inhibiting complement.

[0041] In practice, the quantitative measurement of such cleavage is generally more accurate when carried out by the measurement of body fluid C3a levels rather than of body fluid C3b levels, since C3a remains in the fluid phase whereas C3b is rapidly cleared. C3a levels in a body fluid can be measured by methods well known in the art such as, for example, by using a commercially available C3a EIA kit, e.g., that sold by Quidel Corporation, San Diego, Calif., according to the manufacturer's specifications. Particularly specific antibodies capable of inhibiting complement such as an anti-C5 antibody produce essentially no reduction in body fluid C3a levels following complement activation when tested in such assays.

[0042] Certain antibodies of the disclosure will prevent the cleavage of C5 to form C5a and C5b, thus preventing the generation of the anaphylatoxic activity associated with C5a and preventing the assembly of the membrane

Samsung Bioepis Exhibit No. 1002
Page 1965

attack complex associated with C5b. As discussed above, in a particular embodiment, these anti-C5 antibodies will not impair the opsonization function associated with the action of C3b.

[0043] A preferred method of inhibiting complement activity is to use a monoclonal antibody which binds to complement C5 and inhibits cleavage. This decreases the formation of both C5a and C5b while at the same time allowing the formation of C3a and C3b which are beneficial to the recipient. Such antibodies which are specific to human complement are known (U.S. Patent 6,355,245). These antibodies disclosed in U.S. Patent 6,355,245 include a preferred whole antibody (now named eculizumab). A similar antibody against mouse C5 is called BB5.1 (Frei et al., Mol. Cell. Probes. 1:141-149 (1987)). Antibodies to inhibit complement activity need not be monoclonal antibodies. They can be, e.g., polyclonal antibodies. They may additionally be antibody fragments. An antibody fragment includes, but is not limited to, an Fab, F(ab'), $F(ab')_2$, single-chain antibody, and Fv. Furthermore, it is well known by those of skill in the art that antibodies can be humanized (Jones et al., Nature 321:522-5 (1986)), chimerized, or deimmunized. The antibodies to be used in the present disclosure may be any of these. It is preferable to use humanized antibodies.

[0044] In specific embodiments, a therapeutic agent of the disclosure comprises an antibody or antibody fragment. Antibodies and fragments thereof may be made by any conventional method, such as those methods described herein. Antibodies are found in multiple forms, e.g., IgA, IgG, IgM, etc. Additionally, antibodies can be engineered in numerous ways. They can be made as single-chain antibodies (including small modular immunopharmaceuticals or SMIPs™), Fab and $F(ab')_2$ fragments, etc. Antibodies can be humanized, chimerized, deimmunized, or fully human. Numerous publications set forth the many types of antibodies and the methods of engineering such antibodies. For example, see U.S. Patent Nos. 6,355,245; 6,180,370; 5,693,762; 6,407,213; 6,548,640; 5,565,332; 5,225,539; 6,103,889; and 5,260,203.

[0045] This invention provides fragments of anti-C5 antibodies, which may comprise a portion of an intact antibody, preferably the antigen-binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies (Zapata et al., Protein Eng. 8:1057-1062 (1995)); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

[0046] Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment of an antibody yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

[0047] "Fv" refers to the minimum antibody fragment that contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although likely at a lower affinity than the entire binding site.

[0048] The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments that have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

[0049] "Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains that enables the scFv to form the desired structure for antigen binding. For a review of scFv see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore, eds. (Springer-Verlag: New York, 1994), pp. 269-315.

[0050] SMIPs are a class of single-chain peptide engineered to include a target binding region, effector domain (CH2 and CH3 domains). *See, e.g.,* U.S. Patent Application Publication No. 20050238646. The target binding region may be derived from the variable region or CDRs of an antibody, e.g., an anti-C5 antibody of the application. Alternatively, the target binding region is derived from a protein that binds C5.

[0051] The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., Proc. Natl. Acad. Sci. USA, 90: 6444-6448 (1993).

[0052] It is well known that the binding to a molecule (or a pathogen) of antibodies with an Fc region assists in the processing and clearance of the molecule (or pathogen). The Fc portions of antibodies are recognized by

Samsung Bioepis Exhibit No. 1002
Page 1966

specialized receptors expressed by immune effector cells. The Fc portions of IgG1 and IgG3 antibodies are recognized by Fc receptors present on the surface of phagocytic cells such as macrophages and neutrophils, which can thereby bind and engulf the molecules or pathogens coated with antibodies of these isotypes (C. A. Janeway et al., Immunobiology 5th edition, page 147, Garland Publishing (New York, 2001)).

[0053] This disclosure also provides monoclonal anti-C5 antibodies. A monoclonal antibody can be obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are often synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. Monoclonal antibodies may also be produced in transfected cells, such as CHO cells and NS0 cells. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies and does not require production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present disclosure may be made by the hybridoma method first described by Kohler et al., Nature 256:495-497 (1975), or may be made by recombinant DNA methods (*see, e.g.*, U.S. Patent Nos. 4,816,567 and 6,331,415). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., Nature 352:624-628 (1991) and Marks et al., J. Mol. Biol. 222:581-597 (1991), for example.

[0054] A description of the preparation of a mouse anti-human-C5 monoclonal antibody with specific binding characteristics is presented in U.S. Patent Application Publication No. 20050226870. Wurzner et al., Complement Inflamm. 8:328-340 (1991), describe the preparation of other mouse anti-human-C5 monoclonal antibodies referred to as N19-8 and N20-9.

[0055] Other antibodies specifically contemplated are "oligoclonal" antibodies. As used herein, the term "oligoclonal" antibodies" refers to a predetermined mixture of distinct monoclonal antibodies. *See, e.g.,* PCT publication WO 95/20401; U.S. Patent Nos. 5,789,208 and 6,335,163. In one embodiment, oligoclonal antibodies consisting of a predetermined mixture of antibodies against one or more epitopes are generated in a single cell. In other embodiments, oligoclonal antibodies comprise a plurality of heavy chains capable of pairing with a common light chain to generate antibodies with multiple specificities (*e.g.*. PCT publication WO 04/009618). Oligoclonal antibodies are particularly useful when it is desired to target multiple epitopes on a single target molecule (e.g., C5). In view of the assays and epitopes disclosed herein, those skilled in the art can generate or select antibodies or mixtures of antibodies that are applicable for an intended purpose and desired need.

[0056] In certain embodiments that include a humanized and/or chimeric antibody, one or more of the CDRs are derived from an anti-human C5 antibody. In a specific embodiment, all of the CDRs are derived from an anti-human C5 antibody. In another specific embodiment, the CDRs from more than one anti-human C5 antibody are mixed and matched in a chimeric antibody. For instance, a chimeric antibody may comprise a CDR1 from the light chain of a first anti-human C5 antibody combined with CDR2 and CDR3 from the light chain of a second anti-human C5 antibody, and the CDRs from the heavy chain may be derived from a third anti-human C5 antibody. Further, the framework regions may be derived from one of the same anti-human C5 antibodies, from one or more different antibodies, such as a human antibody, or from a humanized antibody. Human or humanized antibodies are specific for administration to human patients.

[0057] In certain embodiments, single chain antibodies, and chimeric, humanized or primatized (CDR-grafted) antibodies, as well as chimeric or CDR-grafted single chain antibodies, comprising portions derived from different species, are also encompassed by the present disclosure as antigen-binding fragments of an antibody. The various portions of these antibodies can be joined together chemically by conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques. For example, nucleic acids encoding a chimeric or humanized chain can be expressed to produce a contiguous protein. See, e.g., U.S. Pat. Nos. 4,816,567 and 6,331,415; U.S. Pat. No. 4,816,397; European Patent No. 0,120,694; WO 86/01533; European Patent No. 0,194,276 B1; U.S. Pat. No. 5,225,539; and European Patent No. 0,239,400 B1. See also, Newman et al., BioTechnology 10:1455-1460 (1992), regarding primatized antibody. See, e.g., Ladner et al., U.S. Pat. No. 4,946,778; and Bird et al., Science 242:423-426 (1988), regarding single chain antibodies.

[0058] In addition, functional fragments of antibodies, including fragments of chimeric, humanized, primatized or single chain antibodies, can also be produced. Functional fragments of the subject antibodies retain at least one binding function and/or modulation function of the full-length antibody from which they are derived. Preferred functional fragments retain an antigen-binding function of a corresponding full-length antibody (such as for example, ability of anti-C5 antibody to bind C5).

[0059] General methods for the immunization of animals (in this case with C5 and/or C5b, etc.), isolation of antibody producing cells, fusion of such cells with immortal cells (e.g., myeloma cells) to generate hybridomas secreting monoclonal antibodies, screening of hybridoma supernatants for reactivity of secreted monoclonal

Samsung Bioepis Exhibit No. 1002
Page 1967

antibodies with a desired antigen (in this case the immunogen or a molecule containing the immunogen), the preparation of quantities of such antibodies in hybridoma supernatants or ascites fluids, and for the purification and storage of such monoclonal antibodies, can be found in numerous publications. These include: Coligan, et al., eds. Current Protocols In Immunology, John Wiley & Sons, New York, 1992; Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988; Liddell and Cryer, A Practical Guide To Monoclonal Antibodies, John Wiley & Sons, Chichester, West Sussex, England, 1991; Montz et al., Cellular Immunol. 127:337-351 (1990); Wurzner et al., Complement Inflamm. 8:328-340 (1991); and Mollnes et al., Scand. J. Immunol. 28:307-312 (1988).

Pharmaceutical Formulations and Uses

[0060] Methods of administration of small molecules, proteins, and nucleic acids are well-known to those of skill in the art. Methods of administration of antibodies are well-known to those of skill in the art. To achieve the desired inhibition, the antibodies can be administered in a variety of unit dosage forms. The dose will vary according to the particular antibody. For example, different antibodies may have different masses and/or affinities, and thus require different dosage levels. Antibodies prepared as Fab fragments will also require differing dosages than the equivalent intact immunoglobulins, as they are of considerably smaller mass than intact immunoglobulins, and thus require lower dosages to reach the same molar levels in the patient's blood. The dose will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician. Dosage levels of the antibodies for human subjects are generally between about 1 mg per kg and about 100 mg per kg per patient per treatment, and preferably between about 5 mg per kg and about 50 mg per kg per patient per treatment. In terms of plasma concentrations, the antibody concentrations are preferably in the range from about 25 μg/mL to about 500 μg/mL. However, greater amounts may be required for extreme cases and smaller amounts may be sufficient for milder cases.

[0061] Administration of the anti-C5 antibodies will generally be performed by an intravascular route, e.g., via intravenous infusion by injection. Other routes of administration may be used if desired but an intravenous route will be the most preferable. Formulations suitable for injection are found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, Pa., 17th ed. (1985). Such formulations must be sterile and non-pyrogenic, and generally will include a pharmaceutically effective carrier, such as saline, buffered (e.g., phosphate buffered) saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions, and the like. The formulations may contain pharmaceutically acceptable auxiliary substances as required, such as, tonicity adjusting agents, wetting agents, bactericidal agents, preservatives, stabilizers, and the like.

[0062] Administration of the antibodies capable of inhibiting complement such as an anti-C5 antibody will generally be performed by a parenteral route, typically via injection such as intra-articular or intravascular injection (e.g., intravenous infusion) or intramuscular injection. Other routes of administration, e.g., oral (p.o.), may be used if desired and practicable for the particular antibody capable of inhibiting complement to be administered. Antibodies capable of inhibiting complement such as an anti-C5 antibody can also be administered in a variety of unit dosage forms and their dosages will also vary with the size, potency, and in vivo half-life of the particular antibody capable of inhibiting complement being administered. Doses of antibodies capable of inhibiting complement such as an anti-C5 antibody will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician.

[0063] In certain embodiments, a typical therapeutic treatment includes a series of doses, which will usually be administered concurrently with the monitoring of clinical endpoints with the dosage levels adjusted as needed to achieve the desired clinical outcome. In certain embodiments, treatment is administered in multiple dosages over at least a week. In certain embodiments, treatment is administered in multiple dosages over at least a month. In certain embodiments, treatment is administered in multiple dosages over at least a year. In certain embodiments, treatment is administered in multiple dosages over the remainder of the patient's life.

[0064] The frequency of administration may also be adjusted according to various parameters. These include the clinical response, the plasma half-life of the therapeutic of the disclosure, and the levels of the antibody in a body fluid, such as, blood, plasma, serum, or synovial fluid. To guide adjustment of the frequency of administration, levels of the therapeutic of the disclosure in the body fluid may be monitored during the course of treatment.

[0065] In certain embodiments, the frequency of administration may be adjusted according to an assay measuring cell-lysing ability of complement present in one or more of the patient's body fluids. The cell-lysing ability can be measured as percent hemolysis in hemolytic assays of the types described herein. A 10% or 25% or 50% reduction in the cell-lysing ability of complement present in a body fluid after treatment with the antibody capable of inhibiting complement used in the practice of the application means that the percent hemolysis after treatment is 90, 75, or 50 percent, respectively, of the percent hemolysis before treatment.

[0066] For the treatment of antibody mediated neuropathies by systemic administration of an antibody capable of inhibiting complement such as an anti-C5 anti-

Samsung Bioepis Exhibit No. 1002
Page 1968

body (as opposed to local administration), administration of a large initial dose is specific, i.e., a single initial dose sufficient to yield a substantial reduction, and more preferably an at least about 50% reduction, in the hemolytic activity of the patient's serum. Such a large initial dose is preferably followed by regularly repeated administration of tapered doses as needed to maintain substantial reductions of serum hemolytic titer. In another embodiment, the initial dose is given by both local and systemic routes, followed by repeated systemic administration of tapered doses as described above. For administration of an anti-C5 antibody to humans, see, e.g., Hillmen et al., N. Engl. J. Med. 350:552-559 (2004). The levels of antibody administered in Hillmen et al. are based on half-life of the antibody and are relevant to administration of an anti-C5 antibody for other indications such as for treating neuropathies.

[0067] Formulations particularly useful for antibody-based therapeutic agents are also described in U.S. Patent App. Publication Nos. 20030202972, 20040091490 and 20050158316. In certain embodiments, the liquid formulations of the application are substantially free of surfactant and/or inorganic salts. In another specific embodiment, the liquid formulations have a pH ranging from about 5.0 to about 7.0. In yet another specific embodiment, the liquid formulations comprise histidine at a concentration ranging from about 1 mM to about 100 mM. It is also contemplated that the liquid formulations may further comprise one or more excipients such as a saccharide, an amino acid (e.g., arginine, lysine, and methionine) and a polyol. Additional descriptions and methods of preparing and analyzing liquid formulations can be found, for example, in PCT publications WO 03/106644, WO 04/066957, and WO 04/091658.

[0068] Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the pharmaceutical compositions of the application.

[0069] In certain embodiments, formulations of the subject antibodies are pyrogen-free formulations which are substantially free of endotoxins and/or related pyrogenic substances. Endotoxins include toxins that are confined inside microorganisms and are released when the microorganisms are broken down or die. Pyrogenic substances also include fever-inducing, thermostable substances (glycoproteins) from the outer membrane of bacteria and other microorganisms. Both of these substances can cause fever, hypotension and shock if administered to humans. Due to the potential harmful effects, it is advantageous to remove even low amounts of endotoxins from intravenously administered pharmaceutical drug solutions. The Food & Drug Administration ("FDA") has set an upper limit of 5 endotoxin units (EU) per dose per kilogram body weight in a single one hour period for intravenous drug applications (The United States Pharmacopeial Convention, Pharmacopeial Forum 26 (1):223 (2000)). When therapeutic proteins are administered in amounts of several hundred or thousand milligrams per kilogram body weight, as can be the case with monoclonal antibodies, it is advantageous to remove even trace amounts of endotoxin.

[0070] Formulations of the subject antibodies include those suitable for oral, dietary, topical, parenteral (e.g., intravenous, intraarterial, intramuscular, subcutaneous injection), ophthalmologic (e.g., topical or intraocular), inhalation (e.g., intrabronchial, intranasal or oral inhalation, intranasal drops), rectal, and/or intravaginal administration. Other suitable methods of administration can also include rechargeable or biodegradable devices and controlled release polymeric devices. Stents, in particular, may be coated with a controlled release polymer mixed with an agent of the application. The pharmaceutical compositions of this disclosure can also be administered as part of a combinatorial therapy with other agents (either in the same formulation or in a separate formulation).

[0071] The amount of the formulation which will be therapeutically effective can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems. The dosage of the compositions to be administered can be determined by the skilled artisan without undue experimentation in conjunction with standard dose-response studies. Relevant circumstances to be considered in making those determinations include the condition or conditions to be treated, the choice of composition to be administered, the age, weight, and response of the individual patient, and the severity of the patient's symptoms. For example, the actual patient body weight may be used to calculate the dose of the formulations in milliliters (mL) to be administered. There may be no downward adjustment to "ideal" weight. In such a situation, an appropriate dose may be calculated by the following formula: Dose (mL) = [patient weight (kg) x dose level (mg/kg)/ drug concentration (mg/mL)]

[0072] To achieve the desired treatment results, anti-C5 antibodies can be administered in a variety of unit dosage forms. The dose will vary according to the particular antibody. For example, different antibodies may have different masses and/or affinities, and thus require different dosage levels. Antibodies prepared as Fab' fragments or single chain antibodies will also require differing dosages than the equivalent native immunoglobulins, as they are of considerably smaller mass than native immunoglobulins, and thus require lower dosages to reach the same molar levels in the patient's blood.

[0073] Other therapeutics of the disclosure can also be administered in a variety of unit dosage forms and

Samsung Bioepis Exhibit No. 1002
Page 1969

their dosages will also vary with the size, potency, and *in vivo* half-life of the particular therapeutic being administered.

[0074] Doses of therapeutics of the disclosure will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician.

[0075] The formulations of the application can be distributed as articles of manufacture comprising packaging material and a pharmaceutical agent which comprises the antibody capable of inhibiting complement and a pharmaceutically acceptable carrier as appropriate to the mode of administration. The packaging material may include a label which indicates that the formulation is for use in the treatment of antibody mediated neuropathies. Although antibodies are preferred, especially anti-C5 antibodies which have already been shown to be safe and effective at decreasing the accumulation of downstream complement components in persons, the use of other complement inhibitors is also contemplated by this disclosure. The pharmaceutical formulations and uses of the disclosure may be combined with any known complement inhibitors or antibody mediated neuropathy treatments known in the art (supra).

[0076] Treatments of the application may be used in combination with other treatments of the application or known treatments for antibody mediated neuropathies. Treatments of the application may be co-administered with other treatments that treat symptoms of antibody mediated neuropathies. Administration of the therapeutics of the disclosure will generally be performed by a parenteral route, typically via injection such as intra-articular or intravascular injection (e.g., intravenous infusion) or intramuscular injection. Other routes of administration, e.g., oral (p.o.), may be used if desired and practicable for the particular antibody capable of inhibiting complement to be administered.

[0077] In case of conflict, the present application, including any definitions herein, will control.

[0078] The present methods are described with reference to the following Examples, which are offered by way of illustration and are not intended to limit the disclosure in any manner. Standard techniques well known in the art or the techniques specifically described below are utilized.

EXAMPLES

EXAMPLE 1. ECULIZUMAB PREVENTS COMPLEMENT-MEDIATED STRUCTURAL AND FUNCTIONAL LESIONS IN THE IN VITRO MFS MODEL

[0079] In the *in vitro* MFS model, treatment of diaphragm nerve-muscle preparations with CGM3 anti-GQ1b mAb and normal human serum (NHS), in the presence of an irrelevant isotype matched mAb (100 μg/ml), that served as a negative control for eculizumab, induced complement activation at NMJs. This was morphologically evidenced by C3c and MAC deposition (FIGs. 1A, 1B, 1D, 1E and 1F), as well as damaged perisynaptic Schwann cells (pSCs) and motor nerve terminals shown by ethidium homodimer-1 (EthD-1) staining and loss of neurofilament (NF) staining, respectively (FIGs. 1C, 1E and 1F). These features were identical to those reported before (Goodyear et al. J. Clin. Invest 104:697-708 (1999); Halstead et al. Brain 127:2109-2123 (2004); O'Hanlon et al. Brain 124:893-906 (2001)). The addition of 100 μg/ml eculizumab to the NHS as complement source completely prevented MAC deposition (FIGs. 1B, ID, 1E and 1F; $p<0.001$) and terminal axonal NF loss (FIGs. 1C and 1F; $p<0.001$), while the deposition of the earlier complement component C3c was not affected (FIGs. 1A and 1D; $p= 0.2$). Furthermore, damage to pSCs was abolished as only 2% of the 1439 NMJs investigated had one or more EthD-1 positive nuclei, compared with 33% of 1529 NMJs investigated in control mAb-treated tissue (FIG. 1 E; p<0:001).

[0080] These immunohistological observations paralleled the electrophysiological and functional observations. At CGM3 pre-incubated NMJs, NHS (40%) in the presence of the control mAb induced a large increase in the frequency of miniature endplate potentials (MEPPs, the postsynaptic events arising from spontaneous release of single acetylcholine quanta from the presynaptic motor nerve terminal), as previously observed (Goodyear et al. J. Clin. Invest 104:697-708 (1999)). This effect was almost completely prevented by 100 μg/ml eculizumab. The MEPP frequency was about 0.7 s^{-1} before and after 50 μg/ml CGM3 incubation and rose to 34.0 ± 4.6 s^{-1} in the presence of NHS with 100 μg/ml control mAb added. By contrast the MEPP frequency was only 3.1 ± 1.3 s^{-1} in the presence of NHS with 100 μg/ml eculizumab added (FIGs. 2A and 2B; n=5 muscles; p<0.001).

[0081] Eculizumab did not alter the muscle fiber resting membrane potential or MEPP amplitude, rise- and decay times (data not shown). As described before (Goodyear et al. J. Clin. Invest 104:697-708 (1999); Plomp et al. Ann. Neurol. 45:189-199 (1999)), asynchronous twitching of individual muscle fiber occurs during NHS incubation (median visual score 4; n= 5 muscles). Eculizumab prevented such high grade of twitching with only a median score of I being observed (FIG. 2C; n=5 muscles, p<0.01, Mann-Whitney test), which equals the low basal levels observed in the observation periods before and after CGM3 incubation.

[0082] The final result of anti-CGM3/complement-mediated presynaptic damage is block of synaptic transmission at the NMJ due to the inability to release acetylcholine (Goodyear et al. J. Clin. Invest 104:697-708 (1999)). Eculizumab completely prevented this effect. No 'silent' NMJs (i.e. absence of MEPPs and nerve-stimulation evoked muscle action potentials) were encountered, whereas in the control mAb condition 19 ± 2.6 % of the total number of NMJs sampled were observed to be silenced (FIG. 2D; n=5 muscles). Furthermore, we quan-

Samsung Bioepis Exhibit No. 1002
Page 1970

tified the protective effect of eculizumab on muscle paralysis in the *in vitro* MFS model. Eculizumab inhibited loss of nerve stimulation-induced contraction force of hemi-diaphragm preparations in a concentration dependent manner following 200 μg/ml CGM3 and 33% NHS treatment (FIGs. 2E and 2F). In the control experiments without eculizumab, NHS induced almost complete loss of contraction force within 90 min. Addition of 3 or 6 μg/ml eculizumab to the NHS slowed down the rate of contraction force loss considerably (50% loss was observed at 39 and 60 min, respectively, compared to 27 min in the control condition; FIG. 2F). Higher eculizumab concentrations of 9 and 12 μg/ml were almost completely protective (>90% of the initial contraction left after 90 min) while 25, 50 and 100 μg/ml completely prevented any CGM3/NHS-induced loss of contraction. A Boltzmann sigmoidal curve fitted through the obtained concentration-effect data points yielded an EC_{50} of 7.1 μg/ml for eculizumab under these conditions (FIG. 2G).

[0083] These immunohistological and functional analyses show that eculizumab efficiently prevents complement-mediated pathophysiological effects in the *in vitro* MFS model.

EXAMPLE 2. ECULIZUMAB DOSE RESPONSE CURVE *IN VIVO*.

[0084] Next the *in vivo* benefits of eculizumab in this murine model of Guillain-Barré syndrome were examined. Mice were passively immunized with anti-ganglioside antibody or PBS (as a negative control) followed by concomitant injections of NHS (intraperitoneal) and eculizumab (intravenous) at 0, 50, 100, 200, and 400 μg/mouse in PBS. Complement C3 was richly deposited at the NMJ of all mice receiving anti-ganglioside antibody. An increasing dose of eculizumab results in a dose-dependent reduction in MAC deposition at the NMJ (FIG. 3A). Examination of the neurofilament signal demonstrates preservation of axonal integrity at all doses of eculizumab investigated when compared to PBS treated baseline control. (*n*=3 for each dose) (FIG. 3B).

EXAMPLE 3. ECULIZUMAB PROTECTS AGAINST NEUROPATHY AND RESPIRATORY PARALYSIS IN AN IN VIVO MFS MODEL

[0085] In order to determine the *in vivo* efficacy of eculizumab, we generated an *in vivo* MFS mouse model through intraperitoneal injection of CGM3 anti-GQ1b antibody and NHS as complement source. At 16 h after the CGM3 injection, a dose of 200 μg eculizumab or control mAb was administered systemically, via the tail vein, followed by intraperitoneal injection of 0.5 ml NHS). The protocol of different injection routes of eculizumab and NHS was applied to avoid the immediate inhibition of C5 in the NHS by eculizumab within the confinement of the peritoneal cavity. Mice treated with CGM3, NHS and control mAb (n=10) developed a general weak appearance, in some cases a low-back posture, invaginated abdominal flanks and breathing difficulties (panting) within 2 h following the NHS injection. Intravenous eculizumab injection completely prevented the development of these symptoms (n=11). We quantified weakness with grip-strength measurement in CGM3 pre-treated mice, before and 2h after injection of control mAb or eculizumab and NHS (FIG. 4A). Control group mice (n=5) pulled 56.0 ± 5.7 gjust before the control mAb and NHS injection, while the eculizumab group mice (n=5) pulled 54.3 ± 8.4 g (p= 0.87) at this stage. The pulling force 2h after control mAb/NHS injection was 22% lower (43.7 ± 4.5 g; p<0.005). Eculizumab ameliorated such an effect (8% reduction; p=0.07). Respiratory disturbance was assessed with whole-body plethysmography continuously after mAb/NHS injection for a period of 6h (FIGs. 4B and 4C; n=5 mice per group). The tidal volume before CGM3 injection was similar in both groups (0.21 ± 0.01 and 0.22 ± 0.03 ml in the control mAb and eculizumab group, respectively). A reduction of approximately 50% (p<0.001) was observed in the control mAb group at 4 and 6 h after NHS injection. Such a decrease was largely prevented by eculizumab (only 17% reduction at these time points, FIG. 4B). Similarly, respiration rate was depressed in the control mAb group by approximately 30% (p<0.01) at 4 and 6 h post-NHS injection. In the eculizumab group, this reduction was only 7% (FIG. 4C). Both treatment groups showed an equal initial reduction of about 40% of the respiration rate when measured 2 h post mAb/NHS, compared to the rate before CGM3 injection, which apparently was due to the intraperitoneal injection regimen. Example traces of the respiration signals obtained are shown in FIG. 4D.

[0086] The hemi-diaphragm phrenic nerve preparations were dissected from *in vivo* CGM3/NHS treated mice at 4 h following NHS injection and performed electrophysiological measurements at NMJs, muscle contraction experiments, and detailed immunohistological analyses. Visual inspection indicated that only a very small part of the muscles from control mAb-treated mice contracted upon supramaximal electrical stimulation of the phrenic nerve, as compared to a complete contraction of muscles obtained from eculizumab-treated mice. In *in vitro* contraction experiments in tissues harvested from passive immunizations, this effect was quantified (FIGs. 4E and 4F). Twitch and tetanic (40Hz) tension were 0.07 ± 0.06 and 0.92 ± 0.61 g, respectively, in the control mAb group (n=4), while in the eculizumab group (n=4) these were 1.36 ± 0.29 and 9.26 ± 0.65 g, respectively, equalling the values of muscles from non-NHS treated age-matched mice (data not shown). With electrophysiological analysis we tested whether paralysis was caused by NMJ dysfunction. In dissected muscles from the control mAb group 66 ± 7% of the sampled NMJs had been 'silenced', i.e. there were no detectable MEPPs and no muscle action potentials upon nerve stimulations. In the eculizumab group only 3 ± 2% had been 'silenced' (FIGs. 4G and 4H; p<0.001). Immunohistological analy-

Samsung Bioepis Exhibit No. 1002
Page 1971

sis showed that CGM3 was deposited equally at NMJs of the two treatment groups (data not shown). C3c deposits were identified at NMJs of both groups, albeit with a somewhat less intensity in the eculizumab group (FIGs. 5A and 5D; $p<0.001$). MAC was clearly deposited at NMJs of muscles from control mAb-treated mice but virtually absent in the eculizumab-treated group (FIGs. 5B, 5D and 5E). Eculizumab prevented loss of terminal integrity at NMJs of control-mAb-treated mice, as evidenced by the NF signal intensity and pattern overlying the endplate region, i.e. terminal axonal branching clearly remaining intact (FIGs. 5C and 5E). In addition, electron microscopy showed the presence of a well-preserved presynaptic ultrastructure at the NMJ of eculizumab-treated mice in comparison with that of control-mAb-treated mice which showed characteristic terminal swelling, synaptic vesicle depletion and swollen mitochondria (FIG. 5F) (O'Hanlon et al. Brain 124:893-906 (2001)). These electrophysiological, functional and immunohistological data show that complement-mediated terminal motor neuropathy occurs in our *in vivo* MFS model and that diaphragm paralysis contributes to the observed respiratory deficits. Most importantly, *in vivo* eculizumab treatment effectively prevented these *in vivo* neuropathological defects.

EXAMPLE 4. METHODS

Mice

[0087] Male Balb/c mice (3-6 weeks old, 10-25 g) were obtained from Harlan (UK or NL). In some muscle contraction experiments (see below) male and female GM2/GD2-synthase *null*-mutant mice (Bullens et al. J. Neurosci. 22:6876-6884 (2002)) were used at 12-22 weeks of age (19-37 g). All animal experiments were carried out in accordance to UK Home Office guidelines (UK PPL60/3096), Dutch law and Glasgow and Leiden University guidelines.

Monoclonal antibodies and normal human serum

[0088] The IgM anti-GQ1b ganglioside mAb, CGM3 was derived from mice inoculated with a GT1a-bearing *Campylobacter jejuni* lipooligosaccharide (Goodyear et al. J. Clin. Invest 104:697-708 (1999)). CGM3 reacts with gangliosides GQ1b, GD3 and GT1a that all share the terminal disialylgalactose structure. Previous studies have shown that CGM3 has similar ganglioside specificity and induced identical complement-dependent pathogenic effects as human MFS sera (Goodyear et al. J. Clin. Invest 104:697-708 (1999)). CGM3 concentration was measured using quantitative ELISA (Bethyl Laboratories, Texas, USA). Normal human serum (NHS) was taken from a single donor stock that had been freshly frozen and stored in multiple aliquots at -70°C to preserve complement activity. Prior to experimental use, CGM3 and NHS were dialyzed for 24 h at 4°C against Ringer's solution (116 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM NaH_2PO_4, 23 mM $NaHCO_3$, 11 mM glucose, pH 7.4), pre-gassed with 95% O_2/5% CO_2. The humanized anti-human C5 mAb eculizumab and a non-specific isotype-matched control mAb ALXN3300 were obtained from Alexion Pharmaceuticals (Cheshire, USA) and stored at 4°C

In vitro MFS model

[0089] The *in vitro* model for MFS using CGM3 and NHS has been described before (Goodyear et al. J. Clin. Invest 104:697-708 (1999); O'Hanlon et al. Brain 124:893-906 (2001)). In short, mouse hemi-diaphragms with phrenic nerve attached (or triangularis sterni muscle in some cases for illustrative NMJ immunohistology) were dissected and mounted in Ringer's medium at room temperature (20-22 °C). Untreated small control sections were removed from each muscle preparation prior to any incubations and snap frozen on dry ice for subsequent baseline immunohistological analysis. Muscles were incubated with CGM3 (50 μg/ml) for 2-2.5 h at 32°C, then for 30 min at 4°C and then equilibrated for 10 mins at room temperature, rinsed in Ringer's medium and subsequently exposed to 33 or 40% NHS in Ringer's medium for 1 h at room temperature. Eculizumab (100 μg/ml) or the control mAb (100 μg/ml) was mixed with NHS 10 min prior to the incubation of the muscle preparation. *In vitro* electrophysiological measurements at the NMJ (see below) and scoring of muscle fiber twitchings, a hallmark of uncontrolled transmitter release at NMJs in this model (Jacobs et al. Muscle Nerve 25:549-558 (2002); O'Hanlon et al. Brain 124:893-906 (2001)), were performed before and after CGM3 incubation, and during the 1 h NHS incubation. Muscle strips were frozen on dry ice and stored at -20°C, both before and after incubation with NHS. NMJs and terminal motor axons were assessed for levels of IgM, C3c, MAC, NF and for pSC viability (see below).

In vivo MFS model

[0090] Balb/c mice (3-4 weeks old, 10-15 g) were injected intraperitoneally with 1.5 mg CGM3, followed 16 h later by an intraperitoneal injection of 0.5 ml 100% NHS. Mice were observed for a further 4-6 h and analyzed with whole-body plethysmography (see below) and grip-strength testing as described before (Kaja et al. Eur J. Neurosci 25:2009-2020 (2007)). The mice were then killed by CO_2 asphyxiation and hemi-diaphragm muscle tissue was dissected and analyzed in electrophysiological and muscle contraction experiments (see below) or further processed for immunohistological analyses (see below). The effect of eculizumab on *the in vivo* symptoms and the subsequently analyzed electrophysiological, functional and histological lesions was tested in this model by injecting 200 μg eculizumab or control mAb in the tail vein, shortly before intraperitoneal NHS injection. In-

Samsung Bioepis Exhibit No. 1002
Page 1972

itial dose-range finding experiments (data not shown) indicated that eculizumab at doses of >50 μg/mouse protected against neuropathy.

Plethysmography

[0091] Non-invasive whole-body plethysmography (EMMS, Hants, UK) was employed to demonstrate changes in breathing parameters in *in vivo* experiments. Baseline data were collected prior to onset of experiment. Mice were injected with CGM3 followed by NHS with eculizumab or control mAb 16 h later (protocol as above) and monitored continuously for 6 h. Flow derived parameters of breath frequency and tidal volume were collected from 25 accepted breaths and averaged over 1 h periods.

In vitro electrophysiological analysis of NMJs and visual scoring of muscle contractility

[0092] Left and right hemi-diaphragms with their phrenic nerve attached were dissected and pinned out in a silicone rubber-lined dish in 1.5 ml Ringer's medium at room temperature. Intracellular recordings of miniature endplate potentials (MEPPs, the postsynaptic events arising from spontaneous release of single acetylcholine quanta from the presynaptic motor nerve terminal) were made at NMJs at 20-22°C. Muscle fibers were impaled near the NMJ with a 10-20 MΩ glass micro-electrode filled with 3 M KCl, connected to a GeneClamp 500B amplifier (Axon Instruments/Molecular Devices, Union City, CA, USA) for amplifying and filtering (10 kHz low-pass) of the signal. Signals were digitized, stored and analyzed (off-line) using a Digidata 1322A interface, Clampex 9.2 and Clampfit 9.2 programs (all from Axon Instruments/Molecular Devices), Mini Analysis 6.0 (Synaptosoft, Fort Lee, USA) and routines programmed in Matlab (The MathWorks Inc., Natick, MA, USA).

[0093] The occurrence of spontaneous asynchronous fiber twitches that are induced by a high MEPP frequency resulting from CGM3/complement-mediated presynaptic damage (Plomp et al. Ann. Neurol. 45:189-199 (1999)) was scored visually every 5 min (O'Hanlon et al. Brain 124:893-906 (2001)). The tissue scored 0 for no activity, 1 for the twitching of <10 fibers, 2 for a small amount of twitching across the tissue, 3 for a moderate amount and 4 for an extensive amount. The average score during each measuring period was calculated. The final result of CGM3/complement-mediated presynaptic damage is a silencing of synaptic transmission at the NMJ. The number of'silent' NMJs (i.e. without detectable MEPPs and occurrence of phrenic nerve-stimulation evoked muscle action potentials) was expressed as percentage of the total number of NMJs sampled (8-41) in a muscle under each experimental condition.

Muscle contraction experiments

[0094] The protective efficacy of eculizumab on *in vitro* CGM3/NHS-induced loss of nerve stimulation-evoked contraction force of the hemi-diaphragm preparation was tested. We used diaphragm from GM2/GD2-synthase null-mutant mice because earlier studies showed a somewhat higher sensitivity of muscle of this strain to CGM3/NHS-induced loss of contraction (Bullens et al. J. Neurosci. 22:6876-6884 (2002)). Left and right phrenic nerve/hemi-diaphragm preparations that had been pre-incubated with 200 μg/ml CGM3 for 3 h at 32 °C were mounted in a silicone-rubber lined dish containing 2.25 ml Ringer's medium at room temperature (20-22 °C), continuously bubbled with 95% O_2 / 5% CO_2. The ribcage side of the muscle was thoroughly fixed to the bottom of the dish by multiple small pins and the central tendon was connected via a metal hook and a string to a force transducer that was connected to an amplifier and digitizing equipment, as described earlier (Kaja et al. Eur J. Neurosci 25:2009-2020 (2007)). Supramaximal stimuli (usually ~10 V) of 100 μs duration were delivered every 5 min for 3 s at 40 Hz from a Master-8 programmable stimulator (AMPI, Jerusalem, Israel). Basic tension was adjusted with a vernier control to obtain maximal stimulated tetanic contraction force (usually about 10 g). Stability of the elicited contraction was monitored for 30-50 min. Subsequently, the medium was replaced by NHS (33%) to which eculizumab (3, 6, 9, 12, 25, 50 or 100 μg/ml) had been added and admixed for ~10 min prior to addition and the effect was monitored for 100-200 min, under continuous gentle bubbling with 95% O_2 /5% CO_2. In the control experiments without added eculizumab, 100 μg/ml of the control mAb was added to the NHS. The amplitude of contractions was cursor-measured off-line in the Clampfit 9.0 program (Axon Instruments/Molecular Devices, Union City, USA), at 2 s after the start of each nerve stimulation train.

[0095] Tetanic and twitch contraction forces were also measured upon *in vitro* nerve stimulation of left hemi-diaphragm muscles that were dissected from mice that had been subjected to the *in vivo* MFS protocol.

Immunohistological analyses

[0096] Unfixed hemi-diaphragm sections were mounted in Lipshaw's M-1 mounting medium (Pittsburgh, PA, USA), and longitudinal cryostat sections (8-20 μm) were cut onto 3-aminopropyltriethoxysilane coated slides, air-dried, then stored at -20°C. To localize NMJs, TRITC and bodipy-labelled α-bungarotoxin (α-BTx, diluted 1/750 to 1.3 μg/ml; Molecular Probes, Eugene, Oregon) were used. The intermediate complement component C3c was detected by incubation with FITC-labelled rabbit anti-C3c (1/300; Dako, Ely, UK) for 1 h at 4°C. MAC was detected using mouse anti-human C5b-9 (1/50; Dako) followed by FITC conjugated goat anti-mouse IgG (1/300), both for 1 h at 4°C.

[0097] For NF staining, sections of unfixed tissue were pre-incubated for 1 h at 4°C with TRITC-conjugated α-BTx, rinsed, immersed in ethanol at -20°C for 20 min,

Samsung Bioepis Exhibit No. 1002
Page 1973

then incubated overnight at room temperature with the rabbit polyclonal serum 1211 (1/750; reactive with phosphorylated NF; Affiniti Research Products Ltd. Exeter, UK) followed by FITC conjugated goat anti-rabbit IgG (1/300; Southern Biotechnology Associates) for 3 h at 4°C. All detection antibodies were diluted in phosphate-buffered saline (PBS).

[0098] pSC viability was assessed using EthD-1, a membrane impermeant dye that labels with red fluorescence the nucleic acids of membrane-permeabilized cells (Molecular Probes, Eugene, Oregon, USA). In brief, nerve-muscle preparations were exposed to Ringer's containing 2 μM EthD-1. The tissue was incubated in the dark at room temperature for 1 h, rinsed in Ringer's solution and frozen for immunohistology. NMJs were identified in 15 μm cryostat sections by staining with bodipy conjugated α-BTx (1.3 μg/ml), and the percentage of NMJs with EthD-1 positive nuclei at NMJs was calculated.

[0099] For illustrations, whole mount triangularis sterni muscles were incubated with CGM3 (50 μg/ml) and fluorochrome-conjugated α-BTx (TRITC/FITC/CY5) (2 μg/ml; 1:500), followed by NHS plus eculizumab or control mAb. Preparations were incubated with fluorescently conjugated α-BTx and various combinations of the following: mouse anti-C5b-9 (1:40; Dako), anti-C3c-FITC (1:200; Dako), or EthD-1 (2 μM) in Ringer's medium for 1 h at room temperature, rinsed in Ringer's medium, followed by fixation for 20 min in 4% formaldehyde in PBS. Unreactive aldehyde groups were quenched, by incubating with 0.1 M glycine for 10 min. The antibodies were then reapplied in PBS and agitated overnight at room temperature. For staining of intracellular antigens, muscle was fixed in 4% formaldehyde for 20 min followed by 10 min in 0.1M glycine and then incubated in a permeabilizing solution of 0.5% Triton-X100 in PBS for 30 min at RT, and either rabbit anti-S 100 (1:150; Dako) or rabbit anti-NF (1:150; Chemicon, Hampshire, UK) diluted in permeabilizing solution applied overnight at room temperature. Tissue was rinsed in PBS and where necessary incubated in the following fluorescently conjugated antibodies diluted 1:300; anti-rabbit IgG-FITC, anti-mouse IgG-FITC or -TRITC and agitated for 7 h at RT in the dark. Tissue was rinsed in PBS and mounted in Citifluor mounting medium (Citifluor Products, Canterbury, UK).

Image acquisition, quantitation and statistical analysis

[0100] Digital images were captured using both Zeiss Pascal confocal laser scanning microscope and Zeiss Axio Imager Z1 with ApoTome. Image-analysis measurements were made using Scion Image (Scion Corporation, Frederick, Maryland, USA) image analysis software. For quantitative analysis of IgM, C3c, MAC and NF, 3 staining runs of each marker were performed on tissue from at least 3 individual hemi-diaphragms, and quantified as previously described (O'Hanlon et al. Brain 124:893-906 (2001)). All studies were observer blinded. For immunohistological analysis of non-parametric data, statistical comparisons were made using Mann-Whitney Test employing a 1% level of significance. For comparison of EthD-1 positive pSC at the NMJ, chi-squared test was used at 1% level of significance.

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[0101]

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Samsung Bioepis Exhibit No. 1002
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Samsung Bioepis Exhibit No. 1002
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Claims

1. An antibody or antigen-binding fragment thereof for use in the treatment of an anti-ganglioside or anti-glycolipid antibody mediated neuropathy in a mammal, wherein the antibody or antigen-binding fragment thereof binds to:

   (i) complement component C5 and inhibits the cleavage of C5 into fragments C5a and C5b; or
   (ii) C5b,

   wherein said antibody or antigen-binding fragment thereof inhibits the formation of the membrane attack complex; and
   wherein said anti-ganglioside or anti-glycolipid antibody mediated neuropathy is selected from the group consisting of: acute motor axonal neuropathy; acute inflammatory demyelinating polyneuropathy; Bickerstaff's brainstem encephalitis; acute ophthalmoparesis; ataxic Guillain-Barre syndrome; pharyngeal-cervical-brachial weakness; chronic neuropathy syndromes with anti-glycolipid antibodies; anti-MAG IgM paraproteinemic neuropathy; chronic sensory ataxic neuropathy with anti-disialosyl antibodies; IgM, IgG and IgA paraproteinemic neuropathy; motor neuropathy with anti-GM1 and anti-GM2 antibodies; chronic inflammatory demyelinating neuropathy (CIDP); multifocal motor neuropathy (MMN); and multifocal acquired demyelinating sensory and motor neuropathy (MADSAM).

2. The antibody or antigen-binding fragment thereof for use according to claim 1, wherein said anti-ganglioside or anti-glycolipid antibody binds to a glycolipid selected from the group consisting of: GM1, GM1(NeuGc), GM1b, GalNAc-GM1b, GD1a, GalNAc-GD1a, GD1b, 9-*O*-acetyl GD1b, GD3, GT1a, GT1b, GQ1b, GQ1bα, LM1, galactocerebroside, and SGPG.

3. The antibody or antigen-binding fragment thereof for use according to claim 1 or 2, wherein said mammal is a human.

4. The antibody or antigen-binding fragment thereof for use according to any one of claims 1 to 3, wherein said antibody or antigen-binding fragment thereof is selected from the group consisting of: a polyclonal antibody, a monoclonal antibody or antibody fragment, a diabody, a chimerized or chimeric antibody or antibody fragment, a humanized antibody or antibody fragment, a deimmunized human antibody or

Samsung Bioepis Exhibit No. 1002
Page 1976

antibody fragment, a fully human antibody or antibody fragment, a single chain antibody, an Fv, an Fab, an Fab', and an $F(ab')_2$.

5. The antibody or antigen-binding fragment thereof for use according to any one of claims 1 to 4, wherein said antibody or antigen-binding fragment thereof inhibits the cleavage of C5 into fragments C5a and C5b.

6. The antibody or antigen-binding fragment thereof for use according to claim 5, wherein said antibody is eculizumab.

7. The antibody or antigen-binding fragment thereof for use according to claim 5, wherein said antibody is pexelizumab.

8. The antibody or antigen-binding fragment thereof for use according to claim 6, wherein said treatment is chronic.

9. The antibody or antigen-binding fragment thereof for use according to claim 7, wherein said treatment comprises treating an acute episode.

10. The antibody or antigen-binding fragment thereof for use according to any one of claims 1 to 9, wherein said treatment comprises administering the antibody or antigen-binding fragment thereof systemically to said mammal.

11. The antibody or antigen-binding fragment thereof for use according to any one of claims 1 to 9, wherein said treatment comprises administering the antibody or antigen-binding fragment thereof locally to said mammal.

12. The antibody or antigen-binding fragment thereof for use according to any one of claims 1 to 11, wherein said treatment results in:

    (a) decreased neural injury in said mammal; or
    (b) a decreased amount and/or extent of an undesirable physiological condition resulting from excess nerve injury.

13. The antibody or antigen-binding fragment thereof for use according to claim 12, wherein said undesirable physiological condition is selected from the group consisting of: ophthalmoplegia, ataxia, areflexia, abnormal muscle coordination, paralysis of the eye muscles, aching pain in the muscles, absence of the tendon reflexes, numbness and tingling felt in the lower extremities, weakness of the distal muscles of the lower extremities, a footdrop, weakness involving the entire lower extremities, weakness involving the upper extremities, weakness in the muscles of respiration, and death.

14. The antibody or antigen-binding fragment thereof for use according to any one of claims 1 to 13, wherein said treatment results in:

    (a) decreased membrane attack complex (MAC) formation at presynaptic neuromuscular junctions;
    (b) restoration of a normal frequency of miniature endplate potentials;
    (c) restoration of synaptic transmission at the neuromuscular junctions; or
    (d) inhibition of loss of terminal integrity at the neuromuscular junctions.

Patentansprüche

1. Antikörper, oder Antigen-bindendes Fragment davon, zur Verwendung in der Behandlung einer Anti-Gangliosid- oder Anti-Glycolipid-Antikörper-vermittelten Neuropathie in einem Säuger, wobei der Antikörper oder das Antigen-bindende Fragment davon an:

    (i) Komplement-Komponente C5 bindet und die Spaltung von C5 in die Fragmente C5a und C5b hemmt; oder
    (ii) C5b bindet,

    wobei der Antikörper oder das Antigen-bindende Fragment davon die Ausbildung des Membranangriffskomplexes hemmt; und
    wobei die Anti-Gangliosid- oder Anti-Glycolipid-Antikörper-vermittelte Neuropathie ausgewählt ist aus: akuter Neuropathie des motorischen Axons; akuter entzündlicher demyelinisierender Polyneuropathie; Bickerstaffs Hirnstamm-Enzephalitis; akuter Ophthalmoparese; ataktischem Guillain-Barre-Syndrom; Rachen-Halswirbel-Brachialis-Schwäche; chronischen Neuropathie-syndromen mit Anti-Glycolipid-Antikörpem; Anti-MAG IgM Paraprotein-Neuropathie; chronischer sensorischer ataktischer Neuropathie mit Anti-Disialosyl-Antikörpern; IgM-, IgG- und IgA-Paraprotein-Neuropathie; Motor-Neuropathie mit Anti-GM1- und Anti-GM2-Antikörpern; chronischer entzündlicher demyelinisierender Neuropathie (CIDP); multifokaler motorischer Neuropathie (MMN); und multifokaler erworbener demyelinisierender sensorischer und motorischer Neuropathie (MADSAM).

2. Antikörper, oder Antigen-bindendes Fragment davon, zur Verwendung nach Anspruch 1, wobei der Anti-Gangliosid- oder Anti-Glycolipid-Antikörper an ein Glycolipid ausgewählt aus: GM1, GM1(NeuGc), GM1b, GalNAc-GM1b, GD1a, GalNAc-GD1a,

Samsung Bioepis Exhibit No. 1002
Page 1977

GD1b, 9-O-azetyl-GD1b, GD3, GT1a, GT1b, GQ1b, GQ1bα, LM1, Galactocerebrosid und SGPG bindet.

3. Antikörper, oder Antigen-bindendes Fragment davon, zur Verwendung nach Anspruch 1 oder 2, wobei der Säuger ein Mensch ist.

4. Antikörper, oder Antigen-bindendes Fragment davon, zur Verwendung nach einem der Ansprüche 1 bis 3, wobei der Antikörper oder das Antigen-bindende Fragment davon ausgewählt ist aus: einem polyklonalen Antikörper, einem monoklonalen Antikörper oder Antikörperfragment, einem Diabody, einem chimärisierten oder chimären Antikörper oder Antikörperfragment, einem humanisierten Antikörper oder Antikörperfragment, einem de-immunisierten menschlichen Antikörper oder Antikörperfragment, einem komplett menschlichen Antikörper oder Antikörperfragment, einem Einzelketten-Antikörper, einem Fv, einem Fab, einem Fab' und einem $F(ab')_2$.

5. Antikörper, oder Antigen-bindendes Fragment davon, zur Verwendung nach einem der Ansprüche 1 bis 4, wobei der Antikörper oder das Antigen-bindende Fragment davon die Spaltung von C5 in die Fragmente C5a und C5b hemmt.

6. Antikörper, oder Antigen-bindendes Fragment davon, zur Verwendung nach Anspruch 5, wobei der Antikörper Eculizumab ist.

7. Antikörper, oder Antigen-bindendes Fragment davon, zur Verwendung nach Anspruch 5, wobei der Antikörper Pexelizumab ist.

8. Antikörper, oder Antigen-bindendes Fragment davon, zur Verwendung nach Anspruch 6, wobei die Behandlung chronisch ist.

9. Antikörper, oder Antigen-bindendes Fragment davon, zur Verwendung nach Anspruch 7, wobei die Behandlung ein Behandeln einer akuten Episode umfasst.

10. Antikörper, oder Antigen-bindendes Fragment davon, zur Verwendung nach einem der Ansprüche 1 bis 9, wobei die Behandlung das systemische Verabreichen des Antikörpers oder des Antigen-bindenden Fragments davon an den Säuger umfasst.

11. Antikörper, oder Antigen-bindendes Fragment davon, zur Verwendung nach einem der Ansprüche 1 bis 9, wobei die Behandlung das lokale Verabreichen des Antikörpers oder des Antigen-bindenden Fragments davon an den Säuger umfasst.

12. Antikörper, oder Antigen-bindendes Fragment davon, zur Verwendung nach einem der Ansprüche 1 bis 11, wobei die Behandlung zu:

    (a) reduzierter neuraler Verletzung in dem Säuger führt; oder
    (b) einer reduzierten Menge und/oder einem reduzierten Ausmaß eines unerwünschten physiologischen Zustands führt, der aus übermäßiger Nervenverletzung resultiert.

13. Antikörper, oder Antigen-bindendes Fragment davon, zur Verwendung nach Anspruch 12, wobei der unerwünschte physiologische Zustand ausgewählt ist aus: Ophthalmoplegie, Ataxie, Areflexie, abnormale Muskelkoordination, Lähmung der Augenmuskeln, schmerzhaftes Leiden der Muskeln, Fehlen des Sehnenreflexes, Taubheit und kribbelndes Gefühl in den unteren Extremitäten, Schwäche in den distalen Muskeln der unteren Extremitäten, Steppergang, Schwäche, die die gesamten unteren Extremitäten betrifft, Schwäche, die die oberen Extremitäten betrifft, Schwäche in den Atemmuskeln, und Tod.

14. Antikörper, oder Antigen-bindendes Fragment davon, zur Verwendung nach einem der Ansprüche 1 bis 13, wobei die Behandlung zu:

    (a) reduzierter Ausbildung des Membranangriffskomplexes (MAC) an präsynaptischen neuromuskulären Endplatten;
    (b) Wiederherstellung einer normalen Frequenz von Miniatur-Endplattenpotentialen;
    (c) Wiederherstellung synaptischer Übertragung an neuromuskulären Endplatten; oder
    (d) Hemmung des Verlusts der Unversehrtheit der Termini an neuromuskulären Endplatten

führt.

Revendications

1. Anticorps ou l'un de ses fragments de liaison de l'antigène pour une utilisation dans le traitement d'une neuropathie médiée par des anticorps anti-gangliosides ou anti-glycolipides chez un mammifère, où l'anticorps ou l'un de ses fragments de liaison de l'antigène se lie:

    (i) au composant C5 du complément et inhibe le clivage de C5 en fragments C5a et C5b; ou
    (iii) à C5b,

    où ledit anticorps ou l'un de ses fragments de liaison de l'antigène inhibe la formation du complexe d'attaque membranaire; et
    où ladite neuropathie médiée par des anticorps anti-gangliosides ou anti-glycolipides est choisie dans le

Samsung Bioepis Exhibit No. 1002
Page 1978

groupe constitué de: la neuropathie axonale motrice aiguë; la polyneuropathie démyélinisante inflammatoire aiguë; l'encéphalite du tronc cérébral de Bickerstaff; l'ophtalmoparésie aiguë; le syndrome de Guillain-Barré ataxique; la faiblesse pharyngo-cervico-brachiale; les syndromes de neuropathie chronique avec des anticorps anti-glycolipides; la neuropathie paraprotéinémique à IgM anti-MAG; la neuropathie ataxique sensorielle chronique avec des anticorps anti-disialosyles; la neuropathie paraprotéinémique à IgM, IgG et IgA; la neuropathie motrice avec des anticorps anti-GM1 et anti-GM2; la neuropathie démyélinisante inflammatoire chronique (CIDP); la neuropathie motrice multifocale (MMN); et la neuropathie sensorielle et motrice démyélinisante acquise multifocale (MADSAM).

2. Anticorps ou l'un de ses fragments de liaison de l'antigène pour une utilisation selon la revendication 1, où lesdits anticorps anti-gangliosides ou anti-glycolipides se lient à un glycolipide choisi dans le groupe constitué de: GM1, GM1(NeuGc), GM1b, GalNAc-GM1b, GD1a, GalNAc-GD1a, GD1b, 9-O-acétyl-GD1b, GD3, GT1a, GT1b, GQ1b, GQ1bα, LM1, galactocérébroside, et SGPG.

3. Anticorps ou l'un de ses fragments de liaison de l'antigène pour une utilisation selon la revendication 1 ou 2, où ledit mammifère est un être humain.

4. Anticorps ou l'un de ses fragments de liaison de l'antigène pour une utilisation selon l'une quelconque des revendications 1 à 3, où ledit anticorps ou l'un de ses fragments de liaison de l'antigène est choisi dans le groupe constitué de: un anticorps polyclonal, un anticorps monoclonal ou un fragment de celui-ci, un diabody, un anticorps chimérisé ou chimérique ou un fragment de celui-ci, un anticorps humanisé ou un fragment de celui-ci, un anticorps humain désimmunisé ou un fragment de celui-ci, un anticorps totalement humain ou un fragment de celui-ci, un anticorps à chaîne unique, un Fv, un Fab, un Fab', et un $F(ab')_2$.

5. Anticorps ou l'un de ses fragments de liaison de l'antigène pour une utilisation selon l'une quelconque des revendications 1 à 4, où ledit anticorps ou l'un de ses fragments de liaison de l'antigène inhibe le clivage de C5 en fragments C5a et C5b.

6. Anticorps ou l'un de ses fragments de liaison de l'antigène pour une utilisation selon la revendication 5, où ledit anticorps est l'éculizumab.

7. Anticorps ou l'un de ses fragments de liaison de l'antigène pour une utilisation selon la revendication 5, où ledit anticorps est le pexélizumab.

8. Anticorps ou l'un de ses fragments de liaison de l'antigène pour une utilisation selon la revendication 6, où ledit traitement est chronique.

9. Anticorps ou l'un de ses fragments de liaison de l'antigène pour une utilisation selon la revendication 7, où ledit traitement comprend le traitement d'un épisode aigu.

10. Anticorps ou l'un de ses fragments de liaison de l'antigène pour une utilisation selon l'une quelconque des revendications 1 à 9, où ledit traitement comprend l'administration de l'anticorps ou de l'un de ses fragments de liaison de l'antigène de façon systémique audit mammifère.

11. Anticorps ou l'un de ses fragments de liaison de l'antigène pour une utilisation selon l'une quelconque des revendications 1 à 9, où ledit traitement comprend l'administration de l'anticorps ou de l'un de ses fragments de liaison de l'antigène localement audit mammifère.

12. Anticorps ou l'un de ses fragments de liaison de l'antigène pour une utilisation selon l'une quelconque des revendications 1 à 11, où ledit traitement conduit à:

    (a) une diminution des lésions neurales chez ledit mammifère; ou
    (b) une diminution de la quantité et/ou de l'étendue d'une condition physiologique indésirable résultant d'un excès de lésions nerveuses.

13. Anticorps ou l'un de ses fragments de liaison de l'antigène pour une utilisation selon la revendication 12, où ladite condition physiologique indésirable est choisie dans le groupe constitué de: une ophtalmoplégie, une ataxie, une aréflexie, une coordination musculaire anormale, une paralysie des muscles des yeux, une douleur musculaire, l'absence des réflexes tendineux, un engourdissement et une sensation de picotements dans les extrémités inférieures, une faiblesse des muscles distaux des extrémités inférieures, un pied tombant, une faiblesse impliquant les extrémités inférieures entières, une faiblesse impliquant les extrémités supérieures, une faiblesse dans les muscles de la respiration, et le décès.

14. Anticorps ou l'un de ses fragments de liaison de l'antigène pour une utilisation selon l'une quelconque des revendications 1 à 13, où ledit traitement conduit à:

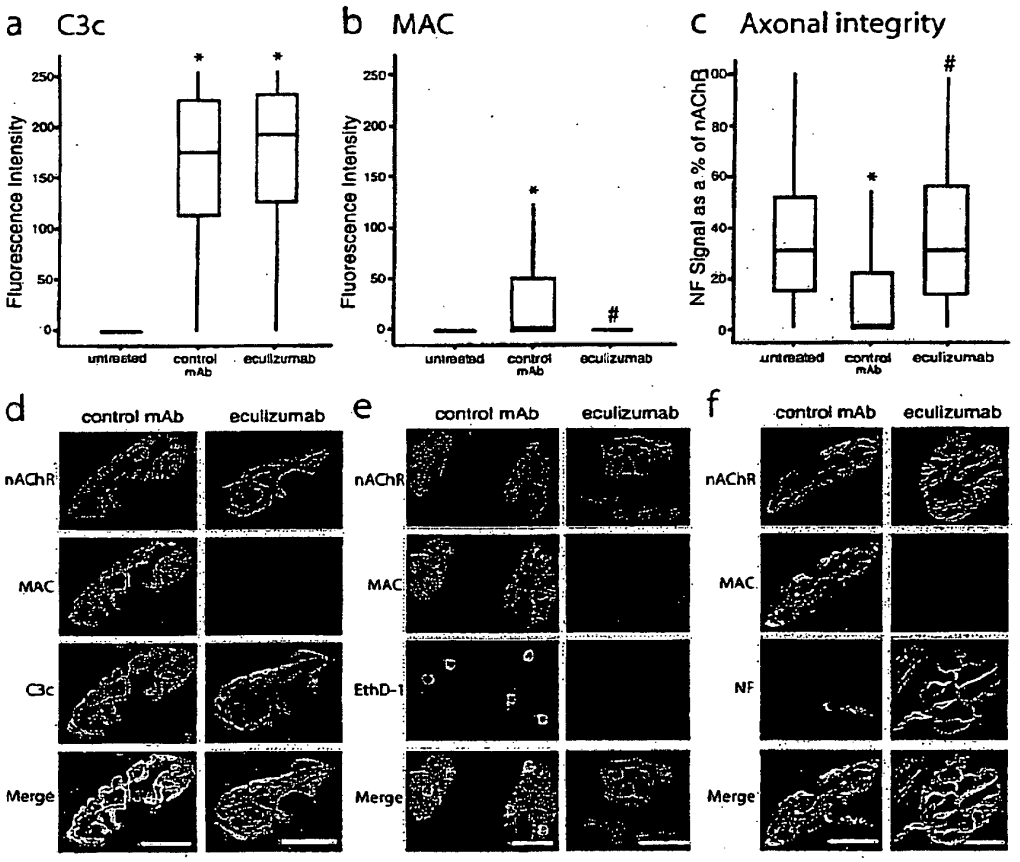
    (a) une diminution de la formation du complexe d'attaque membranaire (MAC) au niveau des jonctions neuromusculaires présynaptiques;

Samsung Bioepis Exhibit No. 1002
Page 1979

(b) la restauration d'une fréquence normale des potentiels miniatures de plaque terminale;
(c) la restauration de la transmission synaptique au niveau des jonctions neuromusculaires; ou
(d) l'inhibition de la perte d'intégrité terminale au niveau des jonctions neuromusculaires.

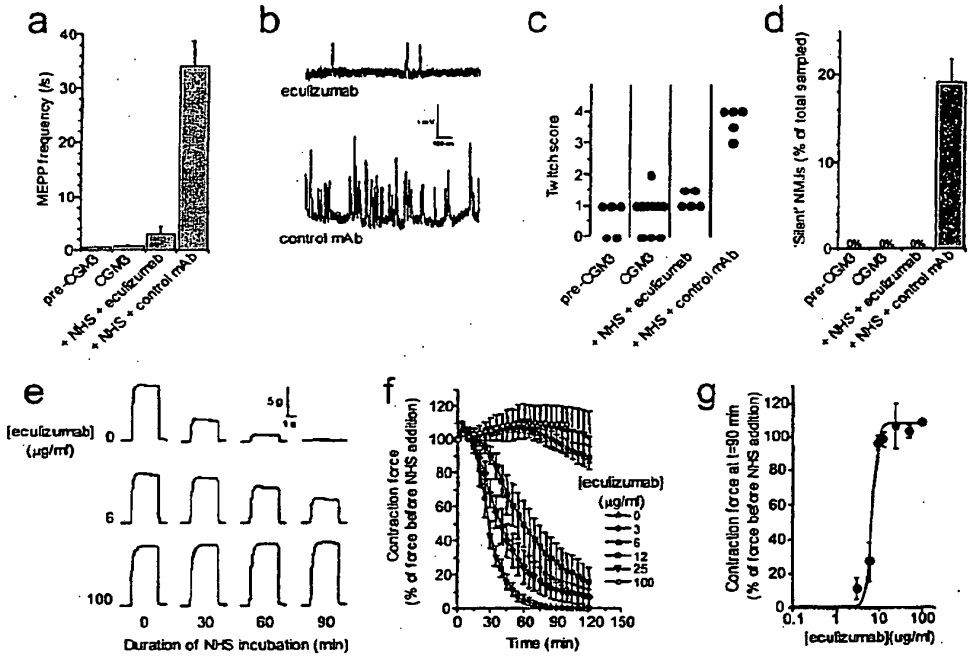
Samsung Bioepis Exhibit No. 1002
Page 1980

Figure 1



Samsung Bioepis Exhibit No. 1002
Page 1981

Figure 2



Samsung Bioepis Exhibit No. 1002
Page 1982

Figure 3A

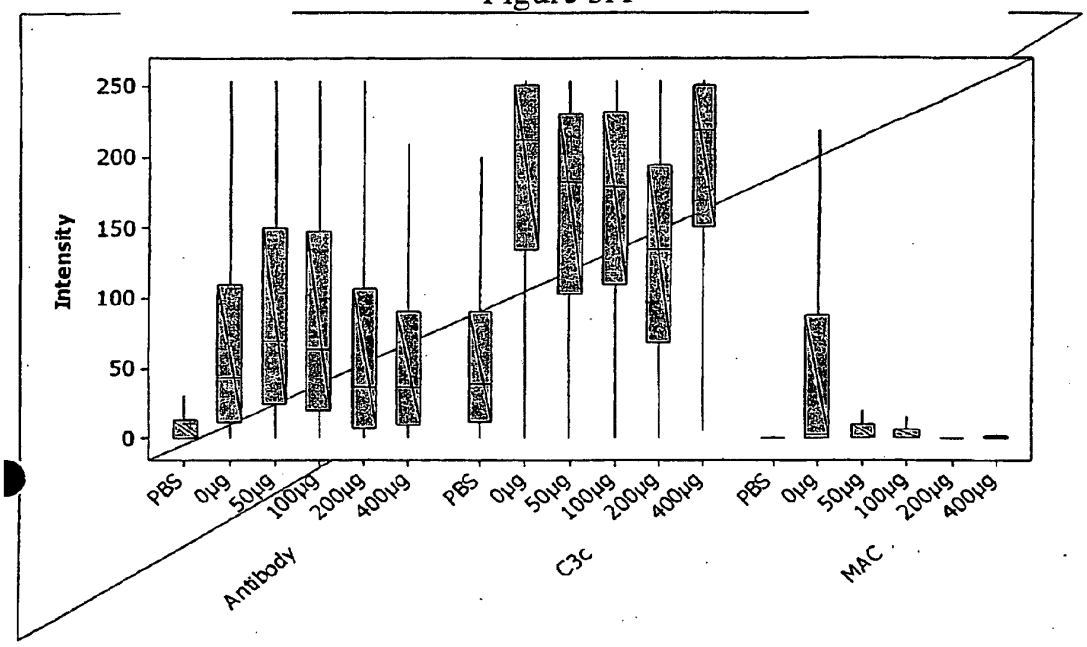
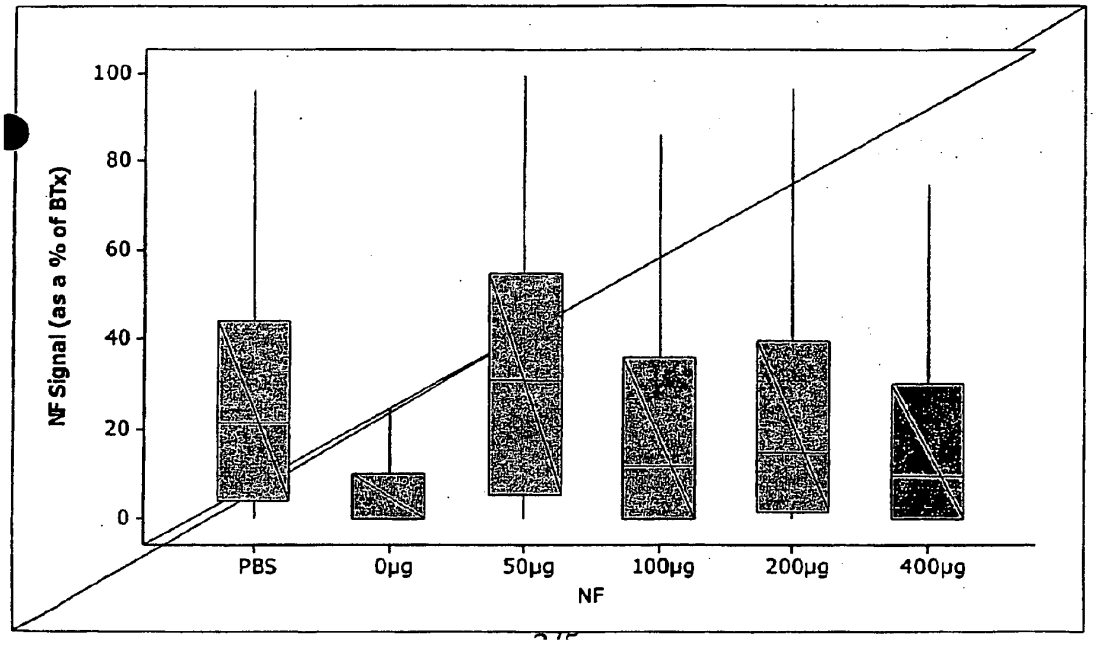
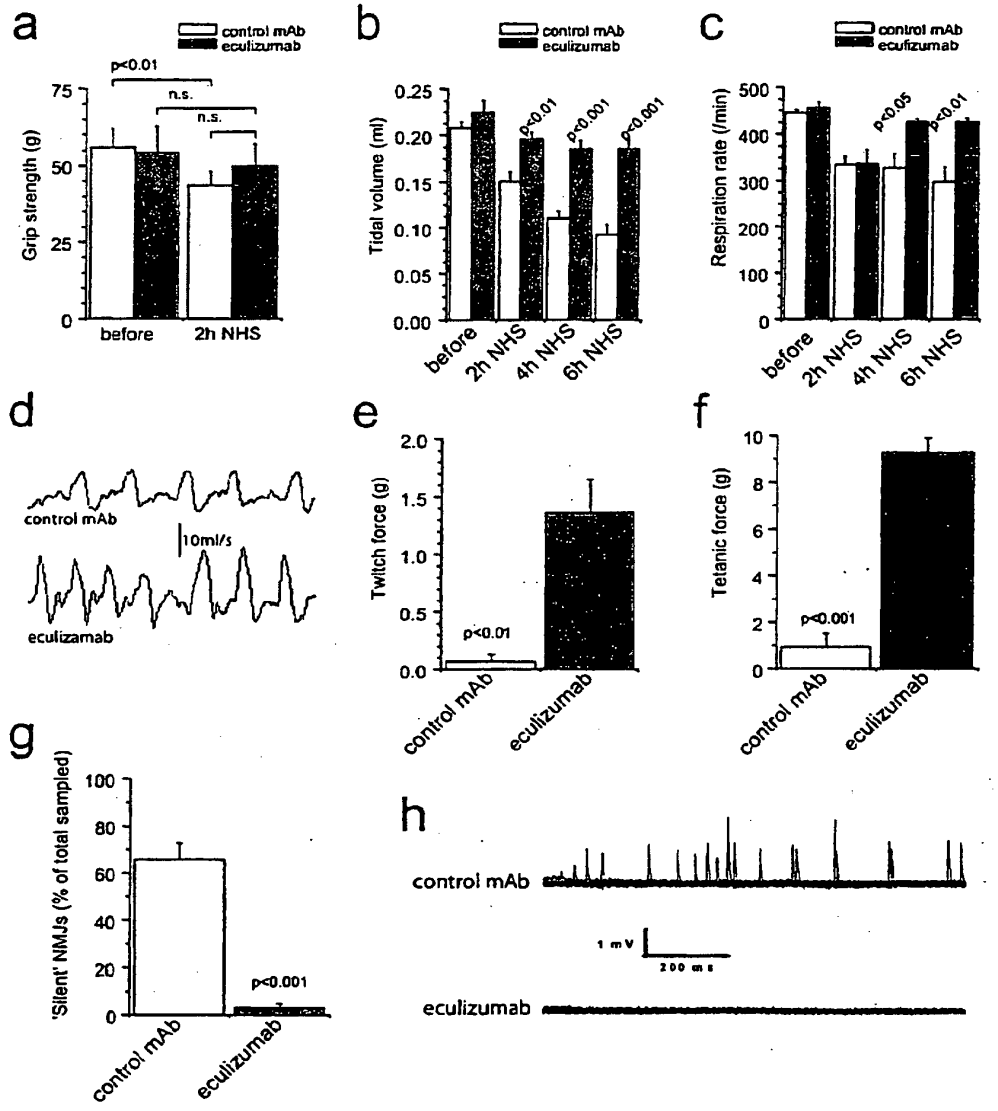


Figure 3B



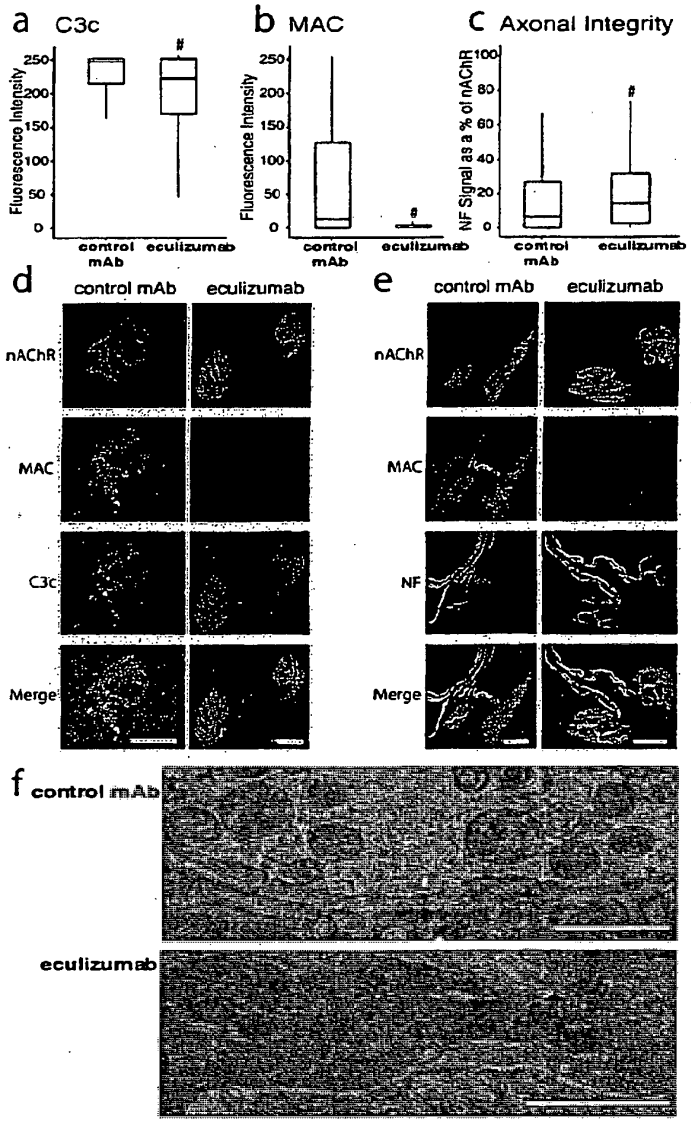
Samsung Bioepis Exhibit No. 1002
Page 1983

Figure 4



Samsung Bioepis Exhibit No. 1002
Page 1984

Figure 5



Samsung Bioepis Exhibit No. 1002
Page 1985

REFERENCES CITED IN THE DESCRIPTION

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Samsung Bioepis Exhibit No. 1002
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(71) Applicant: **ALEXION PHARMACEUTICALS, INC.** [US/US]; 352 Knotter Drive, Cheshire, CT 06410 (US).

(72) Inventors: **BOWDISH, Katherine, S.**; 13754 Boquita Drive, Del Mar, CA 92014 (US). **FREDERICKSON, Shana**; 520 N. Granados Ave., Solana Beach, CA 92075 (US). **RENSHAW, Mark**; 12725 Moran Way, San Diego, CA 92129 (US).

(74) Agent: **FARBER, Mark**; Alexion Pharmaceuticals, Inc., 352 Knotter Drive, Cheshire, CT 06410 (US).

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(54) Title: RATIONALLY DESIGNED ANTIBODIES

(57) Abstract: Antibodies or fragments thereof having at least two CDR regions replaced or fused with biologically active peptides are described. Compositions containing such antibodies or fragments thereof are useful in therapeutic and diagnostic modalities.

RATIONALLY DESIGNED ANTIBODIES

Related Applications

This application is a continuation-in-part of U.S. Application Serial No. 10/006,593 filed December 5, 2001 which claims priority to U.S. Provisional Patent Application No. 60/251,448 filed December 5, 2000, and to U.S. Provisional Patent Application No. 60/288,889 filed May 4, 2001, and to U.S. Provisional Patent Application No. 60/294,068 filed May 29, 2001. The disclosures of these three provisional applications are incorporated herein in their entirety by this reference.

TECHNICAL FIELD

The present invention relates to antibody molecules and biologically active peptides as diagnostic and therapeutic reagents.

BACKGROUND OF RELATED ART

Antibodies are produced by B lymphocytes and defend against infection. Antibodies are produced in millions of forms, each with a different amino acid sequence. Antibody molecules are composed of two identical light chains and two identical heavy chains. When digested by the enzyme papain, two identical Fab fragments are produced along with one Fc fragment. When digested with the enzyme pepsin one $F(ab')_2$ fragment is produced. Light and heavy chains consist of constant and variable regions. Within the variable regions are hypervariable regions (aka complementarity determining regions (CDRs)) which form the antigen binding site. The remaining parts of the variable regions are referred to as framework regions.

Important biological functions, such as receptor binding, activation and enzymatic activity, are often attributable to discrete regions of larger protein molecules, comprising a limited number of amino acid residues. Peptides displaying binding, activation or enzymatic activity have also been discovered by screening libraries of peptides generated by the random linking of amino acid residues. These peptides may not correspond to a linear arrangement of amino acids in a larger protein

molecule exhibiting similar biological activity and are referred to as discontinuous peptide epitopes or mimotopes. Certain peptide mimetics have been described and cloned. See, e.g., U.S. Pat. No. 6,083,913 (thrombopoietin (TPO) mimetic), U.S. Pat. No. 5,835,382 (erythropoietin (EPO) mimetic), U.S. Pat. No. 5,830,851 (EPO mimetic) and Wrighton et al, Science, (1996) 273:458-63. Peptide epitopes and mimotopes due to their small size are potentially advantageous over large protein molecules for use as therapeutic reagents. However, the results with these peptides as therapeutics may often be unsatisfactory. One drawback to the use of peptides as therapeutic reagents is that they are generally unstable *in vivo*, i.e., their clearance rates from serum may be quite rapid. In addition, it is difficult to predict the activity, therapeutic or otherwise, of a peptide if it is fused into a larger molecule since conformational changes and other molecular forces may interfere with or totally negate the activity of the peptide. Attempts have been made to introduce certain polypeptides into CDR regions of antibodies. See, e.g., PCT Appln. WO 94/18221. However, as mentioned previously, due to conformational changes which may be caused by surrounding amino acids, the biological activity of active polypeptides may be diminished or negated. Therefore, it is an object herein to provide rationally designed antibodies or fragments thereof which include biologically active peptides for use as diagnostic and therapeutic reagents.

SUMMARY

Provided herein are biologically active recombinant antibodies and fragments thereof that mimic the activity of biologically active peptides, methods of making such antibodies and methods for their use in therapy and diagnosis. These antibodies and fragments thereof do not suffer from some of the disadvantages of isolated peptides, as antibodies naturally have long serum half-lives and are highly specific in binding their target. It has surprisingly been found that incorporation of particular amino acids surrounding a target peptide that has been combined into an antibody molecule actually increases the biological activity of the peptide.

Immunoglobulins or fragments thereof have a peptide of interest inserted into a complementarity determining region (CDR) of an antibody molecule. The antibody molecule serves as a scaffold for presentation of the peptide and confers upon the

Samsung Bioepis Exhibit No. 1002
Page 1992

peptide enhanced stability. The peptide optionally replaces all the amino acids of a CDR region, or may be added to an existing CDR, whereby the original antigen specificity is disrupted, wherein the CDR region is defined by either of the two accepted schemes (See, Kabat et al., Sequences of Proteins of Immunologics Interest, 5th ed (1991), NIH Publication 91-3242 and Chothia et al. J.Mol. Bio (1992) (227)776-98.) Furthermore, additional amino acids may be randomly introduced which flank the peptide and allow for the screening of optimum peptide presentation in the antibody framework. It has been surprisingly found that in certain cases a proline flanking the peptide provides an increase in biological activity.

In particular embodiments an immunoglobulin molecule or fragment has amino acids residues corresponding to one complementarity determining region (CDR) replaced with amino acid residues comprising a biologically active hemopoietic or thrombopoietic peptide. In another particular embodiment, amino acid residues corresponding to at least two complementarity determining regions (CDRs) are each replaced by amino acid residues comprising such a biologically active peptide. In a single immunoglobulin molecule or fragment thereof, one or more complementarity determining regions can be replaced with a peptide; for example, CDR3 of a heavy chain, CDR3 of a light chain, CDR3 of both a heavy and light chain, CDR2 and CDR3 of a heavy chain, or CDR2 and CDR3 of a light chain. Other combinations of replaced CDR regions are possible, including the replacement of CDR1. In addition, instead of replacement of a CDR, one could add the peptide to a native CDR without actual replacement of amino acid residues while still disrupting the original antigen specificity.

Thus, in one aspect, a biologically active peptide is provided with enhanced activity by adding a proline to its carboxy terminus to form a proline-extended biologically active peptide which is used to replace or add to at least a portion of at least one CDR region in an immunoglobulin molecule or fragment thereof. In another aspect, an immunoglobulin molecule or fragment thereof is provided which has either a TPO mimetic peptide or EPO mimetic peptide as a replacement for at least one native CDR region. In this aspect, the TPO mimetic peptide or EPO mimetic peptides may optionally be proline-extended as described herein.

Samsung Bioepis Exhibit No. 1002
Page 1993

In further particular embodiments the immunoglobulin molecule or fragment thereof is an Fab, a ScFv, a heavy chain variable region, a light chain or a full IgG molecule. The immunoglobulin molecule or fragment thereof can also have a dimerization domain, so as to enable immunoglobulin molecules which have only one CDR replaced with a peptide to dimerize and thus activate receptors that require dimerization for activation.

In certain embodiments, the biologically active peptide can be a linear peptide epitope or a discontinuous peptide epitope. Furthermore, the biologically active peptide, when substituted for a CDR region, can have in addition to proline, one, two or more additional flanking amino acid residues proximate to the amino and/or the carboxyl termini of the peptide, which are positioned between the peptide and immunoglobulin framework region residues (i.e., at what was the junction between a CDR and the adjoining framework). The flanking amino acid residues are not typically present in the active peptide. If preferred flanking amino acid residues are already known, the flanking amino acid residues are encoded by codons which designate those specific amino acid residues. However, by initially utilizing codons, such as NNK, NNY, NNR, NNS and the like, which designate multiple amino acid residues, a collection of peptides that differ from one another merely by the flanking residues is generated. The flanking amino acid residues may determine the presentation of the peptide in the immunoglobulin molecule or fragment thereof and thus may influence the binding and/or biological activity exhibited by the peptide. This random collection of flanking amino acids allows for the selection of the best context to display the peptide sequence within the antibody framework that results in specific binding to the target molecule and the exhibition of optimal biological activity. Screening of libraries of immunoglobulins having a common peptide but different flanking amino acid residues can be carried out using binding, growth and activation assays known by those skilled in the art and as described herein.

The peptide replacing the amino acid residues comprising a CDR can be any peptide which specifically binds a target molecule and whose utility could be altered by incorporation in an antibody framework. The peptide could also exhibit a specific activity (e.g., agonist, antagonist, enzymatic, etc.). In a particular embodiment the

Samsung Bioepis Exhibit No. 1002
Page 1994

peptide is an agonist or an antagonist for a cell surface receptor. For example, the cell surface receptor can be for a cytokine, a growth factor, or a growth inhibitor.

In particularly useful embodiments, replacement of at least a portion of a CDR with a peptide provides an antibody that acts as an agonist. The peptide used to replace at least a portion of a CDR may itself have agonist properties. Alternatively, the peptide (although specifically binding to a receptor) may not exhibit agonist activity. Rather, agonist activity might be exhibited only when the peptide is substituted for at least a portion of a CDR and is thus present in the engineered antibody. In such embodiments, the presence or absence of proline flanking the peptide is not critical, but can, in some instances, be preferred.

Thus, in one aspect the present disclosure provides for an agonist antibody comprising an antibody framework engineered to contain at least one biologically active peptide inserted at, or in place of at least a portion of, one or more CDRs. The biologically active peptide may or may not exhibit agonist activity prior to insertion into the antibody framework. In certain embodiments the antibody framework is engineered to contain two peptides capable of dimerizing with each other.

In yet another aspect, the present disclosure provides for an immunoglobulin molecule or fragment thereof comprising a region where amino acid residues corresponding to at least a portion of a complementary determining region (CDR) are replaced with a biologically active peptide, whereby the immunoglobulin molecule or fragment thereof exhibits agonist activity. The biologically active peptide may or may not exhibit agonist activity prior to insertion into the antibody framework. In particularly useful embodiments the immunoglobulin molecule or fragment thereof exhibits c-mpl agonist activity.

In yet another aspect, the present disclosure provides for an immunoglobulin molecule or fragment thereof comprising a biologically active peptide inserted at a complementary determining region (CDR), whereby the immunoglobulin molecule or fragment thereof exhibits agonist activity.

In yet another aspect, the present disclosure provides for an immunoglobulin molecule or fragment thereof comprising a region where amino acid residues corresponding to at least a portion of a complementary determining region (CDR) are

Samsung Bioepis Exhibit No. 1002
Page 1995

replaced with a biologically active peptide, whereby the immunoglobulin molecule or fragment thereof exhibits c-mpl agonist activity.

In further particular embodiments, the peptide replacing the amino acids of a CDR is an agonist TPO mimetic peptide. One such agonist peptide has at least the sequence IEGPTLRQWLAARA (SEQ. ID. NO. 1). Other sequences are possible for TPO agonist mimetic peptides, which can be found using binding, growth and activation assays known by those skilled in the art and as described herein. Agonist TPO mimetic peptides when positioned in CDR regions can have one or more additional amino acid residues at the amino and/or carboxyl termini of the peptide which become covalently bonded to immunoglobulin framework residues. One such TPO mimetic peptide has an additional proline residue added to the carboxyl terminus; IEGPTLRQWLAARAP (SEQ. ID. NO: 2). Other immunoglobulin molecules or fragments thereof have a CDR region replaced by the TPO mimetic peptides comprising the amino acid sequence of SEQ. ID. NOs: 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, and 49 (see Fig. 5).

Another biologically active peptide that can replace the amino acid residues of a CDR is an agonist EPO mimetic peptide. One such EPO agonist peptide has as its amino acid sequence DYHCRMGPLTWVCKPLGG (SEQ. ID. NO: 3). Other amino acid sequences are possible for EPO agonist mimetic peptides, which can be found using binding, growth and activation assays known by those skilled in the art and as described herein. Agonist EPO mimetic peptides when located in CDR regions can also have one or more additional amino acid residues at the amino and/or carboxyl termini of the peptide which become covalently bonded to immunoglobulin residues. Thus, in particular embodiments provided herein are immunoglobulin molecules (IgG) or fragments (e.g., Fab, scFv, heavy or light chains) that have a CDR region replaced with a TPO or EPO mimetic peptide. For example, the TPO peptide can include at least the sequence IEGPTLRQWLAARA (SEQ. ID. NO:1) and may further optionally have an additional proline at the immediate downstream position. The EPO mimetic encompasses at least the sequence DYHCRMGPLTWVCKPLGG (SEQ. ID. NO: 3). Likewise, it may optionally have an additional proline at the immediate downstream position.

Samsung Bioepis Exhibit No. 1002
Page 1996

Any immunoglobulin molecule (antibody) or fragment thereof could potentially provide the framework and have a CDR replaced with a peptide according to the present disclosure. For therapeutic or *in vivo* diagnostic use it is preferable that the antibody is of human origin or humanized, such as an anti-tetanus toxoid immunoglobulin. Furthermore, independent of or in conjunction with the presence of additional flanking amino acids bound to the peptide, one or more amino acid residues in other regions of the immununoglobulin, other CDR region(s) and/or framework regions, can be altered to modify the binding, activity and/or expression displayed by the peptide in the context of the immunoglobulin molecule.

It is contemplated that after construction of biologically active recombinant antibodies and/or fragments thereof, such recombinants can be subjected to randomization methods known in the art to introduce mutations at one or more points in the sequence to alter the biological activity of the antibodies. After generation of such mutants using randomization methods such as those described herein, the resulting recombinants may be assayed for activity using binding, growth, expression and activation assays.

Further provided are nucleic acid molecules encoding immunoglobulin molecules or fragments thereof which have the amino acids of one or more CDR regions replaced by a biologically active peptide. These nucleic acid molecules can be present in an expression vector, which can be introduced (transfected) into a recombinant host cell for expression of these molecules. Also provided are methods of producing an immunoglobulin molecule or fragment thereof containing a biologically active peptide, comprising culturing a recombinant host cell under conditions such that the nucleic acid contained within the cell is expressed.

Also provided are compositions, comprising an immunoglobulin molecule or fragment thereof which has amino acid residues corresponding to a CDR replaced with amino acid residues comprising a TPO or EPO mimetic peptide and a pharmaceutically acceptable carrier.

Further provided are EPO mimetic peptides with additional flanking residues which are suitable for replacement of CDRs. Also provided are nucleic acid molecules encoding these peptides.

Samsung Bioepis Exhibit No. 1002
Page 1997

Further provided are methods of engineering immunoglobulin molecules or fragments thereof to exhibit an agonist activity in which a biologically active peptide replaces at least a portion of one or more CDR regions of light and/or heavy chains. The methods encompass inserting a nucleic acid molecule encoding a biologically active peptide in place of at least a CDR region of a nucleic acid molecule encoding an immunoglobulin heavy or light chain or adding the molecule to the native CDR sequence and then expressing the nucleic acid molecule encoding the immunoglobulin heavy or light chain variable domain along with its complementary variable region domain, such that the two domains associate.

Further provided are methods of engineering immunoglobulin molecules or fragments thereof to exhibit an activity (property) of a biologically active TPO or EPO peptide in which a biologically active peptide replaces one or more CDR regions of light and/or heavy chains. The methods encompass inserting a nucleic acid molecule encoding a biologically active peptide in place of at least a portion of a CDR region of a nucleic acid molecule encoding an immunoglobulin heavy or light chain or adding the molecule to the native CDR sequence; and expressing the nucleic acid molecule encoding the immunoglobulin heavy or light chain variable domain along with its complementarity variable region domain, such that the two chains associate.

In another aspect, this disclosure provides a method for producing in a polypeptide a binding site capable of binding a preselected agent, the method including the steps of introducing a nucleotide sequence that codes for an amino acid residue sequence defining said binding site into a CDR region of a nucleic acid comprising an immunoglobulin heavy or light chain gene by amplifying the CDR region of the immunoglobulin gene, the introduced nucleotide sequence having the formula – X_a-Y-X_b wherein X is the same or different at each occurrence and represents a randomizing trinucleotide, the sum of a and b is 4 or less and Y is a nucleotide sequence that encodes a minimum recognition domain of said binding site. In particularly useful embodiments, amplification is achieved using overlap PCR, however, any known amplification technique could be employed, such as, for example, the methods disclosed in WO94/18221, the disclosure of which is incorporated herein by reference.

Samsung Bioepis Exhibit No. 1002
Page 1998

In yet another aspect, this disclosure provides methods for creation of a library of monoclonal antibodies that can be screened for a desired activity. These methods of making a library include the steps of inserting a nucleic acid molecule encoding a biologically active peptide into, or in place of at least a portion of, one or more CDR regions of a nucleic acid molecule encoding an immunoglobulin heavy or light chain, providing up to a pair of randomizing trinucleotides on either side of the inserted nucleic acid molecule, and expressing a library of monoclonal antibodies. In particularly useful embodiments, a pair of randomizing trinucleotides is provided on both sides of the inserted nucleic acid molecules. The library of monoclonal antibodies thus produced can then be screened for a desired activity.

In a specific embodiment, antibodies and fragments thereof have different amino acids flanking the peptide at the amino and the carboxyl termini where the peptide becomes bound to the antibody scaffold. This, results in a population of antibody molecules or fragments thereof that may differ in the presentation of the peptide. The population is screened for those antibodies that exhibit the biological activity of the peptide. In a preferred embodiment, the amino acid immediately adjacent the peptide is a proline.

If the activity of the biologically active peptide is to activate a target molecule, this may require dimerization of two target molecules (e.g. receptors in the hematopoietic superfamilies). For dimerization to occur, two peptides must be positioned to each bind a target molecule such that the two bound target molecules can then properly associate. This can be accomplished by having two peptides present on the same antibody or fragment thereof or by causing two antibody molecules each containing one peptide to bind together. Thus, for example, a single peptide can be inserted into or substituted for at least a portion of a CDR and then expressed as an immunoglobulin or a $F(ab')_2$ fragment. As another example, two peptides can be inserted into or substituted for at least a portion of one or more CDRs and expresses as any antibody or antibody fragment.

The screening of antibodies or fragments thereof can be accomplished by panning with cells that have surface molecules to which the peptide specifically binds. Solid phase binding using purified target molecules or fragments thereof can also be

Samsung Bioepis Exhibit No. 1002
Page 1999

used. Binding can also be carried out in solution using labeled target molecules. In addition, antibodies or fragments thereof can be screened by the use of biological assays for agonist or antagonist activity of the peptide.

Also provided are libraries of different immunoglobulin molecules or fragments thereof wherein amino acid residues corresponding to a complementarity determining region (CDR) are replaced with amino acid residues comprising a biologically active peptide which has at least one additional amino acid residue at the amino or the carboxyl terminus and the immunoglobulin molecules or fragments thereof differ by the additional amino acid residue of the peptide.

In specific embodiments the biologically active peptide is a TPO mimetic or an EPO mimetic. The antibodies of the library are displayed on phage.

Further provided are methods of stimulating proliferation, differentiation or growth of cells, which include contacting the cells with an effective amount of an immunoglobulin molecule or fragment thereof having one or more CDRs replaced with a biologically active peptide which binds to a receptor on the cells surface. In specific embodiments the biologically active peptide is a TPO mimetic or an EPO mimetic.

In other specific embodiments, is provided a method of stimulating proliferation, differentiation or growth of megakaryocytes by contacting megakaryocytes with an effective amount of an immunoglobulin molecule or fragment thereof having one or more CDRs replaced with a TPO mimetic peptide. Also provided is a method of increasing platelet production, which involves contacting megakaryocytes with an effective amount of an immunoglobulin molecule or fragment thereof having one or more CDR regions replaced with a TPO mimetic peptide. Also provided is a method of stimulating megakaryocytes and/or increasing platelet production in a patient, in which an effective amount of an immunoglobulin molecule or fragment thereof having one or more CDRs replaced with a TPO mimetic peptide is administered to a patient in need thereof. The immunoglobulin molecule and the megagakarocytes can also be contacted in vitro and the resultant cells can be introduced into the patient. In addition, an antibody or fragment thereof having at least one TPO mimetic peptide incorporated therein can be administered to a subject who intends to donate platelets, thus

Samsung Bioepis Exhibit No. 1002
Page 2000

increasing the capacity of a donor to generate platelets to provide a more robust source of such platelets.

Also provided herein is a method of stimulating proliferation, differentiation or growth of hematopoietic cells, comprising contacting the cells with an effective amount of an immunoglobulin molecule or fragment thereof having one or more CDRs replaced with a EPO mimetic peptide.

Also embodied herein is a method of activating a homodimeric receptor protein, by contacting the receptor with an immunoglobulin molecule or fragment thereof having a CDR region replaced with a biologically active peptide that specifically binds the receptor and which has been dimerized. In a further embodiment the receptor is a thrombopoietin receptor.

DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagrammatic representation of the vector pRL4.

Figures 2A and B show the sequence of the human tetanus toxoid antibody framework, light and heavy chains, respectively.

Figure 3 is a diagram depicting the grafting of the TPO mimetic peptide AF12505 into the heavy chain CDR3 region of the tetanus toxoid framework antibody. XX represents flanking random amino acids.

Figure 4 is a diagram of the construction of a peptide cloned into the heavy chain CDR3 region.

Figure 5 represents the amino acid and nucleotide sequences of clones that encode TPO mimetic peptide AF1205 with different random flanking residues.

Figure 6A-C depicts the nucleic acid sequence of plasmid pRL8 (SEQ. ID. NO: 60). pRL8 is a modified version of pRL4 (pRL4 is also known as pComb 3X). The pRL4 was modified between the Spe I and neighboring Sfi I restriction sites (shown by underlining) to include a flexible linker (murine kappa hinge region) followed by a Jun leucine zipper dimerization domain.

Figure 7 is a schematic depiction of a portion of the plasmid pRL8.

Samsung Bioepis Exhibit No. 1002
Page 2001

Figure 8 depicts the nucleic acid sequence of a portion of plasmid pRL8 (SEQ. ID. NO: 52) along with amino acid sequences corresponding to certain delineated nucleic acid sequences (SEQ. ID. NO: 53).

Figure 9 is a chart showing sequences of certain TPO positive clones herein.

Figure 10 is a bar graph showing activity of certain Fab clones containing 2 TPO mimetic peptides.

Figure 11 is a bar graph showing activity of certain Fab clones containing 2 or 3 TPO mimetic peptides.

Figure 12 graphically depicts the activity of Clone 59 as reflected by induction of luciferase activity.

Figure 13A depicts the amino acid sequence and nucleic acid sequence of the 5G1.1-TPO heavy chain (SEQ. ID. NOS: 67 and 68, respectively).

Figure 13B depicts the amino acid sequence and nucleic acid sequence of the 5G1.1 light chain (SEQ. ID. NOS: 69 and 70, respectively).

Figure 14 is a bar graph showing FACS analysis of cMpl receptor binding of purified 5G1.1+ TPO mimetic peptide compared to parental 5G1.1 antibody.

Figure 15 is a bar graph showing comparative activity of 5G1.1 antibody containing the TPO mimetic peptide in connection with cells transfected with a control vector containing no cMpl-R and cells transfected with a vector containing cMpl-R.

Figure 16 shows the sequence of clone 429/Xb4 (SEQ. ID. NO: 116)

Figure 17 is a flow chart showing the initial steps for making vector pRL5-Kappa.

Figure 18 is a flow chart showing additional steps for making vector pRL5-Kappa.

Figure 19 is a map of vector pRL5.

Figure 20 is a schematic of vector pRL5-Kappa.

Figure 21A-I show the nucleic acid sequence of vector pRL5-Kappa.

Figure 22 shows the amino acid sequences for the heavy and light chain of clone 116.

Figure 23 shows the activity of clone 116.

Samsung Bioepis Exhibit No. 1002
Page 2002

Figure 24 shows the sequences of heavy chain clones in accordance with an alternative embodiment of the present disclosure.

DETAILED DESCRIPTION

As used herein, "immunoglobulin" refers to an entire immunoglobulin molecule or molecules that contain immunologically active portions of whole immunoglobulin molecules and includes Fab, F(abɘ)2, scFv, Fv, heavy chain variable regions and light chain variable regions. The terms immunoglobulin and antibody are used interchangeably herein.

Any peptide that exhibits a useful property is suitable for insertion in an antibody framework. Peptide activities and uses include, but are not limited to, binding a receptor, binding a membrane bound surface molecule, binding a ligand, binding an enzyme or structural protein, activating or inhibiting a receptor, targeted drug delivery, or any enzymatic activity. Those peptides whose utility can be increased from the enhanced stability conferred upon them when presented in the context of an immunoglobulin molecule are usually selected. It should be understood that "biological activity" as used herein includes any activity associated with a molecule having activity in a biological system, including, but not limited to, the stimulatory or inhibitory activity triggered by protein-protein interactions as well as the kinetics surrounding such interactions including the stability of a protein-porotein complex. Enhancing or increasing "biological activity" herein is meant to include an increase in overall activity or an increase in any component of overall activity. It should be understood that a peptide may exhibit one biological activity (such as, e.g., simply binding to a target) before insertion into the antibody framework, and a different or enhanced biological activity (such as, e.g., agonist activity) after insertion into the antibody framework.

Many peptides which could benefit from display in the context of an immunoglobulin have been identified and are known to those who practice the art, e.g., EPO and TPO mimetic peptides. Other examples include peptides that bind to receptors which are activated by ligand-induced homo-dimerization including active fragments displaying G-CSF activity, GHR activity and prolactin activity as described in

Samsung Bioepis Exhibit No. 1002
Page 2003

Whitty and Borysenko, *Chem Biol.*, (1999) Apr 6(4):R107-18; other examples of suitable peptides include a nerve growth factor mimetic from the CD loop as described in Zaccaro et al., *Med. Chem.* (2000) 43(19); 3530-40; an IL-2 mimetic as described in Eckenberg, et al., *J. Immunol.* (2000) 165(8):4312-8; glucogon-like peptide-1 as described in Evans et al., *Drugs R.D.* (1999) 2(2): 75-94; tetrapeptide I (D-lysine-L-asparaginyl-L-prolyl-L-tyrosine) which stimulates mitogen activated B cell proliferation as described in Gagnon et al., *Vaccine* (2000) 18(18):1886-92. Peptides which exhibit receptor antagonistic activity are also contemplated. For example, N-terminal peptide of vMIP-II as an antagonist of CXCR4 for HIV therapy as described in Luo et al., *Biochemistry* (2000) 39(44):13545-50; antagonist peptide ligand (AFLARAA) of the thrombin receptor for antithrombotic therapy as described in Pakala et al., *Thromb. Res.* (2000) 100(1): 89-96; peptide CGRP receptor antagonist CGRP (8-37) for attenuating tolerance to narcotics as described in Powell et al., *Br. J. Pharmacol.* (2000) 131(5): 875-84; parathyroid hormone (PTH)-1 receptor antagonist known as tuberoinfundibular peptide (7-39) as described in Hoare et al., *J. Pharmacol. Exp. Ther.* (2000) 295(2):761-70; opioid growth factor as described in Zagon et al., *Int. J. Oncol.* (2000) 17(5): 1053-61; high affinity type I interleukin 1 receptor antagonists as disclosed in Yanofsky, et al., *Proc. Natl. Acad. Sci. USA*, Vol. 93, pp. 7381-7386, July 1996 and Vigers, et al., *J. Biol. Chem.*, Vol 275, No 47, pages 36927-36933, 2000; and acid fibroblast growth factor binding peptide as described in Fan et al., *IUBMB Life* (2000) 49 (6) 545-48. Peptides can also be discovered using methods familiar to those skilled in the art. In order to identify a region of a protein that is involved in a specific biological function, a survey of the shorter peptide fragments making up that protein may reveal the linear peptide epitope responsible. Alternatively by surveying libraries of random peptides, a peptide that represents an optimal linear epitope or a discontinuous epitope may be discovered that mimics the activity of the natural protein. One method for selection is termed peptide phage-display. In this approach, a random peptide epitope library is generated so that peptides are present on the surface of a bacteriophage particle. These collections, or libraries, of peptides can then be surveyed for those able to bind to a specific immobilized target protein. (Pasqualini, R. et al., *j. Cell Biol.*, 130, 1995, 1189-1196; Wrighton, N.C., et al.,

Samsung Bioepis Exhibit No. 1002
Page 2004

Science, 273, 1996, pages 458-463; Cwirla, S.E., et al., *Science*, 276, 1997, pages 1696-1699; Koivunen et al, *J. Biol, Chem.*, 268, 1993, pages 20205-20210; Koivunen et al., *Bio/Technol.*, 13, 1995, pages 265-270; Healy et al., *Biochem.*, 34, 1995, pages 3948-3955; Pasqualini et al., *J. Cell Biol.*, 130, 1995, pages 1189-1196). Alternative peptide selection systems are also possible including cell surface display and ribosomal display.

Peptide mimetics used in accordance with this description are generally less than or equal to the number of amino acid residues that make up a CDR region, although they could be longer.

Any antibody can serve as a scaffold sequence, however typically human antibodies are chosen as human therapeutics is one of the ultimate objectives. Human or humanized antibodies are less likely to cause an adverse immune response in a human patient. The major criteria in selecting an antibody to serve as a framework for insertion of a peptide, is that the replacement of one or more CDRs of the antibody with the peptide must change the antigen specificity. The antibody can be a complete antibody or an Fab, scFv or $F(ab')_2$ fragment or portion thereof.

Alternatively, a library of antibodies can have one or more heavy and/or light chain CDRs replaced with a desired peptide. The resulting library can then be screened to identify antibodies having a desired activity. It should be understood that randomization with in the substituted peptide can also be provided to generate an antibody library.

A useful antibody is the anti-tetanus toxoid (TT) Fab, as it is human and because modification of the HCDR3 is sufficient to change the antigen specificity of the antibody (Barbas et al., *J. Am. Chem. Soc.*, 116, 1994, pages 2161-2162 and Barbas et al., *Proc. Natl. Acad. Sci. USA*, 92, 1995, pages 2529-2533).

Grafting of the DNA sequence of the peptide of choice into an antibody so as to replace the CDR(s) of an antibody with the peptide sequence is carried out using recombinant DNA techniques known to those skilled in the art.

Examples of methods which can be utilized to graft a desired peptide having biological activity in place of a CDR region include, but are not limited to, PCR overlap, restriction enzyme site cloning, site specific mutagenesis and completely synthetic

Samsung Bioepis Exhibit No. 1002
Page 2005

means. For a description of techniques involving overlap PCR, see, e.g., Example 1 herein. Site specific mutagenesis can be accomplished in several ways. One is based on dut/ung Kunkel mutagenesis (Kunkel, T.A., *Proc. Natl. Acad. Sci.* (1985) vol. 82, pp. 488-92). The Muta-Gene *in Vitro* Mutagenesis kit is available from BioRad based on this methodology (cat. # 170-3581 or 170-3580). Several PCR amplification based mutagenesis approaches are also commercially available such as Stratagene's QuickChange Site-Directed Mutagenesis Kit and the ExSite PCR-based Site-Directed Mutagenesis Kit. Another non-PCR method is available from Promega as the GeneEditor *in vitro* Site-Directed Mutagenesis System. Completely synthetic means are also well-known and described, e.g., in Deng, et al., *Methods Mol. Biol.* (1995) 51:329-42; Kutemeler et al., *Biotechniques*, (1994) 17(2): 242-246; Shi et al., *PCR Methods Appl.*, (1993) 3(1): 46-53 and Knuppik et al., *J. Mol. Biol.*, (2000) 11:296(1): 571-86 each incorporated herein by reference. In addition, the above methods used for replacing all or a portion of at least one CDR sequence can be utilized to graft a desired peptide into or adjacent to at least one native CDR sequence without replacing the original CDR sequence. In this manner, a CDR/biologically active peptide mimetic fusion construct is formed.

It is contemplated that flanking sequences may be added to the carboxyl and/or amino terminal ends of the biologically active peptide. Flanking sequences can be useful to reduce structural constraints on the grafted peptide to allow it to more easily adopt a conformation necessary for biological activity. In a preferred embodiment, a flanking region including a proline is covalently attached to the carboxy terminus of the biologically active peptide to create a proline extended biologically active peptide.

In one embodiment, a flanking region can be generated by randomizing two amino acid positions on each side of the peptide graft in order to determine the best sequence. In this manner, a library having members with multiple varied sequences can be generated. The resulting constructs are then tested for biological activity as described below by, e.g., panning techniques. Recombinant proteins can be generated that have random amino acids at specific positions. This can be accomplished by modifying the encoding DNA. When introducing randomization at a specific amino acid's codon position, a preferable deoxyribonucleotide "doping

Samsung Bioepis Exhibit No. 1002
Page 2006

strategy" is $(NNK)_x$ in order to cover all 20 amino acids and to minimize the number of encoded stop codons. Accordingly, N may be A, C, G, or T (nominally equimolar), K is G or T (nominally equimolar), and x is typically up to about 5, 6, 7, or 8 or more, thereby producing libraries of mono-, di-, tri-, quadra-, penta-, hexa-, hepta-, and octa-peptides or more. The third position may also be G or C, designated "S". Thus, NNK or NNS (i) code for all the amino acids, (ii) code for only one stop codon, and (iii) reduce the range of codon bias from 6:1 to 3:1. There are 32 possible codons resulting from the NNK motif: 1 for each of 12 amino acids, 2 for each of 5 amino acids, 3 for each of 3 amino acids, and only one of the three stop codons. Other alternatives include, but are not limited to:

$(NNN)_x$ which would provide all possible amino acids and all stops;

$(NNY)_x$ eliminates all stops and still cover 14 of 20 amino acids;

$(NNR)_x$ covers 14 of 20 amino acids; and

$(NNS)_x$ covers all 20 amino acids and only one stop.

The third nucleotide position in the codon can be custom engineered using any of the known degenerate mixtures. However, the group NNK, NNN, NNY, NNR, NNS cover the most commonly used doping strategies and the ones used herein.

The collection of engineered antibodies that are created during this process can be surveyed for those that exhibit properties of the peptide as, e.g., phage displayed antibodies, essentially as has been described in Barbas, C.F., III, Kang, A.S., Lerner R.A., and Benkovic, S.J., Assembly of combinatorial antibody libraries on phage surfaces: the gene III site, *Proc. Natl. Acad. Sci. USA*, 88, 1991, pages 7978-7982 incorporated herein by reference. This technology allows recombinant antibodies (as complete antibodies, Fab F(ab')$_2$, or scFv) to be expressed on the surface of a filamentous bacteriophage. That same phage will have within it the genes encoding that specific antibody.

It is contemplated that any other known method of introducing randomization into a sequence may be utilized herein. For example, error prone PCR can introduce random mutations into nucleic acid sequences (See, e.g., Hawkins et al., J. Mol. Biol, (1992) 226(3): 889-96). Briefly, PCR is run under conditions which compromise the fidelity of replication, thus introducing random mutations in sequences as those skilled

Samsung Bioepis Exhibit No. 1002
Page 2007

in the art would accomplish. After generation of such random mutants, they can be placed into phage display formats, panned and thus evaluated for activity. Likewise, particular bacteria known to provide random mutations of genes, such as Epicurian Coli® XL1-Red Competent cells (commercially available from Stratagen, La Jolla, CA.), which do so during plasmid replication can be utilized to provide random mutants which are then screened for biological activity in accordance with the present disclosure.

It is also contemplated that randomization may be introduced at any point in the nucleotide sequence after incorporation of an active peptide into the antibody or fragment thereof to alter the overall biological activity of the antibody. In this manner, not only can alterations be made in the biological activity of a peptide mimetic by causing mutations within the peptide's sequence, but mutations in the surrounding scaffold can be incorporated with the resulting constructs being assayed for alterations in biological activity or expression. Indeed, it is contemplated that libraries having repertoires of multiple constructs resulting from such randomization can be generated and assayed.

Single chain libraries can be utilized in accordance with the present disclosure because an entire binding domain is contained on one polypeptide. The light chain variable region is separated from heavy chain variable region by a linker region. The use of short linkers (< 11 amino acids) favors a dimeric complex where V_H of one ScFv associates with V_L of another ScFv molecule and visa versa, these molecules are termed diabodies (Kortt, A.A., Malky, R.L., Caldwell, J.B., Gruen, L.C., Ivanci, N., Lawrence, M.G. et al. *Eur. J. Biochem.* 221:151-157, 1994). This is because folding of monomeric ScFv is impaired with linkers < 11 amino acids (Alfthan, K., Takkinen, K., Sizman, D., Soderlund, H., and Teeri, T.T. *Protein-Eng.* 8:725-731, 1995). Longer linkers (> 11 amino acids) favors folding of monomeric ScFv into a single antigen binding domain, thus precluding dimer formation.

One useful phage display vector is pRL4 which is also known as pComb 3X (see Fig. 1). This vector enables display of chimeric expression products on the surface of packaged phagemid particles. pRL4 is a modified version of pComb3H (Barbas, C.F. III and Burton, D.R. 1994. Monoclonal Antibodies from Combinatorial

Samsung Bioepis Exhibit No. 1002
Page 2008

Libraries. Cold Spring Harbor Laboratory Course Manual, Cold Spring Harbor, N.Y.; Burton, D.R.; Barbas, C.F. III. *Advances in Immunology* 57:191-280, 1994; Lang, I.M., Chuang, T.L., Barbas, C.F. 3rd, Schleef, R.R. *J. Biol. Chem.* 271: 30126-30135, 1996; Rader and Barbas, Phage Display, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2000). The design of pRL4 allows for dimerization of scFv antigen binding domains on the phage surface and in soluble form as detailed below. When the plasmid is transformed into a *supE* bacterial host such as ER2537 (F' *Sup E*, New England Biolabs, Beverly, MA), the amber mutation is suppressed approximately fifty percent of the time. In this way half of the expressed scFvs are fused with the filamentous phage gene III protein (amino acids 230-406) and the other half will be terminated just prior to gene III to produce soluble scFv. Both the scFv-pIII fusion and soluble scFv products have the *Omp A* signal sequence and will be transported to the periplasm where they will be able to form dimeric scFv complexes, termed diabodies (Kortt, A.A., Malby, R.L., Caldwell, J.B., Gruen, L.C., Ivanci, N., Lawrence, M.C. et al. *Eur. J. Biochem.* 221: 151-157, 1994). Diabodies are expected to fold such that the V_H of one scFv will pair with the V_L of a second scFv-pIII resulting in divalent antibody fragments. In a non-*sup E* host, such as TOP1OF' (InVitrogen, Carlsbad, CA), the amber stop codon is recognized yielding soluble scFv diabodies.

In the final single chain expression construct in pRL4, the single chain antibody fragments are cloned downstream of the *E. coli* lacZ promoter, ribosome binding site, and *omp* A leader sequence. These elements allow induction of expression by IPTG, and the secretion out of the cell via the *omp* A leader sequence when expressed in the suppressor strain ER2537. The single chain fragments are fused in frame with filamentous phage gene III (gIII) sequences (amino acids 230-406). The gIII protein product, pIII, is a minor coat protein necessary for infectivity. Upon promoter induction by IPTG, the single chain antibody-pIII fusion is synthesized and transported to the bacterial periplasmic space. In the periplasmic space, the scFv-gene III fusion proteins are inserted into the membrane. Upon superinfection with helper phage, these fragments are exported out of the cell on the surface of phage as pIII-antibody

Samsung Bioepis Exhibit No. 1002
Page 2009

fragments. Other possible proteins to be used for fusion on the surface of phagemids include filamentous coat protein pVIII and other coat proteins.

Fab fragment libraries, that maintain the native antigen recognition site, are useful to ensure that affinity is maintained.

In the final hybrid Fab expression construct in pRL4, the light and heavy chains are cloned as a single SfiI fragment. In this way, the light chain fragments are cloned downstream of the *E. coli* lacZ promoter, ribosome binding site, and *omp* A leader sequence. These elements allow induction of expression by IPTG, and secretion out of the cell via the *omp* A leader sequence. The light chain fragments are followed by a stop codon, a second ribosome binding site, the *E. coli pel* B leader sequence and heavy chain. Hybrid heavy chain genes are fused in frame with filamentous phage gene III (gIII) sequences (amino acids 230-406). An amber stop codon is present at the fusion junction. In a *sup E* bacterial host such as ER2357 (New England Biolabs, Beverly, MA), the amber mutation is suppressed. Upon promoter induction, a single polycistronic message is transcribed and translated as two polypeptides, a light chain and a heavy chain-gene III fusion protein. Following synthesis the polypeptides are transported to the bacterial periplasmic space as directed by the leader sequences. In the periplasmic space the heavy chain-pIII fusion proteins are inserted into the membrane, and the light and heavy chains are associated covalently through disulfide bonds, forming the antigen binding sites. The human constant region CH1 and C_L sequences include the cysteines that form the disulfide bond between heavy and light chains. Upon superinfection with helper phage, these fragments are exported out of the cell on the surface of phage as Fab-cpIII fusion. In a non-*sup E* host, such as TOP1OF' (Invitrogen, Carlsbad, CA), the amber stop codon is recognized yielding soluble Fab fragments. Important features of the pRL4 phage display system used include a purification His 6 tag, an HA epitope tag following the heavy chain, as well as a suppressible amber stop codon which is located between the heavy chain and the phage gene III. The HA tag is recognized by HA.11 antibody (Babco, Berkeley, CA) and 12CA5 antibody (Roche Molecular Biochemical, Indianapolis, Ind.). The His6 tag allows affinity purification of antibody fragments by Nickel-chelate chromatograph (Qiagen, Valencia, CA). The amber stop allows for quick conversion from a fusion

Samsung Bioepis Exhibit No. 1002
Page 2010

Fab-cpIII product (for incorporation on the phage coat) when the stop is suppressed, to the soluble Fab which is made in a non-suppressor bacterial host.

Selection involves isolating from the library the best candidates that specifically bind to the peptides target molecule and display biological activity.

The phage expressing antibody fragments on their surface can be produced and concentrated so that all members of a library can be allowed to bind to the target molecule. The target molecule can be immobilized on a microtiter dish, on whole cells, the membranes of whole cells, or present in solution. Non-specific Ab-phage are washed away, and bound phage particles are released from the antigen, often by the use of low pH. The recovered Ab-phage are infectious and so can be amplified in a bacterial host. Typically, multiple rounds of this sort of selection are performed. Individual antibody fragment clones can then be analyzed as soluble Fabs or scFvs for identification of those that specifically recognize the target molecule.

Prior to any selection strategy, initial libraries are electroporated into host cells, such as ER2537. Library cultures are grown to log phase and superinfected with helper phage, such as VCSM13, a commercially available helper phage (Stratagene, La Jolla, CA). Superinfection provides the remaining phage components needed for packaging plasmids into phagemid particles. Alternatively, phage display without the use of helper phage may be utilized. Following overnight growth, phagemids in the culture supernate are precipitated with polyethylene glycol (PEG). PEG precipitated phage are used in panning (solid phase cell surface, internalization and membrane), FACS sorting, or magnetic sorting to purify specific binding antibodies from non specific binders.

In cell based panning, antibody-phage libraries are incubated with target cells, and the non-adherent phage are removed with multiple washes. A typical panning protocol is as follows:

1. Block phage particles with PBS + 1%BSA or 10% FBS + 4% milk powder + NaN_3, (except when internalized antibodies are assayed).

2. Add target cells to blocked phages (approximately 5 X 10^6 cells).

3. Mix and rotate slowly at 4° C or 37 ° C.

Samsung Bioepis Exhibit No. 1002
Page 2011

4. Wash cells twice with 1 ml ice cold PBS/1%BSA/NaN_3 or room temperature PBS/1%BSA/NaN_3.

5. Specific antibody-phage bound to cells can be eluted by low pH, for example with 76 mM citric acid ph 2.5 in PBS for 5 to 10 minutes at room temperature.

6. Neutralize eluted phage with 1M Tris-HCl pH 7.4.

7. After neutralization, antibody-phage can be used to infect ER2537 bacteria and amplify during overnight growth for the next round of panning.

Generally, 3-4 rounds of panning are performed on each library. Phage ELISAs using commercially available secondary antibody (sheep anti-M13 antibody-HRP) or soluble antibody ELISAs using a commercially available HA. 11 antibody (Babco, Berkeley, CA) that recognizes the HA tag incorporated into each antibody from PRL4 sequences, can be performed following each round of panning to allow estimation of the enrichment of binding antibodies over non-binders. Following the last round of panning, the antibody-phage can be picked as single colonies from agar plates, grown as monoclonal antibody-phage and screened by ELISA for identification of specific binders. FACS analysis may also be utilized. Specifically the antibody-phage are infected into Top10F' bacteria and plated for single colonies. Single colonies are picked form agar plates, grown and induced with IPTG. Soluble antibody is screened by ELISA for identification of specific binders. Screening can be done against live cells, against intact, mildly fixed target cells, or recombinant protein(s).

Methods for whole cell panning have been described previously (Siegel, D.L., Chang, T.Y., Russell, S.L., and Bunya, V.Y. 1997. *J. Immunol. Methods* 206:73-85 incorporated herein by reference). Other techniques for selection which can be applied include fluorescent activated cell sorting (FACs). Alternative methods for selection using libraries include, but are not limited to, ribosome display and plaque hybridization to a labeled antigen.

Following panning to isolate high affinity antibody binders, bioassays for functional screens of agonist antibodies can be carried out. Dimerization is often a prerequisite for activation of many receptors and thus bioassays focus on agonist antibodies that stimulate receptors via promotion of dimerization. As previously described, single chain multivalency is approached in linker design. Fab fragment

Samsung Bioepis Exhibit No. 1002
Page 2012

multivalency can be approached in a number of ways. A number of recent reports in the literature have shown success in dimeric antibody fragment formation which is applicable to phage display (DeKruif, J., and Logtenberg, T. 1996. *J. Biol. Chem.* 271:7630-7634, Pack, P., and Pluckthun, A. 1992. *Biochemistry* 31:1579-1584, and Holliger, P., and Winter, G. 1993. *Current Opin. Biotech.* 4:446-449). Divalent Fabs can be created in at least two ways. In one approach dimerization is achieved by addition of a dimerization domain to pRL4, forming pRL8 (See Figs. 6A-C, 7 and 8). There are a number of dimerization domains (lexA, Zn fingers, fos, jun etc.) that can be utilized in these vectors to obtain multivalency of Fab fragments. Dimerization domains are selected from, but not limited to, the following: jun (DeKruif, J. and Logtenberg, T. *J. Biol. Chem.* 271:7630-7634, 1996; Kostelny, S.A., Cole, M.S., and Tso, J.Y. *J.Immunol.* 148:1547-1553, 1992) the LexA dimerization region (Kim, B. and Little, J.W. *Science* 255:203-206, 1992), the yeast GCN4 dimerization domain (van Heeckeren, W.J., Sellers, J.W., Struhl, K. *Nucleic Acids Res.* 20:3721-3724,1992), Gin invertase from the bacteriophage Mu (Spaeny-Dekking, L., Schlicher, E., Franken, K., van de Putte, P., Goosen, N. *J. Bacteriol.* 34:1779-1786, 1995), *E. coli* NTRC protein dimerization domain (Klose, K.E., North, A.K., Stedman, K.M., Kustu, S. *J. Mol. Biol.* 241:233-245, 1994), and HSV-1 ICP4 dimerization domain (Gallinari, P., Wiebauer, K., Nardi, M.C., Jiricny, J. *J. Virol.* 68:3809-3820, 1994) all incorporated by reference. Also, a high temperature dimer domain from thermus organisms can be utilized (MacBeath, G., Kast, P., Hilvert, D., *Biochemistry* 37:100062-73, 1998 and MacBeath, G., Kast, P., Hilvert, D., *Science* 279:1958-61, 1998). These are functional domains that when incorporated into a molecule allow for dimerization to occur. In addition, dimerization can be achieved in cells through the use of full IgG vectors, or dimerization domains such as CH3 dimerization domains. Those of ordinary skill in the art are familiar with these and other dimerization domains and their use to dimerize proteins.

Additional methods that may be utilized to generate antibody constructs which contain at least two binding sites are known. The antibody or antibody fragments created by each of these approaches could be utilized for testing agonistic antibody activity as described in Example 1 below for whole IgG produced in

Samsung Bioepis Exhibit No. 1002
Page 2013

mammalian cells. These methods include chemical dimerization of Fab, pegylation of Fab, production of Fab'2, generation of whole IgG in bacterial cells, and use of diabodies (scFvs). Importantly, any of the antibody forms generated for analysis of agonistic activity could be used as the final therapeutic product.

Chemical dimerization may be also achieved using a variety of chemical crosslinking reagents. For example, SMCC (Succinimidyl trans-4 (maleimidylmethyl) cyclohexane-1-carboxylate) from Molecular Probes (Eugene, Oregon), Cat # S-1534. This reagent will modify primary amino groups in the antibody. After incubating the antibody with the SMCC at room temperature, the reaction is run over a PD-10 column. This maleimide derivitized Fab can be added to either a second Fab or a separate batch of the same Fab that has been treated with TCEP [(Tris (2-carboxyethyl) phosphine, hydrochloride): Molecular Probes Cat #T-2556] to reduce the thiol groups to SH. The reduction reaction is carried out in the dark for 15 minutes. The conjugation of the maleimide Fab and the thiol reduced Fab occurs at a 1:1 ratio. Dimers are isolated by passing the reaction over a sephadex 200 gel filtration column. Other chemical linkers known to those skilled in the art may be used for dimerization. Production of an Fab' that has an extra cysteine residue engineered into the hinge region has been described, e.g., in U.S. Patent 5,677,425 and Carter, et al.,*BioTechnology*, Vol 10, Feb. 1992, pages 163-167 the disclosures of which are hereby incorporated by reference. That thiol site can be used for conjugation to moieties such as polyethyleneglycol (PEG). Pegylation technology is known, for example, see Koumenis et al, Int. J. Pharm. (2000) 198(1): 83-95, incorporated herein by reference, which makes it possible to link two Fab' molecules together using PEG coupling.

Technology for bacterially producing Fab'2 involves cloning the human IgG hinge region, and optionally part of the CH2, as part of the Fd which includes additional cysteines and is described, e.g., in Better, et al., PNAS USA (1993) 90(2): 457-61, incorporated herein by reference. The additional thiol groups on the Fd hinge can interact and cause two Fab' molecules to dimerize, creating a Fab'2. Fab'2 can be purified directly from the bacterial cells. Additionally, undimerized Fab' from the bacteria can be isolated and chemically converted to Fab'2.

Samsung Bioepis Exhibit No. 1002
Page 2014

As described earlier, the variable regions of the antibody can be cloned as a single chain wherein the variable light (VL) is connected to the variable heavy (VH) by a linker region. If that linker region is short (for example 5-7 amino acids), the folding of the scFv will favor association of two scFvs where the VL of one scFv paris with the VH of the second scFv. In this manner, two antigen binding sites are presented on the diabody.

Antibody constructs which contain two binding sites may be generated using any of these methods in order to test agonist activity and/or be used as the final therapeutic product.

Following the panning or sorting steps of Fab libraries, the library of panned molecules are restricted with Sac I and *Spe* I and cloned into pRL8. Subcloning to pRL8 vector individually or *en masse* following FACS sorting or panning allows expression of dimeric soluble binding Fabs for analysis in bioassays. In pRL8, the antibody fragments are transported to the periplasmic space and form dimers there. The advantage of this approach is that it permits panning of monomeric Fab fragments, favoring high affinity Fabs.

Another approach uses a secondary antibody. pRL4 has the hemagglutinin decapeptide tag recognized by the commercially available HA. 11 antibody (Babco, Berkeley, CA). Fabs identified in FACS sorting or panning to be tested in bioassay are preincubated with HA. 11 which will promote dimerization, prior to addition to bioassays.

Once binding scFv's or Fabs are identified by panning or another selection method, the individual clones, each expressing a unique dimerized antibody fragment on the phage surface are tested for proliferation, differentiation, activation or survival effects on target cells. In addition, soluble dimerized antibody are examined in bioassays.

Biological Assays for Screening for TPO-like Activity

1. Colony formation assays - Megakaryocytic colonies from bone marrow (Megacult C Kit from Stem Cell Technologies Inc., Vancouver BC, Canada).

Samsung Bioepis Exhibit No. 1002
Page 2015

2. Proliferation assays - proliferation of Ba/F3 cells (Cwirla et al. 1997, Science, Vol. 276 pages 1696-1699). The Ba/F3-mpl cell line was established (F. de Sauvage *et al.*, Nature, 369:533 (1994)) by introduction of the cDNA encoding the entire cMpl receptor into the IL-3 dependent murine lymphobiastoid cell line Ba/F3. Stimulation of proliferation of Ba/F3-mpl cells in response to various concentrations of antibodies or TPO was measured by the amount of incorporation of ^{3}H-thymidine as previously described (F. de Sauvage *et al., supra).*

3. Phosphorylation assays - phosphorylation of JAK2 (Drachman et al., J. Biol. Chem., (1999), Vol. 274, pages 13480-13484).

4. Transcriptional based assays - Transiently co-transfect full length cMpl receptor with c-Fos promoter luciferase reporter construct. 24 hour post transfection starve the cells in 0.5% FCS for 24 hours. Stimulate the cells, harvest after 6 hours and take luciferase readings (see also Example 1, Biological Assays section).

Biological Assays for Screening for EPO-like Activity

1. Bone marrow erthroid colony formation in Methylcellulose (Wrighton et al., Science, 1996, Vol. 273 pages 458-463).

2. TF-1 cell (Human erythroleukemia cell line) proliferation. TF-1 cells express both full length and a truncated form of the Epo-R. (J.Cell Physiol., 1989, Vol 140, pages 323-334).

3. The EPO receptor couples directly to JAK2 kinase to induce tyrosine phosphorylation. Epo induces cFos in TF-1 cells. c-Fos transcriptional activation. (Witthuhn et al., Cell, (1993), Vol. 74, pages 227-236).

A number of bioassays can be used in high-throughput screening. Those of ordinary skill in the art are familiar with these and other suitable bioassays. Several non-radioactive assays have been developed in which either DNA synthesis or enzyme activity can be analyzed. For example, an MTT cell proliferation assay (Promega Corporation, Madison, WI) that is based on an assay described by Mosmann (Mossman, T. 1983. *J. Immunol. Methods* 65:55-57 incorporated herein by reference) can be used. This protocol is fast and easy. In the assay, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), a tetrazolium salt, is converted

Samsung Bioepis Exhibit No. 1002
Page 2016

into a blue formazan product by mitochondrial dehydrogenase activity in living cells. The dehydrogenase content, and therefore the amount of colored product produced, is proportional to cell number. The colored product is detectable in an ELISA plate reader at 570nm. Assays are performed in triplicate, *en masse* in 96 well microtiter plates. Briefly, target cells are plated in 100 µl aliquots in culture medium in 96-well plates. Following addition of various concentrations of antibodies, cells are incubated for 48-72 hours at 37°C and 5% CO_2 in a fully humidified atmosphere. MTT is added to each well, and proliferation monitored via ELISA plate reader.

For example, in proliferation assays using TF-1 cells, bacterial cells containing phagemids expressing antibodies are grown overnight at 37°C in 96 well deep well plates in 1 ml of a media that is a mixture of mammalian cell media and bacterial media (in the case of TF-1 cells: RPMI 2.7/SB 0.3/Carb 100ug/ml). TF-1 cells are a human bone marrow erythroleukemia cell line that responds to multiple cytokines (Kitamura, T., Tange, T., Terasawa, T., Chiba, S., Kuwaki, T., Miyagawa, K., Piao, Y.F., Miyazono, K., Urabe, A., Takaku, F., *Cell Physiol.* 140:323-334, 1989; Kitamura, T., Tojo, A., Kuwaki, T., Chiba, S., Miyazono, K., Urabe, A., Takaku, F., *Blood* 73:375-380, 1989; Kitamura, T., Takaku, F., Miyajima, A., *Int. Immunol.* 3:571-577, 1991) On the following day, the overnight cultures are subcultured 1/10 to fresh trays, and placed at 37°C for 2 hours. Following induction with IPTG at 37°C for 4 hours, the plates are centrifuged at 2000 rpm/15' at room temperature. 50 ul each culture supernate are filtered in 96 well filter trays (Millipore) to sterile 96 well assay plates. Mammalian cells are prewashed to remove growth factor and resuspended at a concentration of 1 X 10^5 cells/ml. 50 ul cells are added to each well. Assay plates are incubated in 37°C/5% CO_2 incubator for 72 hours. At 72 hours, the trays are developed by adding 40 ul media/MTS/PMS per well. MTS is an improved more soluble version of MTT. Both assays are based on the cellular conversion of tetrazolium salt. A MTS proliferation assay kit (catalogue number G5421) can be purchased from Promega, Inc. (Madison, WI). Plates are kept at 37°/CO_2 incubator and read at OD_{490} at 1 hour, 4 hours, 8 hours with microplate reader.

The activities of cytokines are often synergistic. Synergy could be manifested through the binding of ligands to two different receptors which then sends the correct

Samsung Bioepis Exhibit No. 1002
Page 2017

signal, or via a priming effect whereby interaction of ligand/receptor primes the cell to respond to a second cytokine. Furthermore, cytokines that act early in lineage development are more often synergistic than cytokines that act at later stages in a developmental pathway. Therefore, suboptimal concentrations of growth factors can be used in these bioassays to examine synergism. Conditions for suboptimal concentrations are determined for each assay. This is done by adding serial dilutions of growth factors, individually and as a mixture, to the assays and determining the levels below which a single factor does not promote a response compared to the mixture, and the level below which the mixture does not promote a response in the bioassay. Bone marrow stromal cells can also be added in bioassays to provide other necessary factors that may play a role in a synergistic response.

In addition, cell proliferation can be examined by monitoring DNA synthesis. A non-radioactive, colorimetric assay that examines 5-bromo-2'-deoxy-uridine (BrdU) incorporation (Roche Molecular Biochemicals, Indianapolis, IN) can be performed in microtiter plate format. Here, cells are cultured in 96-well plates and incubated with BrdU and sub-optimal concentrations of cytokines. The amount of BrdU is determined after labeling with a peroxidase labeled anti-BrdU antibody. Final results are analyzed by ELISA plate reader at 405nm.

A radioactive mitogenesis assay that measures the rate of DNA synthesis as an indication of proliferation (Raines and Ross, *Methods of Enzymol.* 109: 749-773, 1985) can also be used. In these assays, changes in rate of incorporation of [^{3}H]-thymidine in target cells is examined. Again, these assays permit concurrent and rapid screening of many antibody fragments. They have been widely used as a convenient method of assessing the stimulatory and inhibitory effects on the growth of many different cells. Cells are cultured in suspension until they reach exponential growth rate. Cells are then washed free of the medium in which they were cultured, and replated in fresh medium. Cells are aliquoted into 96 well plates in a total volume of 100 ul at a concentration of about 1-2 x 10^5 cells/ml. Dilutions of phage supernatant, soluble dimerized Fab or ScFv antibodies are added and cells are incubated for 18-48 hours in a gassed CO_2 incubator at a temp of 37°C. Following incubation, [^{3}H]thymidine (937kBq) is added to each well and incubated for a further 4 hours. The

Samsung Bioepis Exhibit No. 1002
Page 2018

cells are then removed from the incubator and counted directly in a bench top microplate scintillation counter such as Packard Top Count NXT Instrument (Packard, Meriden, CT). Alternatively cells can be serially transferred to GF/C filters on a Millipore cell harvester (Millipore, Bedford, MA) or similar apparatus. Radioactivity associated with acid-insoluble material retained on the filter is then determined. Dilutions of commercially available growth factors are applied to positive control wells. Negative controls would include supernatants from cells carrying non-insert containing plasmids or irrelevant antibodies treated similarly. The relative growth promoting activities of the standard and the diluents of the phage supernatants under test are compared to quantify the growth promoting activity in the sample.

Activation can be tested for by assaying second messengers or by transcriptional readout assays.

Survival can be assayed, for example, by monitoring apoptosis using assays such as tunnel assays or by other methods known to those who practice the art.

Other useful assays to analyze cellular signal transduction, the activity of kinases and phosphatases and ultimately cellular activities as a result of agonist activity include measurement of the generation of second messengers, e.g. cAMP, Ca+ +, diacylglycerol (DAG), and inositol 1,4,5-triphosphate (IP3). Measurement of spikes in intracellular calcium concentration, intracellular pH and membrane potential in high throughput screening assays can be performed using instruments such as the FLIPR Fluormetric Imaging Plate Reader System (Molecular Devices, Sunnyvale, CA). A number of fluorescent probes are available for examination of second messenger concentrations (Molecular Probes, Eugene OR). Measurement of concentrations of second messengers can also be done on the single cell level (DeBernardi, M.A. and Brooker, G. *Proc. Natl. Acad. Sci USA* 93:4577-4582, 1996). In addition, assays that examine other signaling events such as phosphorylation, apoptosis or levels of RNA or protein of specific genes would be useful. For example. most cytokines have been shown to activate the enzyme PI 3-K (reviewed in Silvennoinen, O., Ihle, J.N. Signaling by the Hematopoietic Cytokine Receptors, R.G. Landes company, Austin, TX 1996). Furthermore, the Jak family of tyrosine kinases have been shown to be central mediators for cytokine receptor signaling (Ihle, J.N., Witthuhn, B.A., Quelle, *F. W.*

Samsung Bioepis Exhibit No. 1002
Page 2019

Annu. Rev. Immunol. 13:369-398, 1995). In addition, several other tyrosine kinases, e.g., members of the Src family, are activated in response to certain cytokine stimulations. In the case of RNA or proteins, e.g., c-Jun and c-Fos are rapidly and transiently upregulated upon cytokine stimulation, while c-Myc induction is slower. These proteins are required for G1 transition and proliferation (reviewed in Silvennoinen, O., Ihle, J.N. Signaling by Hematopoietic Cytokine Receptors, R.G. Landes Company, Austin, TX 1996). High throughput screens that detect increases in these transcripts could be utilized.

In transcriptional read out assays, changes in the transcription of specific genes are observed following exposure of cells to a growth factor ' or growth factor mimetic (agonist or inhibitory antibody). For example, in a myc read-out assay, cells such as IL-3 dependent FDCP-mix cell line is starved of IL-3 growth factor for 8 hours, followed by exposure to growth factor mimetics, or native growth factors for 2 hours at 37°C. At this time, the cells are harvested, RNA is isolated, and reverse transcriptase-polymerase chain reactions (RT-PCR) are performed with primers specific for the myc gene. The RT-PCR reactions are electrophoresed in horizontal agarose gels for quantitation of PCR product. In this case expression of a single gene is being monitored.

Alternatively assay for changes in expression of genes can be monitored using CHIP technology, agonist antibodies could be identified under conditions of high probe sensitivity and a dynamic range. In this way, up to 10,000 or more could be analyzed for changes in expression. Desired genes that could be monitored could include c-myc, c-jun, NF-κB, among others. These genes are downstream of various signal transduction pathways and their expression should change upon a mitogenic response. In one type of commercially available CHIP (Affymetrix, Santa Clara, CA), oligonucleotides from desired test genes can be printed out onto glass surface. Target cells are exposed to test agonist antibodies. RNA is isolated from the cells exposed to test agonist antibodies, copied to cDNA, and *in vitro* transcribed in the presence of biotin. Hybridization of *in vitro* transcribed, biotinylated mRNA is used as probe in the arrays. Chips are then scanned to determine genes that show increases in transcription upon exposure to test agonist antibodies. In another version of CHIP

Samsung Bioepis Exhibit No. 1002
Page 2020

technology (Incyte, Palo Alto, CA), the amount of DNA is not normalized on the glass, therefore, one would set up a competitive hybridization. RNA is isolated from the cells before and after exposure to agonist. cDNA is made from each sample whereby one cDNA reaction has one label incorporated, for example, Cy-3, and the other cDNA population has a different label incorporated, for example Cy-5. Signals are detected and compared on a dual laser scan to collect images.

Visual assays can also be used such as traditional methylcellulose colony forming assays (Stem Cell Technologies, Vancouver BC, Canada). In these assays, colony growth, and morphological changes are scored via light microscope. Visual examination for proliferation or differentiation effects in semi-solid agar cultures or methylcellulose can be performed using the appropriate cell line. Williams Hematology 5 (eds. E. Beutler, M.A. Lichtman, B.S. Coller L T.J. Kipps), McGraw-Hill, Inc., pp L22-L26, 1995). Addition of methylcellulose allows clonal progeny of a single progenitor cell to stay together and facilitates the recognition and enumeration of distinct colonies. All necessary components are added to a basic methylcellulose medium (such as Iscove's MDM, BSA, (-mercaptoethanol, L-glutamine) except colony-stimulating factor supplements and test antibodies (phage supernatants, soluble antibodies) are added to see if they can substitute for growth factors. Cells in methylcellulose culture are incubated for 10-12 days following the addition of antibodies in a 37°C humidified atmosphere of 5% CO_2 in air. After 10-12 days of incubation, colonies are counted using an inverted microscope. After another 8-10 days, colonies are counted again. Comparisons are made between media containing antibodies and controls with and without growth factors. In addition, colonies can be picked from methylcellulose and individual cells examined cytologically by staining with Wright's stain (see Atlas of Hematological Cytology, F.G.J. Hayhoe and R.J. Flemans, Wiley-InterScience 1970).

The receptor-binding affinities of antibody fragments can be calculated (Lfas & Johnson, 1990) from association and dissociation rate constants measured using a BIACORE surface plasmon resonance system (Pharmacia Biosensor). A biosensor chip is activated for covalent coupling of gD-mpl receptor using N-ethyl-N'''-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide

Samsung Bioepis Exhibit No. 1002
Page 2021

(NHS) according to the supplier's (Pharmacia Biosensor) instructions. gD-mpl is buffer-exchanged into 10 mM sodium acetate buffer (pH 4.5) and diluted to approximately 30 µg/mL. An aliquot (5 µL) is injected at a flow rate of 1 µL/min to achieve approximately 400 response units (RU) of coupled protein. Finally, 1M ethanolamine is injected as a blocking agent. For kinetics measurements, 1.5 serial dilutions of antibody are injected in PBS/Tween buffer (0.05% Tween-20 in phosphate buffered saline) at 25°C using a flow rate of 20 µL/min. Equilibrium dissociation constants, K_d s, from SPR measurements are calculated as k_{off}/k_{on}. Standard deviations, s_{on} for k_{on} and s_{off} for k_{off}, are typically obtained from measurements with > 4 protein concentrations (k_{on}) or with > 7 protein concentrations (k_{off}). Dissociation data are fit to a simple AB->A+B model to obtain koff +/- s.d. (standard deviation of measurements). Pseudo-first order rate constant (ks) are calculated for each association curve, and plotted as a function of protein concentration to obtain kon +/- s.e. (standard error of fit).

For conversion of antibody clones into full IgGs, the coding regions for both the light and heavy chains, or fragments thereof, can be separately cloned out of a bacterial vector and into mammalian vector(s). A single vector system, such as pDR1 or its derivatives, can be used to clone both light and heavy chain cassettes into the same plasmid. Alternatively, dual expression vectors where heavy and light chains are produced by separate plasmids can be used. Mammalian signal sequences need to be either already present in the final vector(s) or appended to the 5' end of the light and heavy chain DNA inserts. This can be accomplished by initial transfer of the chains into a shuttle vector(s) containing the proper mammalian leader sequences. Following restriction enzyme digestion, the light chain and heavy chain regions, or fragments thereof, are introduced into final vector(s) where the remaining constant regions for IgG1 are provided either with or without introns. In some cases where introns are used, primer design for PCR amplifying the light and heavy chain variable regions out of pRL4 may need to include exon splice donor sites in order to get proper splicing and production of the antibodies in mammalian cells.

With either vector expression system (single or dual plasmid), the production of antibody heavy and light chains can be driven by promoters that work in mammalian cells such as, but not limited to, CMV, SV40, or IgG promoters. Additionally, the

Samsung Bioepis Exhibit No. 1002
Page 2022

vector(s) will contain a selectable marker for growth in bacteria (such as, but not limited to, ampicillin, chloramphenicol, kanamycin, or zeocin resistance). Selectable markers for mammalian cells (such as, but not limited to, DHFR, GS, gpt, Neomyocin, or hygromyocin resistance) may also be present in the IgG vector(s), or could be provided on a separate plasmid by co-transfection.

Those of ordinary skill in the art using known techniques would be able to synthesize antibodies in other organisms such as yeast, mammalian, insect, and plants (Carlson, J.R. and Weissman, I.L., *Mol. Cell. Biol.,* 8:2647-2650, 1988; Trill, J.J., Shatzman, A.R., Ganguly, S. *Curr. Opin. Biotechnol.* 6:553-560, 1995; Hiatt, A., Cafferkey, R. Bowdish, K. *Nature* 342: 76-78, 1989).

As stated previously, antibodies made in accordance with the disclosure herein provide increased half-life (duration of action) to the activity of small peptides or peptide mimetics such as the TPO mimetic described herein. In another aspect, the serum half-life of an antibody can itself be prolonged by making derivatives that are pegylated. See, e.g., Lee, et al., Bioconjug. Chem (1999) 10(6): 973-81, incorporated herein by reference. Another advantage, e.g., of the TPO mimetic antibody described herein is that normal TPO treatment may result in generation of TPO neutralizing antibodies in patients which interfere with the activity of a patient's naturally occurring TPO. The present TPO mimetic antibody substantially reduces the likelihood that a detrimental immune response will be produced toward native TPO because it has a different amino acid sequence.

The molecules encompassed by the claims can be used in diagnostics where the antibodies or fragments thereof are conjugated to detectable markers or used as primary antibodies with secondary antibodies that are conjugated to detectable markers. Detectable markers, include radioactive and non-radioactive labels and are well-known to those with skill in the art. Common non-radioactive labels include detectable enzymes such as horseradish peroxidase, alkaline phosphatase and fluorescent molecules. Fluorescent molecules absorb light at one wavelength and emit it at another, thus allowing visualization with, e.g., a fluorescent microscope. Spectrophotometers, fluorescence microscopes, fluorescent plate readers and flow sorters are well-known and are often used to detect specific molecules which have

Samsung Bioepis Exhibit No. 1002
Page 2023

been made fluorescent by coupling them covalently to a fluorescent dye. Fluorochromes such as green flurorescent protein, red shifted mutants of green fluorescent protein, amino coumarin acetic acid (AMCA), fluorescein isothiocyanate (FITC), tetramethylchodamine isothiocyanate (TRITC), Texas Red, Cy3.0 and Cy5.0 are examples of useful labels.

The molecules can be used in cell isolation strategies such as fluorescence-activated cell sorting (FACS) if fluorescent markers are used, In fluorescence-activated cell sorting, cells tagged with fluorescent molecules are sorted electronically on a flow cytometer such as a Becton-Dickinson (San Jose, California) FACS IV cytometer or equivalent instrument. The fluorescent molecules are antibodies that recognize specific cell surface antigens. The antibodies are conjugated to fluorescent markers such as fluorescein isothiocyanate (FITC) or Phycoerythrin (PE).

Magnetic sorting is also possible. In magnetic sorting procedures, the antibody is linked directly or indirectly to magnetic microbeads. Cells are precoated with antibodies that recognize cell surface molecules, e.g., receptors involved in proliferation, differentiation, activation or survival. The antibodies are attached to magnetic beads conjugated with a secondary immunoglobulin that binds to the primary antibody displaying the peptide, such as to the HA molecular tag engineered into each antibody. The cells are then removed with a magnet. Magnetic sorting can be positive selection where cells of interest are bound by the antibody and hence the magnet, or negative selection where undesired cells are isolated onto the magnet.

Alternatively, radiolabeled antibodies can be used for diagnostic purposes.

Antibodies and fragments thereof disclosed herein are useful for the amplification of a variety of clinically relevant cell types. Treatment can be *in vivo* or *ex vivo*. For example, agonist antibodies are useful to treat patients suffering from a deficiency in a cell population caused by disease, disorder or treatment related to for example suppression of hematopoiesis where less than the normal number of cells of a given lineage or lineages are present in a patient. The following represent only some examples of the conditions that can be treated with the antibodies containing biologically active peptides disclosed herein, those who practice the art would be able to identify other diseases and conditions that would benefit from such treatment. For

Samsung Bioepis Exhibit No. 1002
Page 2024

example, HIV-infected patients, patients undergoing chemotherapy, bone marrow transplant patients, stem cell transplant patients, and patients suffering from myeloproliferative disorders show subnormal levels of specific hematopoietic lineages.

Thrombocytopenia can be a result of chemotherapy, bone marrow transplantation or chronic disease such as idiopathic thrombocytopenia (ITP) which all result in low platelet levels. The present TPO mimetic antibodies can be used to treat such patients.

Patients undergoing renal dialysis often suffer from treatment related anemia with subnormal levels of red blood cells. In aplastic anemia, bone marrow suppression can cause pancytopenia or may affect only the red blood cells, the white cells, or the platelets. The disclosed antibodies will augment the armamentarium of therapeutic agents for these and other diseases and disorders characterized by deficiencies in specific cell populations, such as hematopoietic cells.

The molecules encompassed by the claimed invention can also be used for *ex vivo* proliferation and differentiation of cells. This is useful for gene therapy purposes, for example for traditional viral vector approaches, and for autologous bone marrow transplants.

In addition, certain antibodies in accordance with the present disclosure can be radiolabeled for radioimmunotherapy or conjugated to toxins to deliver such toxins to specific cell types and result in the killing of those cells.

A biologically active *c-mpl* agonist antibody capable of stimulating proliferation, differentiation and maturation of hematopoietic cells may be used in a sterile pharmaceutical preparation or formulation to stimulate megakaryocytopoietic or thrombopoietic activity in patients suffering from thrombocytopenia due to impaired production, sequestration, or increased destruction of platelets. Thrombocytopenia-associated bone marrow hypoplasia *(e.g.,* aplastic anemia following chemotherapy or bone marrow transplant) may be effectively treated with the disclosed antibodies as well as disorders such as disseminated intravascular coagulation (DIC), immune thrombocytopenia (including HIV-induced ITP and non HIV-induced ITP), chronic idiopathic thrombocytopenia, congenital thrombocytopenia, myelodysplasia, and thrombotic thrombocytopenia.

Samsung Bioepis Exhibit No. 1002
Page 2025

The biologically active *c-mpl* agonist antibodies disclosed herein containing the TPO mimetic peptide may be used in the same way and for the same indications as thrombopoietin (TPO). Thrombopoietin (TPO) stimulates megakaryocytopoiesis and platelet production. These antibodies are expected to have a longer half-life than native or pegylated TPO and thus are used in indications where a longer half-life are indicated.

An example of an assay useful for determining activity of TPO mimetics is the rebound thrombocytosis assay which involves administering to mice a single injection of goat anti-mouse platelet serum to induce acute thrombocytopenia (day 0). On days 5 and 6 mice are injected with test samples. On day 8 platelet counts are determined (^{35}S incorporation into platelets).

EPO mimetic antibodies herein stimulate hematopoiesis in a manner similar to naturally occurring EPO. Such therapy is useful in treating conditions where red blood cell production is compromised such as in chronic renal failure. The biological activity of EPO mimetic antibodies may be determined using *in vitro* or *in vivo* assays.

One *in vitro* assay measures the effect of erythropoietin mimetic antibodies on erythropoiesis in intact mouse spleen cells according to the procedure of Krystal, G., *Exp. Hematol.* 11:649-660 (1983). To screen various embodiments of the EPO mimetic antibodies for activity, for example, *in vitro* or *in vivo*, the EPO mimetic antibodies can be evaluated for the extent of erythropoiesis or receptor binding. Tests to determine biological activity are well-known to those of skill in the art. For example, the biological activity of erythropoietin can be measured as described in, e.g., U.S. Pat. No. 5,614,184 and U.S. Pat. No. 5,580,853 herein incorporated by reference.

The route of antibody administration is in accord with known methods, *e.g.,* injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, subcutaneous, intraocular, intraarterial, intrathecal, inhalation or intralesional routes, topical or by sustained release systems as noted below. The antibody is preferably administered continuously by infusion or by bolus injection. One may administer the antibodies in a local or systemic manner.

The antibodies of the invention may be prepared in a mixture with a pharmaceutically acceptable carrier. Techniques for formulation and administration of

Samsung Bioepis Exhibit No. 1002
Page 2026

the compounds of the instant application may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition. This therapeutic composition can be administered intravenously or through the nose or lung, preferably as a liquid or powder aerosol (lyophilized). The composition may also be administered parenterally or subcutaneously as desired. When administered systematically, the therapeutic composition should be sterile, pyrogen-free and in a parenterally acceptable solution having due regard for pH, isotonicity, and stability. These conditions are known to those skilled in the art.

Briefly, dosage formulations of the compounds of the present invention are prepared for storage or administration by mixing the compound having the desired degree of purity with physiologically acceptable carriers, excipients, or stabilizers. Such materials are non-toxic to the recipients at the dosages and concentrations employed, and may include buffers such as TRIS HCl, phosphate, citrate, acetate and other organic acid salts; antioxidants such as ascorbic acid; low molecular weight (less than about ten residues) peptides such as polyarginine, proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidinone; amino acids such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; counterions such as sodium and/or nonionic surfactants such as TWEEN, PLURONICS or polyethyleneglycol.

When used for *in vivo* administration, the antibody formulation must be sterile and can be formulated according to conventional pharmaceutical practice. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. The antibody ordinarily will be stored in lyophilized form or in solution. Other vehicles such as naturally occurring vegetable oil like sesame, peanut, or cottonseed oil or a synthetic fatty vehicle like ethyl oleate or the like may be desired. Buffers, preservatives, antioxidants and the like can be incorporated according to accepted pharmaceutical practice.

Pharmaceutical compositions suitable for use include compositions wherein one or more rationally designed antibodies are contained in an amount effective to achieve

Samsung Bioepis Exhibit No. 1002
Page 2027

their intended purpose. More specifically, a therapeutically effective amount means an amount of antibody effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated. Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. Therapeutically effective dosages may be determined by using *in vitro* and *in vivo* methods.

An effective amount of antibody to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. In addition, the attending physician takes into consideration various factors known to modify the action of drugs including severity and type of disease, body weight, sex, diet, time and route of administration, other medications and other relevant clinical factors. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. Typically, the clinician will administer antibody until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored by conventional assays.

For any antibody containing a peptide, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the EC_{50} as determined in cell culture *(e.g.,* the concentration of the test molecule which promotes or inhibits cellular proliferation or differentiation). Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the antibody molecules described herein can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.*g.,* for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD_{50} and ED_{50}. Molecules which exhibit high therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such molecules lies preferably within a range of circulating

Samsung Bioepis Exhibit No. 1002
Page 2028

concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See *e.g.*, Fingl *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to provide plasma levels of the antibody which are sufficient to promote or inhibit cellular proliferation or differentiation or minimal effective concentration (MEC). The MEC will vary for each antibody, but can be estimated from *in vitro* data using described assays. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. However, HPLC assays or bioassays can be used to determine plasma concentrations.

Dosage intervals can also be determined using MEC value. Antibody molecules should be administered using a regimen which maintains plasma levels above the MEC for 10-90% of the time, preferably between 30-90% and most preferably between 50-90%.

In cases of local administration or selective uptake, the effective local concentration of the antibody may not be related to plasma concentration.

A typical daily dosage might range from about 1μ/kg to up to 1000mg/kg or more, depending on the factors mentioned above. Typically, the clinician will administer the molecule until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored by conventional assays.

Depending on the type and severity of the disease, from about 0.001 mg/kg to abut 1000 mg/kg, more preferably about 0.01 mg to 100 mg/kg, more preferably about 0.010 to 20 mg/kg of the agonist antibody might be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. For repeated administrations over several days or longer, depending on the condition, the treatment is repeated until a desired suppression of disease symptoms occurs or the desired improvement in the patient's condition is achieved. However, other dosage regimes may also be useful.

Samsung Bioepis Exhibit No. 1002
Page 2029

EXAMPLES

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

Samsung Bioepis Exhibit No. 1002
Page 2030

EXAMPLE 1

Library Construction Of TPO Mimetic Sequences Grafted Into A Human Antibody Framework

An agonist TPO mimetic-peptide IEGPTLRQWLAARA (SEQ. ID. NO: 1) was grafted into the anti-tetanus toxoid (TT) Fab heavy chain CDR3 (HCDR3), replacing the entire HCDR3 sequence GDTIFGVTMGYYAMDV (SEQ. ID. NO: 4). Figure 2A shows the sequence for the human tetanus toxoid antibody employed. Two grafting approaches were taken. In the first approach the agonist peptide was inserted into the H-CDR3 region with two glycines flanking each side. This was to reduce structural constraints on the grafted peptide so that it could more easily adopt the needed conformation. In the second approach, two amino acid positions on each side of the peptide graft were randomized in order that the best presentation of the peptide could be achieved (Figure 3). These two approaches were taken in order to determine whether the peptide alone was sufficient or if specific residues were required for proper presentation of the agonist peptide on the antibody scaffold, thereby conferring its activity to the antibody.

Figure 4 outlines the library construction process. Briefly, the anti-tetanus toxoid Fab was amplified as two fragments. Fragment A was amplified using a forward primer (N-Omp: 5' TAT CGC GAT TGC AGT GGC ACT GGC 3') (SEQ. ID. NO: 5) that annealed to the Omp A leader for the light chain in combination with a backward primer (TPOCDR3-B: 5' GC CAG CCA TTG CCG CAG CGT CGG CCC TTC AAT YNN YNN TCT CGC ACA ATA ATA TAT GGC 3') (SEQ. ID. NO: 6) that annealed at the end of the heavy chain framework region (FR) 3. The reverse primer contained a tail encoding the new CDR3. Fragment B was generated using a forward primer (TPOCDR3-F: 5' CCG ACG CTG CGG CAA TGG CTG GCG GCG CGC GCG NNY NNY TGG GGC CAA GGG ACC ACC GT 3')(SEQ. ID. NO:7) that annealed at the FR4 and the reverse primer Seq-G3Rev (5' TCA AAA TCA CCG GAA CCA GAG C 3') (SEQ. ID. NO: 8) which annealed in the gene III region of the plasmid, downstream of the heavy chain stop signal. The TPOCDR3-F primer also had a tail of bases that encoded the new CDR3 region. TAQ DNA Polymerase (Perkin Elmer) was used in the following PCR program: 94° 30 seconds, then 30 cycles of 94° for 15 sec, 55° for 15

Samsung Bioepis Exhibit No. 1002
Page 2031

seconds, and 72° for 90 seconds, followed by an extension period at 72° for 10 minutes, and a hold at 4°. After the fragments were generated by PCR and gel purified, they were combined for an overlap extension PCR. The new CDR3 primer encoded regions were complementary and provided 23 bases of overlap. Primers N-Omp and SeqG3Rev were used in the overlap PCR protocol to generate the full Fab DNA product. Taq DNA Polymerase (Perkin Elmer) was used in the following PCR program: 94° 30", then 20 cycles of 94° 30", 56° 30", and 72° 3'15", then an extension period of 72° for 15' followed by a 4° hold. After gel purification of the Fab product, an Sfi 1 digest was performed at 50° for 5 hours. Inserts were ligated into Sfi 1digested pRL4 vector overnight. Ligation products were ethanol precipitated, resuspended in H_2O, and then electroporated into competent ER2537 bacteria (suppressor strain, New England Biolabs). Following one hour of shaking in 5 mls SOC, an equal volume of SB was added. Carbenicillin was added to 20ug/ml and the culture shaken for one hour at 37°, followed by one hour at 37° in 50ug/ml carbenicillin. The library culture was transferred into a flask containing 100 mls fresh SB, 50 ug/ml Carbenicillin, and 10^{12} VCS M13 helper phage. After two hours at 37°, kanamycin was added to select for those bacteria that had been infected with helper phage. The following day, the overnight cultures were spun down and the phage in the supernate were precipitated on ice using 4% PEG/0.5 M NaCl. After spinning down the phage, the pellet was resuspended in 1%BSA/PBS, filtered and dialyzed against PBS. Library phage were stored at 4°.

The construction of Fabs containing the non-randomly linked peptide was performed as described above by substituting primers TPOCDR3-B and TPOCDR3-F with alternate specific primers. For PP-(IEGPTLRQWLAARA)-GG (SEQ. ID. NO: 25) grafted antibody, primers used were TPOCDR3g-B (5' GC CAG CCA TTG CCG CAG CGT CGG CCC TTC AAT NGG NGG TCT CGC ACA ATA ATA TAT GGC 3') (SEQ. ID. NO: 9) and TPOCDR3g-F (5' CCG ACG CTG CGG CAA TGG CTG GCG GCG CGC GCG GGN GGN TGG GGC CAA GGG ACC ACC GT 3')(SEQ. ID. NO: 10). For GG-(IEGPTLRQWLAARA)-GG (SEQ. ID. NO: 29) grafted antibody, primers used were TPO-CDR3-ggB (5' GC CAG CCA TTG CCG CAG CGT CGG CCC TTC AAT NCC NCC TCT CGC ACA ATA ATA TAT GGC 3')(SEQ. ID. NO: 11) and TPOCDR3g-F (5'

Samsung Bioepis Exhibit No. 1002
Page 2032

CCG ACG CTG CGG CAA TGG CTG GCG GCG CGC GCG GGN GGN TGG GGC CAA GGG ACC ACC GT 3') (SEQ. ID. NO: 12).

Selection of the TPO Mimetic Peptide Heavy Chain CDR3 Library

In order to select for the optimal peptide display, panning was performed on human platelets. Because platelets express approximately 1800 TPO receptors per cell on their surface (cMpl receptors), they represented a good cell target. In addition, platelets are readily available from a local Blood Bank. To 1ml of concentrated indated human platelets from the Blood Bank, 50 uls of freshly prepared Fab-phage were added in a 15ml conical tube with 0.1% NaN_3. The tube was mixed at room temperature for 1-2 hours. Typically, 10 mls of 50% human serum (taken off the remaining platelets) + 50%{IMDM/10% FBS/0.1% azide/2mM EDTA} was added to the phage/cells. Platelets were pelleted at 5500xg for 5 minutes at room temperature. Supernatant was drained and the pellet was left resting under ~500 uls of the wash for 20 minutes. The platelets were very gently resuspended and then 10mls of 25% human serum (taken off the remaining platelets) +75%{IMDM/10% FBS/0.1% azide/2mM EDTA} was added to the phage/cells. The centrifugation, pellet rest, and resuspension of the platelets was repeated. In panning rounds 3 and 4, a third wash was performed. The washed phage/cells were transferred to an eppindorf tube and spun at 5200xg. Phage were eluted from the platelets 10 minutes at room temperature using acid elution buffer (0.1M HCl, 1mg/ml BSA, and glycine to pH 2.2). Platelets were pelleted at max speed and the eluted phage transferred to a 50 ml conical tube, neutralized with 2M Tris Base. Phage were then allowed to infect fresh ER2537 bacteria for 15 minutes at room temperature and amplified overnight as described above. Four rounds of platelet panning were performed.

After the fourth round of panning, pools of 3 Fab clones expressed as soluble proteins in nonsuppressor bacterial strain TOP10F' (Invitrogen, Carlsbad, CA) were tested by Facs for binding to platelets by utilizing the Fabs' HA epitope tag with rat high affinity anti-HA followed by anti-Rat-FITC (Sigma, St. Louis, Missouri). 25 uls indated concentrated human platelets (washed once with PBS / 5mM EDTA / 2% FBS) were incubated with 100 uls bacterial supernate (60 uls bacterial supernate from three

Samsung Bioepis Exhibit No. 1002
Page 2033

pooled Fab clones were pre-incubated with 40 uls 5% Milk / PSS at 4° for 15 minutes) at room temperature for 20-30 minutes. 1 ml of FACS buffer (PBS / 2% FBS / 5mM EDTA) was added and cells spun down at 5200xg for 5 minutes. Pelleted cells were resuspended in 50 uls of l:10 diluted (in PBS / 1% BSA / 0.1% NaN_3) 2° anti-HA antibody [Rat IgG anti-HA High Affinity clone 3F10 (Roche Molecular Biochemicals)] was added. After 30 minutes at room temperature, the cells were washed with 1 ml FACS buffer as above. Following centrifugation, cells were resuspended in 100 uls of 1:160 diluted (in PBS /1% BSA /0.1% NaN_3) 3° anti-Rat IgG-FITC antibody (Sigma) and incubated 20 minutes at room temperature in the dark. Cells were washed with 1 ml FACS buffer then resuspended in 200 uls FACS buffer for analysis. As a positive control a commercially available sheep anti-IIb/IIIa Ab followed by anti-sheep FITC was used. Many pools of Fabs were clearly positive for binding to platelets by Facs. Follow up Facs analysis was then performed to identify individual clones that bound to the platelets.

Examination Of Individual Candidates By Binding Activity

Several Fabs, as bacterial supernatants, were tested for reactivity to the original antigen tetanus toxoid in order to determine if that binding specificity was retained. The antibody scaffold anti-TT Fab does bind to its antigen TT, but not to BSA. However, four TPO-mimetic peptide grafted Fab clones did not show significant binding to TT or BSA. As seen in previous experiments, the replacement of anti-TT Fab HCDR3 was sufficient to change the specificity of the antibody.

To further examine the binding capabilities of Fabs, Facs analysis was performed on CMK cells, a Megakaryocytic cell line (from German Collection of Microorganisms and Cell Cultures) which also expresses the cMpl receptor. Fab clones that bound CMK cells were then analyzed to verify that the platelet and CMK cell binding was occurring via the cMpl receptor. For that experiment, 293 EBNA cells were transfected with or without the cMpl-R, which had been cloned from Tf-1 cells by RT-PCR. 1 X 10^6 transfected cells were incubated with bacterial supernate from each Fab clone (pre-blocked as described above) for 20-30 minutes at room temperature. Cells were spun down at 2000 rpm for 5 minutes. Pelleted cells were resuspended in

Samsung Bioepis Exhibit No. 1002
Page 2034

90 uls FACS buffer (PBS / 2% FBS / 1mM EDTA) then 10 uls of 2° anti-HA antibody [Rat IgG anti-HA High Affinity clone 3F10 (Boehringer Mannheim Biochemicals) was added for a final 1:10 dilution. After 20 minutes at room temperature, the cells were washed with 1 ml FACS buffer. Following centrifugation, cells were resuspended in 100 uls of 1:50 diluted (in PBS /1% BSA /0.1% NaN_3) 3° anti-Rat IgG-PE antibody (Research Diagnostics Incorporated, RDI) and incubated 20 minutes at room temperature in the dark. Cells were washed with 1 ml FACS buffer then resuspended in 200 uls FACS buffer for analysis. Fabs selected during panning demonstrated strong binding to cells transfected with the cMpl-R but not to control vector transfected cells lacking the cMpl-R. This indicates that cell surface binding was occurring specifically through the cMpl receptor. Anti-TT Fab does not bind to control vector or cMpl-R transfected 293 cells. However, Fab clone X1c shows a shift from 3% binding of control (non-cMpl receptor) transfected cells to 95% binding of cells expressing the cMpl-R.

Examination Of Individual Candidates By Sequence

Sequence analysis of Fab clones which specifically bound to the cMpl receptor (see Fig. 5), revealed the selection of preferred amino acids at the downstream linkage site. The DNA sequence data was analyzed and the amino acid and DNA sequences are as follows:

Samsung Bioepis Exhibit No. 1002
Page 2035

Clone	Binding Properties	SEQ. ID. NO	Sequence
X1a	weak	25	Pro Pro (14 aa peptide) Gly Gly
X1a-11	weak	27	Gly Gly (14 aa peptide) Gly Gly
X1a-13	weak	29	Gly Gly (14 aa peptide) Gly Gly
X1c	strong	31	Trp Leu (14 aa peptide) Pro Val
X2c	weak	33	Met Ile (14 aa peptide*) Val Gly
X3a	strong	35	Val Val (14 aa peptide) Pro Val
X3b	strong	37	Gly Pro (14 aa peptide) Pro Asp
X4b	strong	39	Leu Pro (14 aa peptide) Pro Val
X4c	strong	41	Ser Leu (14 aa peptide) Pro Ile
X5a	strong	43	Thr Met (14 aa peptide) Pro Val
X5c	strong	45	Trp Leu (14 aa peptide) Pro-Val
X7a	weak	47	Thr Arg (14 aa peptide*) Cys Ser
X7b	weak		deletion mutant this clone has lost the peptide
X7c	strong	49	Gln Thr (14 aa peptide) Pro Asp

All clones which demonstrated strong binding, were found to contain a proline just downstream of the 14 amino acid TPO mimetic peptide. Selection by panning of a proline in the downstream linker position represents determination of a surprising amino acid choice which confers improved binding characteristics to the grafted TPO mimetic peptide. Weak binders did not contain this proline although they still contained the TPO mimetic peptide. It should be noted that clone X7a had a silent mutation in this peptide (GCG to GCA retaining the Ala at position 11 in the peptide) and that

Samsung Bioepis Exhibit No. 1002
Page 2036

clone X2c had a mutation in this peptide switching a Thr for Ala at position 11 of the peptide.

Biological Assays

Clones were tested for agonist activity using a transcriptional based assay measuring luciferase activity driven by the c-Fos promoter. Dimerization of the cMpl receptor activates Jak which stimulates the MAP kinase pathway. Thus activation can be measured by assaying luciferase production and activity stimulated by MAP kinase via the cFos promoter. Since dimerization of the cMpl receptor is required for activation, either full IgG or dimerized Fab fragments capable of dimerizing the receptor, could be used to stimulate cMpl receptor activity. Fabs produced in bacteria were dimerized via the HA tag utilizing the 12CA5 anti-HA antibody. Increasing amounts of 12CA5 was added to the bacterial Fabs to dimerize the Fab clones in order that they might in turn dimerize and activate the cMpl receptor. For measurement of agonist activities Fab containing bacterial supernatants (2mls) mixed with 12CA5 were applied to NIH3T3 cells which had been co-transfected with either a control vector or the cMpl receptor and the Fos promoter/luciferase reporter construct. Co-transfections of 3T3 cells were performed by plating NIH 3T3 cells at 3×10^5 cells per 6 cm dish and then transfecting the following day. NIH 3T3 cells were transfected using the Effectine lipofection reagent (Qiagen), transfecting each plate with 0.1 ug pEGFP (a tracer to measure transfection efficiency), 0.2 ug of the Fos promoter/luciferase construct and 0.7 ug of either the empty control vector or the plasmid expressing the cMpl receptor. 3T3 cells were placed in 0.5% serum 24 hours post transfection and incubated for an additional 24 hours in this low serum media to reduce the background activation of the Fos promoter. Antibody supernatants were then applied to these cells for 6 hours. Cells were harvested and luciferase assays performed using 50 ug of cell lysate. No activation was stimulated by the antibodies in the absence of cMpl receptor expression. However, agonist activity was observed in cMpl receptor co-transfected 3T3 cells. This allowed us to demonstrate that the agonist activity observed was through the cMpl receptor and its interaction with the antibody. The data is as follows:

Samsung Bioepis Exhibit No. 1002
Page 2037

Bacterial Supernatants with 12CA5		
CELL Treatment	Relative Fold Luciferase activity	
	CMpl-R transfected	Control transfected
Untreated:	1.0	1.0
+ 10% FCS	3.43	3.0
+ TPA	2.3	2.42
+ TPO	2.32	0.76
+ X4c Sup (alone)	1.03	0.94
+ X4c + 60ul α-HA	1.97	0.84
+ X4c + 30ul α-HA	1.52	0.87
+ X4c + 10ul α-HA	1.22	0.86
+ X4c + 3ul α-HA	0.94	0.68
+ X4c + 1ul α-HA	0.91	1.05
+ X4c + 0.3ul α-HA	1.01	0.95

Activation of cMpl receptor can be tested in a similar manner using full IgGs (converted from Fab as described herein) produced by transient or stable transfection of mammalian cells rather than bacterially produced Fabs dimerized by anti-HA 12CA5. Experimentally transient transfection can be performed essentially as described here. For transfections 2 x 10^6 cells (such as 293 EBNA) would be plated in 6cm dishes for each test sample. The following day each plate would be transfected with 2.5 ug of total DNA (2 ug total of the light chain and heavy chain plasmid(s), 0.25 ug of pAdVAntage (Promega, Madison, Wisconsin), and 0.25 ug of pEGFP) using the

Samsung Bioepis Exhibit No. 1002
Page 2038

Effectine reagent (Qiagen). The 293 cells would be placed in 0.5% serum 24 hours post transfection and incubated for an additional 24 hours in this low serum media to obtain full IgG. Residual growth factors are negligible in this media in stimulating receptors as seen in controls experiments. After 24 hours supernatants would be collected and spun for 5 minutes at 3000rpm to remove any residual cells. For measurement of agonist activities of the full IgGs, 3mls of the conditioned 293 cell supernatants would be applied to NIH3T3 cells as described above.

EXAMPLE 2

Additional Libraries Containing TPO Mimetic Sequences Grafted Into A Human Antibody Framework

Another approach to linking two agonistic peptides together in an antibody framework is to insert the agonist peptide in more than one position within a single Fab fragment. In order to do that, additional libraries containing TPO mimetic sequences grafted into a human antibody framework were constructed. Following selection of peptides properly presented in the context of CDR1, CDR2 or CDR3 of the light chain, or CDR2 of the heavy chain, the binding sequences were combined into a single Fab molecule, for example as listed in Table 1 below, and analyzed for enhanced activity.

TABLE 1

Peptide 1	Peptide 2
H-CDR3	H-CDR2
H-CDR3	L-CDR1
H-CDR3	L-CDR2
H-CDR3	L-CDR3
H-CDR2	L-CDR2
H-CDR2	L-CDR3
L-CDR3	L-CDR2

Four additional libraries have been constructed separately replacing the heavy chain CDR2 as well as the light chain CDR1, CDR2 and CDR3 with the TPO mimetic peptide flanked by 2 random amino acids using an NNK doping strategy. The generation of the libraries was similar to that described for the heavy chain CDR3 TPO

Samsung Bioepis Exhibit No. 1002
Page 2039

peptide library, except that only the chain (heavy or light) being modified to form a library was amplified by PCR and used as the insert. PCR was performed using Expand High Fidelity PCR System (Roche) which contains a mixture of Taq and Pwo Polymerases. The first round of PCR was performed using the program: 94° 30", then 30 cycles of 94° 15", 56° 30", and 72° 2', followed by elongation for 10' at 72° and a 4° hold. Overlap PCR was performed for 10 cycles without primers using the program listed above to allow the full DNA template to be generated by the polymerases. Primers were then added to the PCR reaction tubes for 20 cycles of the same program for amplification.

For the HCDR2 library, the fragment A was created using the forward primer lead VH (5' GCT GCC CAA CCA GCC ATG GCC 3')(SEQ. ID. NO: 13), which annealed at the pel B leader signal located in front of the heavy chain, and the reverse primer HR2 CMPL ANTI (5' AGC CAG CCA CTG GCG CAG GGT TGG GCC TTC GAT MNN MNN TCC CAT CCA CTC AAG CCC TTG 3')(SEQ. ID. NO: 51) that annealed at the end of the heavy chain FR2. The reverse primer contained a tail encoding the new CDR2. Fragment B was created using forward primer HR2 cMpl CODE (5' CCA ACC CTG CGC CAG TGG CTG GCT GCT CGC GCT NNK NNK AGA GTC ACC ATT ACC GCG GAC 3')(SEQ. ID. NO: 14) which annealed at FR3 of the heavy chain and reverse primer N-dp (5' AGC GTA GTC CGG AAC GTC GTA CGG 3')(SEQ. ID. NO: 15) which annealed in the HA epitope tag region of the plasmid, downstream of the heavy chain constant region. The HR2 cMpl CODE primer also had a tail of bases that encoded the new CDR2 region. After the fragments were generated by PCR and gel purified, they were combined for an overlap extension PCR. The new CDR2 primer encoded regions were complementary and provided 24 bases of overlap. Primers leadVH and N-dp were used in the overlap PCR protocol to generate the full heavy chain DNA product. Following gel purification of the heavy chain product, a Xho I / Spe I digest was performed at 37° for 3 hours. Inserts were gel purified and then ligated into Xho I / Spe I digested pRL4 vector containing the anti-TT light chain. Ligation products were precipitated and electroporated into ER2537 bacteria as described above for the generation of the Fab-phage library.

Samsung Bioepis Exhibit No. 1002
Page 2040

The light chain CDR3 library was similarly made using primers for Fragment A of forward primer N-omp and reverse primer LR3 cMpl ANTI (5' AGC CAG CCA CTG GCG CAG GGT TGG GCC TTC GAT MNN MNN ACA GTA GTA CAC TGC AAA ATC 3') (SEQ. ID. NO: 16) and for Fragment B of forward primer LR3 cMpl CODE (5' CCA ACC CTG CGC CAG TGG CTG GCT GCT CGC GCT NNK NNK TTC GGC CAA GGG ACC AAG GTG 3')(SEQ. ID. NO: 17) and reverse primer leadB (5' GGC CAT GGC TGG TTG GGC AGC 3') (SEQ. ID. NO: 18). Primer leadB annealed to the pelB leader sequence located before the VH. The LR3 cMpl ANTI reverse and LR3 cMpl CODE forward primers annealed to the FR3 and FR4 of anti-TT light chain respectively. Both LR3 cMpl primers contain a tail of nucleotides encoding the new CDR3 peptide library, which provides the 24 basepair overlap region for the fusion PCR of Fragment A and Fragment B. Following purification of the light chain PCR products, a Sac I / Xba I digest was performed at 37° for 3 hours. The light chain fragments were then ligated into Sac I / Xba I digested pRL4 containing the anti-TT heavy chain overnight at room temperature. Ligation products were precipitated and electroporated into ER2537 bacteria as described above for the generation of the Fab-phage library.

The construction of the light chain CDR2 library was carried out as described above for the light chain CDR3 library with the exception that specific primers LR2 cMpl ANTI (5' AGC CAG CCA CTG GCG CAG GGT TGG GCC TTC GAT MNN MNN ATA GAT GAG GAG.CCT GGG AGC 3')(SEQ. ID. NO: 19) which annealed at the end of light chain FR2 and primer LR2 cMpl CODE (5' CCA ACC CTG CGC CAG TGG CTG GCT GCT CGC GCT NNK NNK GGC ATC CCA GAC AGG TTC AGT 3')(SEQ. ID. NO: 20) which annealed at the beginning of light chain FR3 were used in place of the LR3 cMpl primers.

The construction of the light chain CDR1 library was also carried out as previously described for the light chain CDR3 library with the exception that specific primers TPOLR1CODE (5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTNNKNNKTGGTACCAGCAGA AACCTGGC 3') (SEQ. ID. NO: 58) which annealed at the beginning of the light chain

Samsung Bioepis Exhibit No. 1002
Page 2041

FR2 and the primer TPOLR1ANTI (5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATMNNMNNGCAGGAGAGGGT GGCTCTTTC 3') (SEQ. ID. NO: 59) which annealed to the end of the light chain FR1 were used in place of the LR3 cMpl primers.

The three additional libraries, which separately replace the heavy chain CDR2 and the light chain CDR2 and CDR3 with the TPO mimetic peptide flanked by 2 random amino acids using the NNK doping strategy, were separately panned on platelets, as was previously described in Example I for the heavy chain CDR3 replacement library. Four rounds of panning were performed and clones were screened by FACS on platelets and cMpl receptor transfected 293 cells as previously described. Two positive clones were obtained from these screens. These clones had the TPO mimetic peptide in the heavy chain CDR2. Unlike with the heavy chain CDR3 clones neither of the heavy chain CDR2 clones had a proline in downstream position. Instead both were found to contain a tyrosine in the upstream position (See Figure 9-clones HC-CDR2 No. 24 and No. 39).

The libraries, including LCDR1, were separately subjected to another panning experiment using cMpl receptor transfected 293 cells instead of platelets during the panning. The 293 cells were observed to reproducibly transfect at a high efficiency and express very high levels of the functional cMpl-receptor on their surface. Thus these cells represented a good cell target for use in panning. For these experiments different groups of plates of 293 cells were separately and sequentially transfected four days in a row. Each group of plates was then sequentially used for the four separate rounds of panning. Each round of harvesting of the cells and panning occurred two days after transfection. For harvesting, cells were removed from the plates using cell disassociation buffer, spun down at 1500 rpm for 5 minutes and re-suspended in IMDM supplemented with 10% FCS, 0.1% sodium azide and 5mM EDTA at a concentration of 1 x 10^6 cell per ml (3x10^6 for LC-CDR1). In the round one pan, 3 x 10^{11} phage from each library were separately applied to 2 ml of cells (6x10^6 for LC-CDR1 and 2 x 10^6 cells for all others) and rotated in a 15 ml conical tube for two hours at room temperature. Cells were washed twice using 10 mls of the IMDM/10%

Samsung Bioepis Exhibit No. 1002
Page 2042

FCS/0.1% sodium azide/5mM EDTA buffer. Phage were eluted in acid and amplified as previously described in Example 1. In round two 4 x 10^6 cells (6x10^6 for LC-CDR1) were used in 2 ml of buffer and 3 x 10^{11} phage from the amplified round one eluted phage was combined with 3 x 10^{11} phage from the un-panned library and added to the cells. Washing, elution and amplification proceeded similar to round one. In round three 4 x 10^6 cells (6x10^6 for LC-CDR1) were used in 2 ml of buffer and 3 x 10^{11} phage from the amplified round two eluted phage were used. Cells were washed three times prior to elution. In round four, 4 x 10^6 cells (6x10^6 for LC-CDR1) were again used in 2 ml of buffer and 3 x 10^{11} phage from the amplified round three eluted and amplified phage were used. Cells were again washed three times prior to elution. At least thirty individual clones were screened by FACS on cMpl receptor transfected 293 cells as previously described. 12 positive clones were obtained from the heavy chain CDR2 library and 25 positive clones were obtained from the light chain CDR2 library, and 14 positive clones were obtained from the light chain CDR1 library. Clones were further analyzed by DNA sequence. The selected flanking amino acid residues for the positive clones are depicted in the attached Figure 9. It is of interest to note that the light chain CDR2 grafted Fabs have a strong selection for a proline (Pro) upstream of the TPO mimetic peptide.

EXAMPLE 3

Combinations of the TPO mimetic peptide grafted Fab clones from Figure 9 have been generated. Thus a single antibody might contain multiple copies of the TPO mimetic peptide within a single light or heavy chain. Alternatively, both the light and heavy chains might contain peptide grafts giving multiple copies within a single Fab. Positive clones selected from the same CDR library were pooled. New libraries were constructed by combining the pool of one TPO mimetic peptide containing CDR with the pool of another. Combinations where one of the TPO mimetic peptides is in the light chain and the other is in the heavy chain are made using simple cloning techniques using the pooled plasmid DNAs, and the unique restriction sites flanking the heavy (Xho I-SpeI) and light chains (Sac I-Xba I). For example, the plasmid DNA for the H-CDR3 peptide grafted heavy chains were combined and digested by Xho I

Samsung Bioepis Exhibit No. 1002
Page 2043

and Spe I. The purified heavy chain inserts were ligated into the Xho I/Spe I digested plasmid containing the L-CDR2 grafts. The resulting library contained heavy chains with CDR3 peptide grafts and light chains with CDR2 peptide grafts. It should be understood that individual clones could also be combined rather that using pools of clones for the pairing of two peptide containing CDRs. For example, a single heavy chain clone with a CDR3 peptide graft was paired with several individual light chain CDR1 clones to create Fabs with multiple copies of TPO mimetic peptides.

Combinations where two TPO mimetic peptides were combined within a given heavy chain was performed using overlap PCR to generate the fragment for cloning. Two overlapping primers which bind between CDR2 and CDR3, and flanking primers, such as "N omp" and "lead B" primers from the light chain and "Lead VH" and "Ndp" primers for the heavy chain were used. For example, to combine H-CDR2 and H-CDR3 the first PCR was performed using lead VH (a primer that anneals in the vector at the heavy chain pelB leader signal) and a reverse primer annealing at FR3 using the H-CDR2 pooled plasmid DNA as the template. The sequence of that primer was 5' CCA TGT AGG CTG TGC CCG TGG ATT 3' (SEQ. ID. NO: 63). In a separate reaction, the pooled plasmids containing the H-CDR3 grafts underwent PCR with a forward primer annealing in FR3 (which is complementary to the above FR3- reverse primer) and N-dp (which anneals in the vector at an epitope tag sequence). The sequence of that primer was 5' CCA CGG GCA CAG CCT ACA TGG AGC 3' (SEQ. ID. NO: 64). The first PCR products were purified then combined in an overlap PCR reaction, where fusion of the two fragments occurred through the complementary FR2 sequences. The full heavy chain product was cloned by Xho I/Spe I into a plasmid containing the anti-tetanus toxoid light chain. In all, five classes of Fabs bearing multiple copies of TPO mimetic peptides were created as detailed in Table 2 below.

Samsung Bioepis Exhibit No. 1002
Page 2044

TABLE 2

Peptide 1	Peptide 2	Peptide 3
H-CDR3	H-CDR2	
H-CDR3	L-CDR2	
H-CDR2	L-CDR2	
H-CDR3	H-CDR2	L-CDR2
H-CDR3	L-CDR1	

20 clones from each combination library and four individual clones of H-CDR3 plus L-CDR1 were tested for biological activity in a luciferase reporter assay as previously described. See Figures 10 and 11. In both experiments, the negative controls can include uninduced cells, cells treated with an irrelevant Fab (anti-tetanus toxoid), cells treated with a Fab clone that only weakly binds cMpl receptor, and X4b and/or X1c Fabs which do bind the cMpl receptor but only have a single binding domain and so can not activate the receptor. The positive control was the addition of TPO. All remaining samples were from the newly formed combination libraries. As can be observed, several clones have significant activity as Fabs. This indicates that incorporation of multiple TPO mimetic peptides into a single Fab molecule is able to bind two receptors and cause receptor activation.

In fact, using Fab 59 the agonistic activity obtained can be as high as native TPO activity. Clone 59 containing two TPO mimetic peptides (HC CDR3 sample x4c and LC CDR2 sample 19 as identified in Fig. 9) and a His6 tag was partially purified from a bacterial periplasmic prep by FPLC using nickel column chromatography. The activity of this Fab was measured and found to be approximately equivalent to that of TPO (see Figure 12), as estimated by cMpl-R specific induction of luciferase activity in direct comparison to known concentrations of TPO. A quantitative western blot was performed in order to determine the Fab 59 antibody concentrations.

These Fabs, or various other two CDR combinations, could be used as a therapeutic product. Alternatively, these clones could be converted to framework germline sequences (either with or without codon optimization) for use as a therapeutic agent so long as activity was maintained.

Samsung Bioepis Exhibit No. 1002
Page 2045

EXAMPLE 4

The TPO mimetic peptide graft in Fab clone X4b has been transplanted into the heavy chain CDR3 of another antibody framework, 5G1.1. Construction of 5G1.1 is described in U.S. Appln. Ser. No. 08/487,283, incorporated herein by reference. The sequence was cloned into 5G1.1 in such a fashion as to replace the native CDR3 with 5' ttg cca ATT GAA GGG CCG ACG CTG CGG CAA TGG CTG GCG GCG CGC GCG cct gtt 3' (SEQ. ID. NO: 65). The peptide graft translated into amino acids is Leu Pro Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Pro Val (SEQ. ID. NO: 66). The 5G1+peptide was produced as a whole IgG antibody (See Figures 13A and 13B).

Purified 5G1.1+peptide antibody as well as the parental 5G1.1 were analyzed for their ability to bind to cMpl receptor by FACS analysis. Binding to receptor expressing and non-receptor expressing 293 cells was compared. See Figure 14. The FACS staining was performed essentially as described previously herein, with the exception that the detection was done using PE conjugated F(ab')2 fragment of goat anti-human IgG (H+L). The negative controls of 3° only anti-tetanus toxoid irrelevant Fab, and Fab X1a which binds weakly to cMpl receptor all showed very little staining. However, binding Fabs X1c and X4b showed strong staining as did the 5G1.1+peptide. None of those clones demonstrated binding to the non-receptor expressing cells indicating that the cell staining is occurring through specific recognition of the cMpl receptor. The parental 5G1.1 without the TPO mimetic peptide did not show staining to any of the cells tested.

The ability of the 5G1.1+ peptide whole IgG to activate the cMpl receptor using the luciferase reporter assay has been determined (see Figure 15). The results herein indicate that the configuration of a whole IgG causes steric limitations in its ability to productively bring the two cMpl receptors together for activation. The activity of the 5G1.1 full IgG construct containing the TPO mimetic peptide in the heavy chain CDR3 positions, was only weakly activating and required approximately 100-200 fold higher molar concentrations as compared to TPO, to stimulate equivalent activity. As was previously observed with the binding experiments, activation by the 5G1.1 containing the peptide was observed only when the cMpl-R was expressed on the cell surface.

Samsung Bioepis Exhibit No. 1002
Page 2046

No receptor specific binding or activity was observed with the parental 5G1.1 not containing the peptide. These results demonstrate that binding and activity of the TPO mimetic peptide and selected amino acid flanking sequences is not limited to or specific for the Tetanus Toxoid antibody framework, but can be applied to other antibody frameworks. Thus the flanking amino acid sequences that were selected during panning are specific for presentation of the TPO mimetic peptide within a given CDR position, but not for amino acid sequence of the antibody framework.

EXAMPLE 5

Library Construction Of Epo Mimetic Sequences Grafted Into A Human Antibody Framework

An agonist EPO mimetic-peptide DYHCRMGPLTWVCKPLGG (SEQ. ID. NO: 3) (designated EMP2 in Wrighton et al. 1996) was grafted separately into the anti-tetanus toxoid Fab heavy and light chain CDR3 region creating two antibody libraries as XXDYHXRMGPLTWVXKPLGGXX (SEQ. ID. NO: 71). Randomized positions were generated using an NNK doping strategy. As with the TPO mimetic peptide, two amino acids flanking the EPO mimetic peptide were randomized in order to select for the optimum presentation of the peptide. In addition the cysteine residues, which formed a disulfide bridge in the original cyclic peptide, were randomized. This was done not only because the CDRs already form loop structures and so the disulfide bridge was not necessary to constrain the peptide, but also because the cysteines might in fact disrupt the normal disulfide bonds of the antibody.

The CDR 3 of the anti-TT antibody heavy chain was completely replaced by the EPO peptide library graft. The generation of the library was essentially as described for the TPO heavy chain CDR2 library. Two alternate primers were used for the HCDR3 library: the reverse primer HR3 EPO ANTI (5' CAC CCA GGT CAG TGG GCC CAT GCG MNN ATG ATA GTC MNN MNN TCT CGC ACA ATA ATA TAT GGC 3')(SEQ. ID. NO: 21) that annealed at the end of the heavy chain FR3, and forward primer HR3 EPO CODE (5' CGC ATG GGC CCA CTG ACC TGG GTG NNK AAA CCA CTG NNK NNK TGG GGC CAA GGG ACC ACG GTC 3')(SEQ. ID. NO: 22) which annealed at FR4 of the heavy chain.

Samsung Bioepis Exhibit No. 1002
Page 2047

The light chain CDR3 EPO peptide library was constructed essentially as described above for the light chain CDR3 TPO peptide library using reverse primer LR3 EPO ANTI (5' CAC CCA GGT CAG TGG GCC CAT GCG MNN ATG ATA GTC MNN MNN ACA GTA GTA CAC TGC AAA ATC 3') (SEQ. ID. NO: 23) that annealed at the end of light chain FR3 and forward primer LR3 EPO CODE (5' CGC ATG GGC CCA CTG ACC TGG GTG NNK AAA CCA CTG NNK NNK TTC GGC CAA GGG ACC AAG GTG 3') (SEQ. ID. NO: 24) which annealed to FR4 of the light chain.

Selection Of The Epo Mimetic Peptide Heavy Chain Cdr3 Library And Light Chain Cdr3 Library

Selection for peptide presentation was performed by solid phase panning on the soluble EPO receptor. In this method, 1ug of purified human EPO-soluble R (hEPO-sR from R&D Systems, Minneapolis, MN cat#307-ER-050) was immobilized on a microtiter dish overnight at 4°. After washing off free hEPO-sR, the plates were blocked with 1% BSA/PBS for one hour at 37°. Phage, prepared as described above, were added to the wells and incubated two hours at 37°. Washes were performed using PBS/0.5% Tween 20 for 5' at room temperature per wash. 1, 5, 10, and 10 washes were performed in the first, second, third and fourth panning rounds respectively. After the washing steps were completed, the bound Fab-phage were eluted with 30 uls of elution buffer for 10' at room temperature. Eluted phage were then neutralized and amplified as described in Example 1.

EXAMPLE 6

A library was generated by the insertion of a TPO mimetic peptide and previously selected flanking amino acids (NP-IEGPTLRQWLAARA-RG) (SEQ. ID. NO: 61) into a collection of human kappa gene fragments, in this case the CDR2 of the light chain. Stocks of human kappa light chains from multiple human peripheral blood lymphocyte (PBL) donors had been previously generated and cloned into pBluescript II SK+. Those constructs served as the source of antibody gene fragments.

Samsung Bioepis Exhibit No. 1002
Page 2048

Antibody Gene Banks

Total RNA from human PBLs was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH) followed by mRNA purification with Oligotex mRNA purification System (QIAGEN, Valencia, CA) according to kit instructions. First strand cDNA was made using SuperScript RTase II cDNA Synthesis Kit (Life Technologies, Rockville, Maryland) with a modified oligo dT primer. The sequence of the primer was 5' TAGGATGCGGCCGCACAGGTC(T_{20}) 3' (SEQ. ID. NO: 62). Samples were cleaned up over a PCR purification Kit spin column (QIAGEN, Valencia, CA) according to kit directions. Light chain products were amplified using the reverse "Not I" primer and forward primers which annealed at the framework 1 (FR1) position of Kappa chains on the 1st strand cDNA. The "Not I" primer had sequence which was identical to the 5' end of the modified oligo dT primer (5' TAGGATGCGGCCGCACAGGTC 3')(SEQ. ID. NO: 72). The set of Kappa FR1 primers used were:

XVB Vk1a CACGCGCACAACACGTCTAGARACATCCAGATGACCCAG (SEQ. ID. NO: 73)

XVB Vk1b CACGCGCACAACACGTCTAGAGMCATCCAGTTGACCCAG (SEQ. ID. NO: 74)

XVB Vk1c CACGCGCACAACACGTCTAGAGCCATCCRGATGACCCAG (SEQ. ID. NO: 75)

XVB Vk1d CACGCGCACAACACGTCTAGAGTCATCTGGATGACCCAG (SEQ. ID. NO: 76)

XVB Vk2a CACGCGCACAACACGTCTAGAGATATTGTGATGACCCAG (SEQ. ID. NO: 77)

XVB Vk2b CACGCGCACAACACGTCTAGAGATRTTGTGATGACTCAG (SEQ. ID. NO: 78)

Samsung Bioepis Exhibit No. 1002
Page 2049

XVB Vk3a CACGCGCACAACACGTCTAGAGAAATTGTGTTGACRCAG (SEQ. ID. NO: 79)

XVB Vk3b CACGCGCACAACACGTCTAGAGAAATAGTGATGACGCAG (SEQ. ID. NO: 80)

XVB Vk3c CACGCGCACAACACGTCTAGAGAAATTGTAATGACACAG (SEQ. ID. NO: 81)

XVB Vk4a CACGCGCACAACACGTCTAGAGACATCGTGATGACCCAG (SEQ. ID. NO: 82)

XVB Vk5a CACGCGCACAACACGTCTAGAGAAACGACACTCACGCAG (SEQ. ID. NO: 83)

XVB Vk6a CACGCGCACAACACGTCTAGAGAAATTGTGCTGACTCAG (SEQ. ID. NO: 84)

XVB Vk6b CACGCGCACAACACGTCTAGAGATGTTGTGATGACACAG (SEQ. ID. NO: 85)

A typical amplification reaction contained 2 µls cDNA reaction, dNTPs, "Not I" reverse primer, one of the XVB forward primers, Opti-prime buffer #5 (Stratagene, La Jolla, CA), and Expand High Fidelity polymerase mixture (Roche Molecular Biochemicals, Indianapolis, IN). Samples were heated to 94°C for 2 minutes, then carried through 10 cycles of 94°C for 15 seconds, 56°C for 30 seconds, and 72°C for 1 minute, followed by 20 cycles of 94°C for 15 seconds, 56°C for 30 seconds, and 72°C for (1 minute + 5 seconds/cycle). The cycles were followed by an extended incubation at 72°C (7 minutes) prior to 4°C hold. Products were ethanol precipitated and then gel purified. Fragments of approximately 850bp were isolated and then digested with Xba I and Sac I. The resulting kappa products were ligated into pBluescript II SK+ that had likewise been digested with Xba I and Sac I. The ligation products were electroporated into Top10F' (Invitrogen, Carlsbad, CA) and grown overnight. The bacterial pellet was used to isolate the Kappa library DNA with QIAGEN's MAXIprep DNA isolation kit.

Samsung Bioepis Exhibit No. 1002
Page 2050

Construction Of Framework Library With TPO Mimetic Peptide

For construction of the TPO light chain framework library, equal amounts of four different kappa light chain libraries from four different patients were used as the starting template for the PCR reactions (25ng total per reaction). The TPO mimetic peptide and selected flanking amino acids were incorporated into the light chains by overlap PCR. In the first round of PCR a set of reverse primers (VK ANTI primers) which bound to the kappa light chain FR2 were separately combined with the forward T7 seq-F primer (5'-ATTAATACGACTCACTATAGGG-3') (SEQ. ID. NO: 86) to synthesize the N terminal piece of the light chain and part of the TPO mimetic peptide within the LC CDR2 position. A second set of forward primers (VK CODE primers), which bound to FR3, were combined separately with the T3 reverse primer (5'-AATTAACCCTCACTAAAGGG-3') (SEQ. ID. NO: 87) to synthesize the rest of the TPO mimetic peptide within the LC CDR2 position and the C terminal half of the light chain by PCR. Separate reactions were performed for each pair of primer combinations in duplicate.

VK6ANTI
5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATCGGGTTCCTGATGAGGAGC TTTGGRG-3' (SEQ. ID. NO: 88)

VK5ANTI
5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATCGGGTTTTGAATAATGAAAA TAGCAG-3' (SEQ. ID. NO: 89)

VK4ANTI
5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATCGGGTTGTAAATGAGCARCT TAGGAG-3' (SEQ. ID. NO: 90)

VK3ANTI
5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATCGGGTTATAGATGAGGAGC CTGGGMG-3' (SEQ. ID. NO: 91)

VK2AANTI
5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATCGGGTTATAAATTAGGCGCC TTGGAG-3' (SEQ. ID. NO: 92)

Samsung Bioepis Exhibit No. 1002
Page 2051

VK2BANTI
5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATCGGGTTATAGATYAGGAGCT
GTGGAG-3' (SEQ. ID. NO: 93)

VK1AANTI
5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATCGGGTTATAGATCAGGAGCT
TAGGA-3' (SEQ. ID. NO: 94)

VK1BANTI
5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATCGGGTTRTAGATCAGGAGCT
TAGGG-3' (SEQ. ID. NO: 95)

VK1CANTI
5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATCGGGTTATAGATCAGGGACT
TAGGG-3' (SEQ. ID. NO: 96)

VK1DANTI
5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATCGGGTTATAGATCAGGYGCT
TAGGG-3' (SEQ. ID. NO: 97)

VK6CODE
5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTCGTGGTGGGGTCCCCTCGA
GGTTCAG-3' (SEQ. ID. NO: 98)

VK5CODE
5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTCGTGGTGGAATCCCACCTC
GATTCAG-3' (SEQ. ID. NO: 99)

VK4CODE
5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTCGTGGTGGGGTCCCTGACC
GATTCAG-3' (SEQ. ID. NO: 100)

VK3ACODE
5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTCGTGGTGGCATCCCAGMCA
GGTTCAG-3' (SEQ. ID. NO: 101)

VK3BCODE
5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTCGTGGTGGTATCCCAGCCA
GGTTCAG-3' (SEQ. ID. NO: 102)

VK2ACODE
5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTCGTGGTGGAGTSCCAGAYA
GGTTCAG-3' (SEQ. ID. NO: 103)

Samsung Bioepis Exhibit No. 1002
Page 2052

VK2BCODE
5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTCGTGGTGGGGTCCCWGACA
GRTTCAG-3' (SEQ. ID. NO: 104)

VK1ACODE
5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTCGTGGTGGGGTCCCATCAA
GGTTCAG-3' (SEQ. ID. NO: 105)

VK1BCODE
5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTCGTGGTGGGGTCCCATCTC
GGTTCAG-3' (SEQ. ID. NO: 106)

Fragments from the first rounds of PCR were gel purified. Those purified fragments were then combined, in an antibody family specific manner, in overlap PCR reactions to generate the full light chain. Reactions for each family were performed in triplicate using 40ng of both the N-terminal and C-terminal piece of the light chain in each reaction. The reactions were run for 10 cycles prior to the addition of the T3 and T7 Seq-F primers, followed by an additional 25 cycles after primer addition. The full length LC fusion PCR products were gel purified, digested with Sac I and Xba I, and then again gel purified. The light chain inserts were then ligated into an appropriate phage display vector, which had been similarly digested with Xba I and Sac I and gel purified. The pRL5-kappa vector used had restriction sites which were compatible with the LC fragments and contained the remaining Kappa constant region from the native Sac I site to the C-terminal Cys. In addition, the anti-tetanus toxoid heavy chain was inserted into the vector by Xho I and Spe I for Fab production.

The ligation mixture was transformed by electroporation into XL-1 Blue bacteria (Stratagene, La Jolla CA) and amplified. The library was panned four rounds on 293 EBNA cells transfected with the cMpl-R in a manner similar to that previously described. Clones obtained during panning were screened for binding by FACs analysis on 293 EBNA cells transfected with or without the cMpl-R as previously described. A number of clones, which specifically bound the cMpl-R, were obtained. DNA fingerprinting of the resulting light chains by digestion with Bst N1 indicated that the clones could be divided into 5 different groups. Partial sequencing of 8 of these clones showed that frameworks from at least two different kappa light chain families were selected during the panning (VK1 and VK3). In an initial test, 3 of the light chain

Samsung Bioepis Exhibit No. 1002
Page 2053

framework clones were combined with the heavy chain of X4b to create a Fab with 2 TPO mimetic peptides. These clones induced activation of the cMpl-R in luciferase assays as previously described. The level of activation using bacterial supernatants of one such clone 429/X4b (see Figure 16) was approximately 10-20 fold lower than that observed with TPO, as estimated by comparing activity to known concentrations of TPO and using quantitative western blots to determine the concentration of the antibody in the supernatant. Additional clones can be screened in a similar fashion on order to identify clones with greater activity.

These Fabs, or various other LC, HC or intrachain CDR combinations, could be used as a therapeutic product. Alternatively, these clones could be converted to framework germline sequences (either with or without codon optimization) for use as a therapeutic agent so long as activity was maintained.

Modification of the Phage Display Vectors pAX131 and pRL5

The Not I site in pAX131 was removed by digesting the vector with Not I, using Klenow polymerase to fill in the Not I overhangs, and then re-ligating the vector. See Figure 17. Further modification was made by digesting pAX 131 with EcoR I and Xba I. An insert that replaced the elements removed by such digestion was generated using overlapping oligonucleotides with the following changes: conversion of the Sac I site to a new Xba I site (single underline in primer sequences below), conversion of the original Xba I site to a Not I site (double underline in the primer sequences below), and ending the insert with a Spe I overhang which is compatible with the vector's Xba I digest generated overhang. Ligation of the EcoR I / Spe I insert into the EcoR I / Xba I cut vector resulted in an Spe I / Xba I hybrid which will no longer cut with either Spe I or Xba I at that site. The sequence of the oligos used were: "EcoSpe" 5' AA TTC AAG GAG TTA ATT ATG AAA AAA ACC GCG ATT GCG ATT GCG GTG GCG CTG GCG GGC TTT GCG ACC GTG GCC CAG GCG GCC TCT AGA ATC TGC GGC CGC a 3'(SEQ. ID. NO: 107), and "SpeEco" 5' ct agt GCG GCC GCA GAT TCT AGA GGC CGC CTG GGC CAC GGT CGC AAA GCC CGC CAG CGC CAC CGC AAT CGC AAT CGC GGT TTT TTT CAT AAT TAA CTC CTT G 3' (SEQ. ID. NO: 108).

Samsung Bioepis Exhibit No. 1002
Page 2054

The intermediate vector created was pAX131Xba/Not. The human kappa constant region was inserted between the Xba I and Not I sites generating pAX131-kappa (see Figure 18). The human kappa constant region was PCR amplified from human cDNA using primers that introduced the upstream Xba I site and in the downstream position a TAA stop codon followed by a Not I site. The primers used were CKXba I (5' GGA GTC TAG ATA ACT GTG GCT GCA CCA TCT GTC TTC 3') (SEQ. ID. NO: 109) and CKNotI (5' AGG AGC GGC CGC TTA ACA CTC TCC CCT GTT GAA GCT C 3') (SEQ. ID. NO: 110).

The light chain cloning modifications incorporated into pAX131-kappa were shuttled into the pRL5 vector (modified to have its Not I site removed as described above) by moving the EcoR I to Xho I fragment. See Figures 19 and 20. This vector was designated as pRL5-kappa. (See Figures 21A-I.)

EXAMPLE 7

(core H-CDR2 library)

An additional HC CDR2 library was constructed where a core sequence of the TPO mimetic peptide (GPTLRQWL) (SEQ. ID. NO: 112) flanked by a single random amino acid on each side was inserted into the heavy chain partially replacing the CDR2 (GXGPTLRQWLXYAQKFQG) (SEQ. ID. NO: 113). The construction of the heavy chain partial CDR2 replacement library was also carried out as previously described for the heavy chain CDR2 library with the exception that the specific primer 8HCR2anti0 (5'CAGCCACTGGCGCAGGGTTGGGCCMNNCCCTCCCATCCACTCAAGCCC-3') (SEQ. ID. NO: 114) which annealed at the end of the heavy chain FR2 and the primer 8HCR2code(5'GGCCCAACCCTGCGCCAGTGGCTGNNKTACGCACAGAAATTCCAG GGCAGAGTCACCATT-3') (SEQ. ID. NO: 115) which annealed to the beginning of the heavy chain FR3 were used in place of the previously described HR2 cMpl primers. The heavy chain partial CDR2 replacement library was panned four rounds on 293 EBNA cells transfected with the cMpl-R as described previously. These clones can now be screened for positive binding clones by FACs analysis as previously described.

Samsung Bioepis Exhibit No. 1002
Page 2055

EXAMPLE 8

An individual clone was created by combining the heavy chain CDR3 clone X4b (See Figures 5 and 9), with the heavy chain CDR2 clone 19 (see Figure 9) to create a new Fab clone containing two TPO mimetic peptides. This clone was further modified by codon optimization and conversion to germline sequences. This new clone, which was named 116 (Figure 22) was purified to 95% homogeneity. The specific activity of the purified 116 Fab was then tested using the luciferase based assays as previously described. The specific activity of the purified 116 was found to be approximately 30 fold lower than TPO (Figure 23).

EXAMPLE 9

A third heavy chain CDR2 library was constructed replacing part of the tetanus toxoid (TT) heavy chain CDR2 with the TPO mimetic peptide flanked by two random amino acids on each side.

Computer modeling indicated that it might be beneficial to stability and folding of the heavy chain to retain four amino acids of the original TT HC CDR2. Specifically, the first three amino acids of the TT heavy chain CDR2 (GLY-ILE-PHE) and the last amino acid of the heavy chain CDR2 (GLY). Thus, the final product would contain these original four amino acids with the TPO mimetic peptide and random flanking amino acids positioned between the first three original amino acids and last original amino acid: GIFXXIEGPTLRQWLAARAXXG (SEQ. ID NO. 119).

The construction of the heavy chain partial CDR2 replacement library was also carried out as described for the previous 2 heavy chain CDR2 libraries with the exception that the specific primer HCR2+ANTI (5'AGCCAGCCACTGGCGCAGGGTTGGGCCTTCGATMNNMNNGAAGATCCCTCCC ATCCACTC-3') (SEQ. ID NO. 120) which annealed at the end of the heavy chain FR2 and the primer HCR2+CODE (5'CCAACCCTGCGCCAGTGGCTGGCTGCTCGCGCTNNKNNKGGCAGAGTCACCA TTACCGCG-3') (SEQ. ID NO. 121) which annealed to the beginning of the heavy chain FR3 were used in place of the previously described HR2 cMPL primers. The heavy chain partial CDR2 replacement library was panned four rounds on 293 EBNA

Samsung Bioepis Exhibit No. 1002
Page 2056

cells transfected with the cMPL-R as described previously. A number of clones with strong binding characteristics were obtained and sequenced (Figure 24).

The clones of the round four pool from panning are used as the starting point for insertion of a second TPO mimetic peptide. For example, this library has been combined with the clone X4b containing a TPO mimetic peptide in the heavy chain CDR3 by sub-cloning, to create clones, which contain two TPO mimetic peptides (one in heavy chain CDR2 and one in heavy chain CDR3). These new clones are screened for TPO mimetic activity. In addition, the second peptide may be placed in light chain CDR1, CDR2 or CDR3, or heavy chain CDR1.

It will be understood that various modifications may be made to the embodiments disclosed herein. Therefore, the above description should not be construed as limiting, but merely as exemplifications of preferred embodiments. Those skilled in the art will envision other modifications within the scope and spirit of the claims appended herein.

Samsung Bioepis Exhibit No. 1002
Page 2057

WHAT IS CLAIMED IS:

1. An immunoglobulin molecule or fragment thereof comprising a region where amino acid residues corresponding to at least a portion of a two complementarity determining regions (CDRs) are replaced with a peptide mimetic selected from the group consisting of an EPO mimetic and a TPO mimetic.

2. An immunoglobulin molecule or fragment thereof according to claim 1 further comprising at least one flanking sequence including at least one amino acid covalently linked to at least one end of the peptide mimetic.

3. An immunoglobulin molecule or fragment thereof according to claim 2 wherein the at least one flanking sequence includes a flanking sequence having a proline that is covalently linked to the peptide mimetic.

4. An immunoglobulin molecule or fragment thereof according to claim 1 wherein the immunoglobulin molecule fragment is selected from the group consisting of Fab fragment, $F(ab')_2$ fragment and ScFv fragment.

5. An immunoglobulin molecule or fragment thereof according to claim 1 wherein the immunoglobulin molecule is a full IgG molecule.

6. An immunoglobulin molecule or fragment thereof according to claim 1 wherein the two CDRs are both located on a heavy chain.

7. An immunoglobulin molecule or fragment thereof according to claim 1 wherein the CDRs are a CDR3 of a heavy chain and a CDR2 of a heavy chain.

8. An immunoglobulin molecule or fragment thereof according to claim 1 wherein the TPO mimetic comprises SEQ. ID. NO. 1.

Samsung Bioepis Exhibit No. 1002
Page 2058

9. An immunoglobulin molecule or fragment thereof according to claim 1 wherein a heavy chain CDR3 and CDR2 are replaced with a peptide and the heavy chain comprises a variable region of SEQ. ID. NO. 125.

10. An immunoglobulin molecule or fragment thereof according to claim 1 wherein the immunoglobulin molecule or fragment thereof is human.

11. An immunoglobulin molecule or fragment thereof according to claim 1 wherein the immunoglobulin molecule or fragment thereof is anti-tetanus toxoid.

12. Nucleic acid encoding an immunoglobulin molecule or fragment thereof according to claim 1.

13. An expression vector comprising nucleic acid according to claim 12.

14. A host cell transformed with an expression vector according to claim 13.

15. A method of producing an immunoglobulin molecule or fragment thereof comprising culturing a host cell according to claim 14 under conditions suitable for expression of the immunoglobulin or fragment thereof.

16. A composition comprising an immunoglobulin or fragment thereof according to claim 1 and a pharmaceutically acceptable carrier.

17. An immunoglobulin molecule or fragment thereof comprising a region where amino acid residues corresponding to at least a portion of a complementarity determining region (CDR) is replaced with a peptide mimetic including SEQ. ID NO. 126, wherein X at each occurrence represents any amino acid.

Samsung Bioepis Exhibit No. 1002
Page 2059

18. An immunoglobulin molecule or fragment thereof according to claim 17 wherein the immunoglobulin molecule or fragment thereof is human.

19. An immunoglobulin molecule or fragment thereof according to claim 17 wherein the immunoglobulin molecule or fragment thereof is anti-tetanus toxoid.

20. Nucleic acid encoding an immunoglobulin molecule or fragment thereof according to claim 17.

22. An expression vector comprising nucleic acid according to claim 20.

23. A host cell transformed with an expression vector according to claim 22.

24. A method of producing an immunoglobulin molecule or fragment thereof comprising culturing a host cell according to claim 23 under conditions suitable for expression of the immunoglobulin or fragment thereof.

25. A composition comprising an immunoglobulin or fragment thereof according to claim 17 and a pharmaceutically acceptable carrier.

Samsung Bioepis Exhibit No. 1002
Page 2060

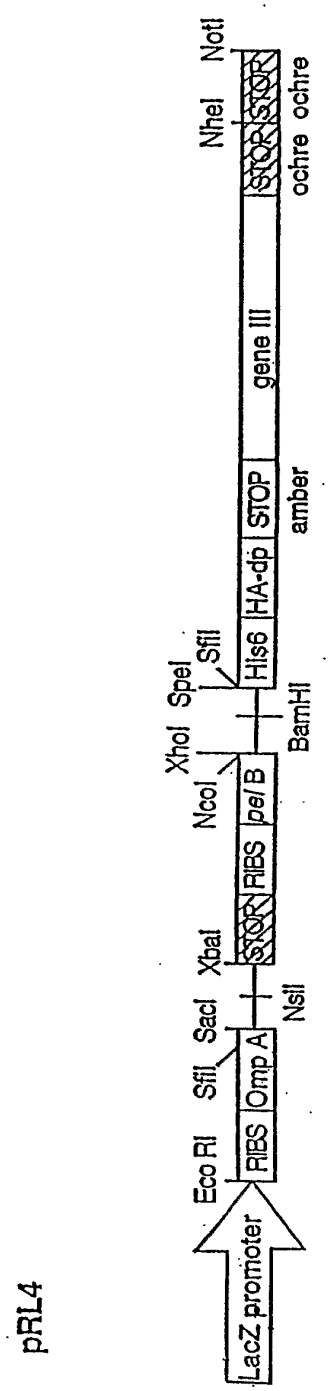


Fig. 1

Samsung Bioepis Exhibit No. 1002
Page 2061

Human Antibody sequence (TT sequence) (SEQ ID NO: 54)
Heavy Chain: cloning sites Xho I and Spe I are underlined

```
1                                                   11
gag gtg cag ctg CTC GAG CAG TCT GGG GCT GAG GTG AAG AAG CCT GGG TCC TCG GTG AAG
glu val gln leu leu glu gln ser gly ala glu val lys lys pro gly ser ser val lys
21                                                  31
GTC TCC TGC AGG GCT TCT GGA GGC ACC TTC AAC AAT TAT GCC ATC AGC TGG GTG CGA CAG
val ser cys arg ala ser gly gly thr phe asn asn tyr ala ile ser trp val arg gln
41                                                  51
GCC CCT GGA CAA GGG CTT GAG TGG ATG GGA GGG ATC TTC CCT TTC CGT AAT ACA GCA AAG
ala pro gly gln gly leu glu trp met gly gly ile phe pro phe arg asn thr ala lys
61                                                  71
TAC GCA CAA CAC TTC CAG GGC AGA GTC ACC ATT ACC GCG GAC GAA TCC ACG GGC ACA GCC
tyr ala gln his phe gln gly arg val thr ile thr ala asp glu ser thr gly thr ala
81                                                  91
TAC ATG GAG CTG AGC AGC CTG AGA TCT GAG GAC ACG GCC ATA TAT TAT TGT GCG AGA GGG
tyr met glu leu ser ser leu arg ser glu asp thr ala ile tyr tyr cys ala arg gly
101                                                 111
GAT ACG ATT TTT GGA GTG ACC ATG GGA TAC TAC GCT ATG GAC GTC TGG GGC CAA GGG ACC
asp thr ile phe gly val thr met gly tyr tyr ala met asp val trp gly gln gly thr
121                                                 131
ACG GTC ACC GTC TCC GCA GCC TCC ACC AAG GGC CCA TCG GTC TTC CCC CTG GCA CCC TCC
thr val thr val ser ala ala ser thr lys gly pro ser val phe pro leu ala pro ser
141                                                 151
TCC AAG AGC ACC TCT GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC AAG GAC TAC TTC CCC
ser lys ser thr ser gly gly thr ala ala leu gly cys leu val lys asp tyr phe pro
161                                                 171
GAA CCG GTG ACG GTG TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC GTG CAC ACC TTC CCG
glu pro val thr val ser trp asn ser gly ala leu thr ser gly val his thr phe pro
181                                                 191
GCT GTC CTA CAG TCC TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG ACC GTG CCC TCC AGC
ala val leu gln ser ser gly leu tyr ser leu ser ser val val thr val pro ser ser
201                                                 211
AGC TTG GGC ACC CAG ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC AGC AAC ACC AAG GTG
ser leu gly thr gln thr tyr ile cys asn val asn his lys pro ser asn thr lys val
221                                                 231
GAC AAG AAA GTT GAG CCC AAA TCT TGT GAC AAA act agt
asp lys lys val glu pro lys ser cys asp lys thr ser
```

Fig. 2A

Samsung Bioepis Exhibit No. 1002
Page 2062

Human Antibody Sequence (TT sequence) (SEQ ID NO: 55)

Light Chain: cloning sites Sac I and Xba I are underlined

```
1                                   11
gag ctc acg cag tct cca ggc acc ctg tct ttg tct ccA ggg gaa aga gcc acc ctc tcc
glu leu thr gln ser pro gly thr leu ser leu ser pro gly glu arg ala thr leu ser
21                                  31
tgc agg gcc agt cac agt gtt agc agg gcc tac tta gcc tgg tac cag cag aaa cct ggc
cys arg ala ser his ser val ser arg ala tyr leu ala trp tyr gln gln lys pro gly
41                                  51
cag gct ccc agg ctc ctc atc tat ggt aca tcc agc agg gcc act ggc atc cca gac agg
gln ala pro arg leu leu ile tyr gly thr ser ser arg ala thr gly ile pro asp arg
61                                  71
ttc agt ggc agt ggg tct ggg aca gac ttc act ctc acc atc agc aga ctg gag cct gaa
phe ser gly ser gly ser gly thr asp phe thr leu thr ile ser arg leu glu pro glu
81                                  91
gat ttt gca gtg tac tac tgt cag cag tat ggt ggc tca ccg tgg ttc ggc caa ggg acC
asp phe ala val tyr tyr cys gln gln tyr gly gly ser pro trp phe gly gln gly thr
101                                 111
AAG GTG GAA CTC AAA CGA ACT GTG GCT GCA CCA TCT GTC TTC ATC TTC CCG CCA TCT GAT
lys val glu leu lys arg thr val ala ala pro ser val phe ile phe pro pro ser asp
121                                 131
GAG CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG TGC CTG CTG AAT AAC TTC TAT CCC AGA
glu gln leu lys ser gly thr ala ser val val cys leu leu asn asn phe tyr pro arg
141                                 151
GAG GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC CTC CAA TCG GGT AAC TCC CAG GAG AGT
glu ala lys val gln trp lys val asp asn ala leu gln ser gly asn ser gln glu ser
161                                 171
GTC ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC AGC CTC AGC AGC ACC CTG ACG CTG AGC
val thr glu gln asp ser lys asp ser thr tyr ser leu ser ser thr leu thr leu ser
181                                 191
AAA GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC TGC GAA GTC ACC CAT CAG GGC CTG AGC
lys ala asp tyr glu lys his lys val tyr ala cys glu val thr his gln gly leu ser
201                                 211
TTG CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG TGT TAG Ttc tag a
leu pro val thr lys ser phe asn arg gly glu cys AMB
```

Fig. 2B

Samsung Bioepis Exhibit No. 1002
Page 2063

Method of grafting peptide into antibody with random sequences surrounding peptide sequence

FR3 TPO Mimetic Peptide

(SEQ ID NO: 56) → Y Y C A R X X I E G P T L R Q

(SEQ ID NO: 57) → TATTAT-TGT-GCG-AGA-NNR-NNR-ATT-GAA-GGG-CCG-ACG-CTG-CGG-CAA-

FR4

W L A A R A X X W G Q G T

TGG-CTG-GCG-GCG-CGC-GCG-NNY-NNY-TGG-GGC-CAA-GGG-ACC-

The TPO mimetic peptide was grafted into the heavy chain CDR3 region of the tetanus toxoid antibody. The peptide was flanked on either side by two random amino acids, shown as "X"s in the figure.

Fig. 3

Samsung Bioepis Exhibit No. 1002
Page 2064

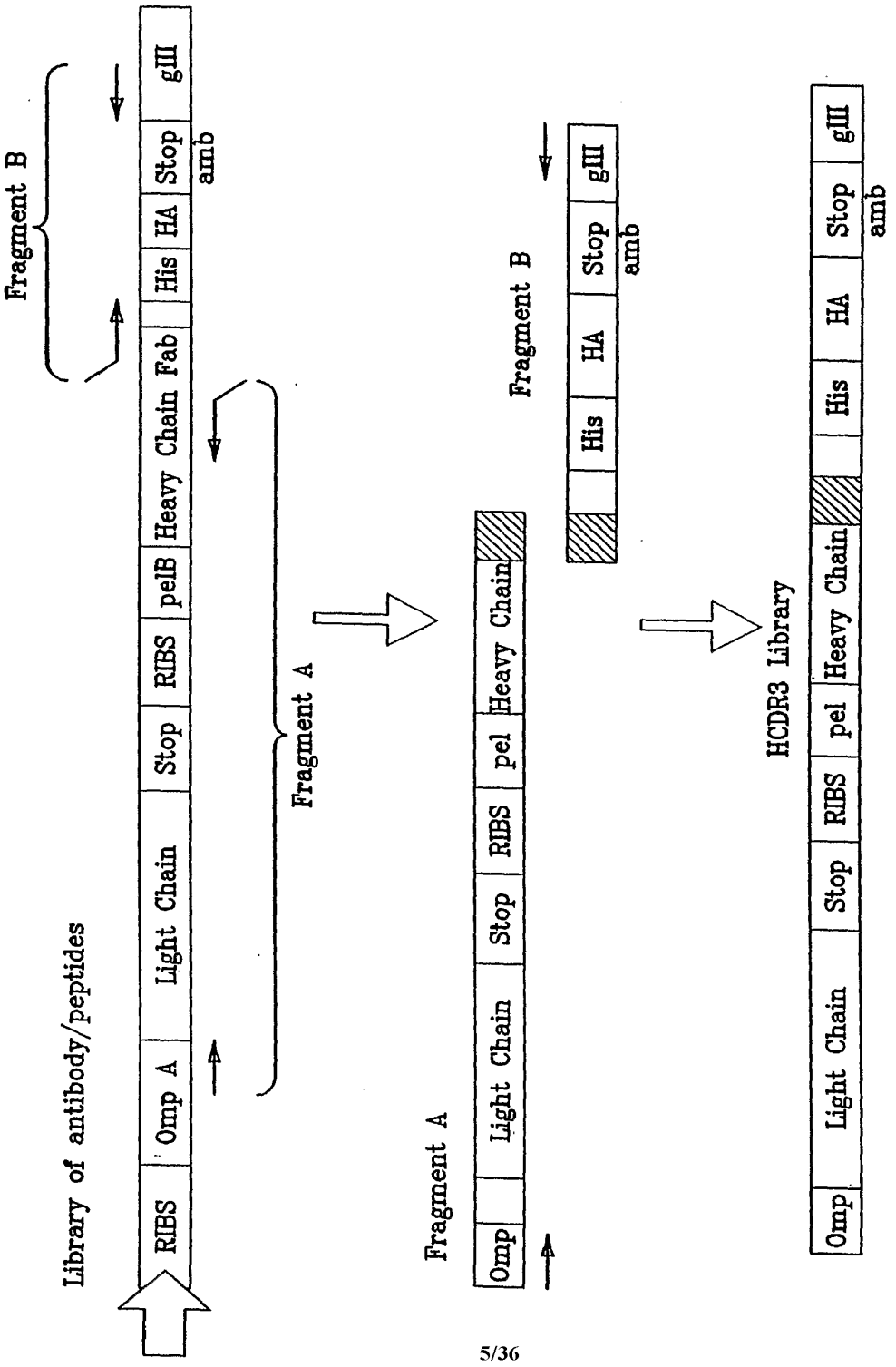


FIG. 4

Samsung Bioepis Exhibit No. 1002
Page 2065

CLONE	AMINO ACID SEQUENCE	SEQ ID NO.
X1a	Pro-Prp-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-Gly-Gly CCG-CCC-ATT-GAA-GGG-CCG-ACG-CTG-CGG-CAA-TGG-CTG-GCG-GCG-CGC-GCG-GGA-GGC	25 26
X1a-11	Gly-Gly-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Ala-Arg-Ala-Gly-Gly GGG-GGT-ATT-GAA-GGG-CCG-ACG-CTG-CGG-CAA-TGG-CTG-GCG-GCG-CGC-GCG-GGC-GGA	27 28
X1a-13	Gly-Gly-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-Gly-Gly GGC-GGT-ATT-GAA-GGG-CCG-ACG-CTG-CGG-CAA-TGG-CTG-GCG-GCG-CGC-GCG-GGA-GGC	29 30
X1c	Trp-Leu-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-Pro-Val TGG-CTG-ATT-GAA-GGG-CCG-ACG-CTG-CGG-CAA-TGG-CTG-GCG-GGG-CGC-GCG-CCT-GTC	31 32
X2c	Met-Ile-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-Val-Gly ATG-ATA-ATT-GAA-GGG-CCG-ACG-CTG-CGG-CAA-TGG-CTG-GCG-GCG-CGC-GCG-GTT-GGC	33 34
X3a	Val-Val-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-Pro-Val GTG-GTA-ATT-GAA-GGG-CCG-ACG-CTG-CGG-CAA-TGG-CTG-GCG-GCG-CGC-GCG-CCT-GTT	35 36
X3b	Gly-Pro-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-Pro-Asp GGG-CCG-ATT-GAA-GGG-CCG-ACG-CTG-CGG-CAA-TGG-CTG-GCG-GCG-CGC-GCG-CCC-GAT	37 38
X4b	Leu-Pro-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-Pro-Val TTG-CCA-ATT-GAA-GGG-CCG-ACG-CTG-CGG-CAA-TGG-CTG-GCG-GCG-CGC-GCG-CCT-GTT	39 40
X4c	Ser-Leu-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-Pro-Ile TCA-CTG-ATT-GAA-GGG-CCG-ACG-CTG-CGG-CAA-TGG-CTG-GCG-GCG-CGC-GCG-CCC-ATC	41 42
X5a	Thr-Met-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-Pro-Val ACA-ATG-ATT-GAA-GGG-CCG-ACG-CTG-CGG-CAA-TGG-CTG-GCG-GCG-CGC-GCG-CCC-GTT	43 44
X5c	Thr-Thr-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-Pro-Val ACG-ACA-ATT-GAA-GGG-CCG-ACG-CTG-CGG-CAA-TGG-CTG-GCG-GCG-CGC-GCG-CCT-GTC	45 46
X7a	Thr-Arg-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-Cys-Ser ACA-CGG-ATT-GAA-GGG-CCG-ACG-CTG-CGG-CAA-TGG-CTG-GCG-GCG-CGC-GCG-TGC-AGC	47 48
X7b	no peptide deletion mutant	
X7c	Gln-Thr-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-Pro-Asp CAG-ACA-ATT-GAA-GGG-CCG-ACG-CTG-CGG-CAA-TGG-CTG-GCG-GCG-CGC-GCG-CCT-CAC	49 50

Fig. 5

Samsung Bioepis Exhibit No. 1002
Page 2066

pRL8

(SEQ ID NO: 60)

GGGAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAA
ATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATC
AAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGT
CCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATC
AGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTC
GAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGA
GCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGC
GAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGT
AACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCAGGTGGC
ACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACA
TTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAAT
ATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCC
TTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAA
GTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGG
ATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCA
ATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGA
CGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTG
GTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAA
GAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTT
ACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAAC
ATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAG
CCATACCAAACGACGAGCGTGTACACCACGATGCCTGTAGCAATGGCAACAAC
GTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAAT
TAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGC
CCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGT
CTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGT
AGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACA
GATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAA
GTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGG
ATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGA
GTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTT
GAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCG
CTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAA
GGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAG
CCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGC
TCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTA
CCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTG
AACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGA

Fig. 6A

Samsung Bioepis Exhibit No. 1002
Page 2067

ACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGG
AGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGC
ACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGT
TTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGG
AGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTG
CTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAA
CCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACC
GAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAA
CCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTT
TCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTC
ACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTG
TGGAATTGTGAGCGGATAACAATTGAATTCAGGAGGAATTTAAAATGAAAAA
GACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCCCAG
GCGGCCGAGCTCGGCCATGGCTGGTTGGGCAGCGAGTAATAACAATCCAGCG
GCTGCCGTAGGCAATAGGTATTTCATTATGACTGTCTCCTTGGCGACTAGCTA
GTTTAGAATTCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCC
GCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGG
GGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGC
TTTCCAGTCGGGAAACCTGTCGTGTTACTAATGATGGTGATGGTGATGGCTAG
TTTTGTCACAAGATTTGGGCTCAACTTTCTTGTCCACCTTGGTGTTGCTGGGCT
TGTGATTCACGTTGCAGATGTAGGTCTGGGTGCCCAAGCTGCTGGAGGGCAC
GGTCACCACGCTGCTGAgGGAGTAGAGTCCTGAGGACTGTAGGACAGCCGGG
AAGGTGTGCACGCCGCTGGTCAgGGCGCCTGAgTTCCACGACACcGTCGCCGG
TTCgGGGAAGTAGTCCTTGACCAGGCAGCCCAGGGCCGCTGTGCCCCCAGAG
GTGCTCTTGGAGGAGGGTGCCAGGGGGAAGACCGATGGGCCCTTGGTGGAG
GCTGCGGAGACGGTGACCGTGGTACCAGCAGAAACCTGGCCAGGCTCCCAG
GCTCCTCATCTATGGTACATCCAGCAGGGCCACTGGCATCCCAGACAGGTTC
AGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGACTGGAGC
CTGAAGATTTTGCAGTGTACTACTGTCAGCAGTATGGTGGCTCACCGTGGTTC
GGCCAAGGGACCAAGGTGGAACTCAAACGAACTGTGGCTGCACCATCTGTCT
TCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTG
TGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGG
ATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACAG
CAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGA
CTACGAGAAACACAAAGTCTACGCCTGCGAAGTCacccatcagggcctgagttcgcccgtcac
aaagagcttcaacggaggagagtgttaatTCTAGATAATTAATTAGGAGGAATTTAAAATGAA
ATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCTGCCCAACCAG
CCATGGCCGAGGTGCAGCTGCTCGAGATGAGCGATAAAATTATTCACCTGAC
TGACGACAGTTTTGACACGGATGTACTCAAAGCGGACGGGGCGATCCTCGTC
GATTTCTGGGCAGAGTGGTGCGGTCCGTGCAAAATGATCGCCCCGATTCTGG
ATGAAATCGCTGACGAATATCAGGGCAAACTGACCGTTGCAAAACTGAACAT
CGATCAAAACCCTGGCACTGCGCCGAAATATGGCATCCGTGGTATCCCGACT
CTGCTGCTGTTCAAAAACGGTGAAGTGGCGGCAACCAAAGTGGGTGCACTTG
TCTAAAGGTCAGTTGAAAGAGTTCCTCGACGCTAACCTGGCGTACCCGTACG
ACGTTCCGGACTACGGTTCTACTAGTccgaaaccgtctaccccaccgggctcttcctgcggtggccgc
atcgcccgtctggaggaaaaagtgaaaaccctgaaagctcagaactccgagctggcgtccactgccaacatgctgcgcgaac

Fig. 6B

Samsung Bioepis Exhibit No. 1002
Page 2068

aggtggcacagctgaaacagaaagttatgaaccatggcggttgtgctagtGGCCAGGCCGGCCAGCACCAT
CACCATCACCATGGCGCATACCCGTACGACGTTCCGGACTACGCTTCTTAGG
AGGGTGGTGGCTCTGAGGGTGGCGGTTCTGAGGGTGGCGGCTCTGAGGGAGG
CGGTTCCGGTGGTGGCTCTGGTTCCGGTGATTTTGATTATGAAAAGATGGCAA
ACGCTAATAAGGGGGCTATGACCGAAAATGCCGATGAAAACGCGCTACAGTC
TGACGCTAAAGGCAAACTTGATTCTGTCGCTACTGATTACGGTGCTGCTATCG
ATGGTTTCATTGGTGACGTTTCCGGCCTTGCTAATGGTAATGGTGCTACTGGT
GATTTTGCTGGCTCTAATTCCCAAATGGCTCAAGTCGGTGACGGTGATAATTC
ACCTTTAATGAATAATTTCCGTCAATATTTACCTTCCCTCCCTCAATCGGTTGA
ATGTCGCCCTTTTGTCTTTAGCGCTGGTAAACCATATGAATTTTCTATTGATTG
TGACAAAATAAACTTATTCCGTGGTGTCTTTGCGTTTCTTTTATATGTTGCCAC
CTTTATGTATGTATTTTCTACGTTTGCTAACATACTGCGTAATAAGGAGTCTTA
AGCTAGCTAATTAATTTAAGCGGCCGCAGATCT

Fig. 6C

Samsung Bioepis Exhibit No. 1002
Page 2069

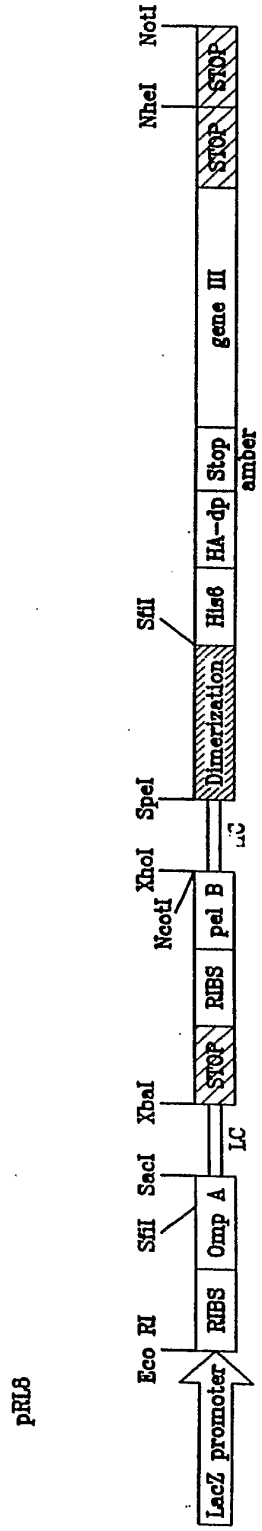


FIG. 7

Samsung Bioepis Exhibit No. 1002
Page 2070

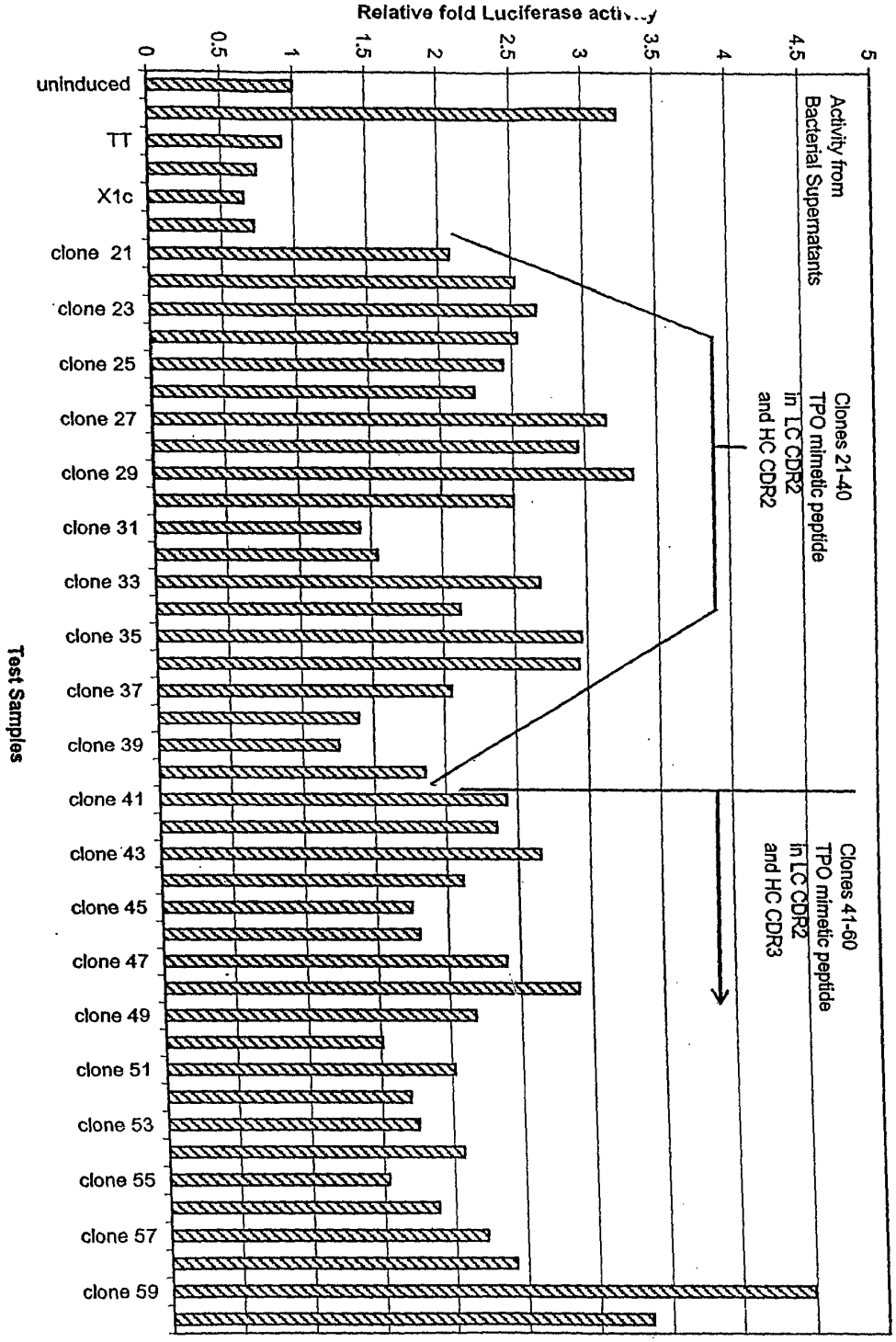
Fig. 8

Samsung Bioepis Exhibit No. 1002
Page 2071

TPO Positive Clones nnk nnk nnk nnk
1 2 IEGPTLRQWLAARA 3 4

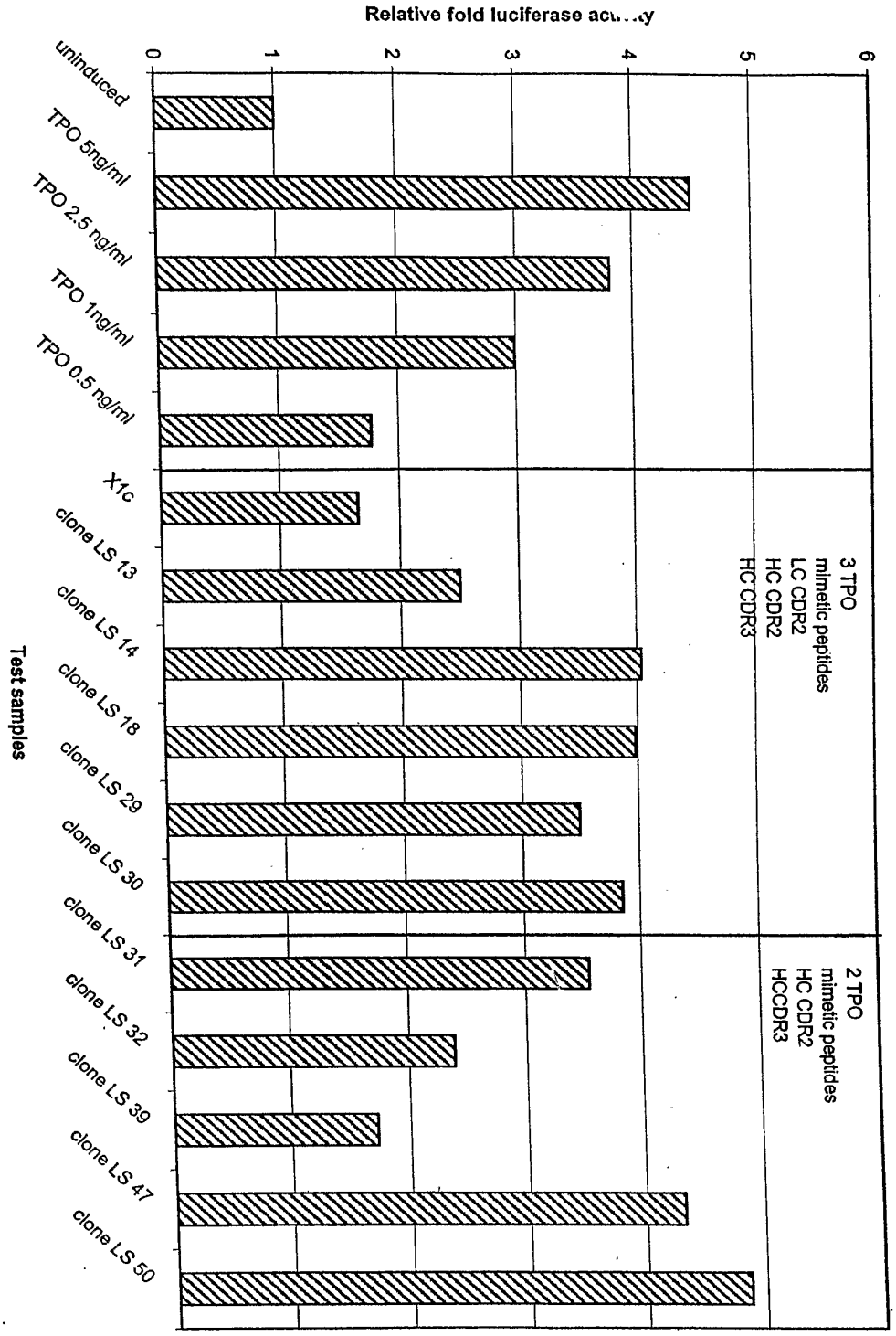
	Sample	nnk 1	nnk 2	nnk 3	nnk 4	Amino Acids 1	Amino Acids 2	Amino Acids 3	Amino Acids 4
HC CDR3	X1c	tgg	ctg	cct	gtc	Trp	Leu	Pro	Val
	X3a	gtg	gta	cct	gtt	Val	Val	Pro	Val
	X3b	ggg	ccg	ccc	gat	Gly	Pro	Pro	Asp
	X4b	ttg	cca	cct	gtt	Leu	Pro	Pro	Val
	X4c	tca	ctg	ccc	atc	Ser	Leu	Pro	Ile
	X5a	aca	atg	ccc	gtt	Thr	Met	Pro	Val
	X5c	acg	aca	cct	gtc	Trp	Leu	Pro	Val
	X7c	cag	aca	cct	cac	Gln	Thr	Pro	Asp
HC CDR2	24	ctt	tat	tct	aat	Leu	Tyr	Ser	Asn
	39	act	tac	ttg	cat	Thr	Tyr	Leu	His
	3	agg	atg	ctc	gag	Arg	Met	Leu	Glu
	7	aag	gaa	tct	aag	Lys	Glu	Ser	Lys
	8	gcg	cat	gtg	cag	Ala	His	Val	Gln
	10	cag	gag	att	agt	Gln	Glu	Ile	Ser
	11	cgg	aat	aat	ccg	Arg	Asn	Asn	Pro
	19	cag	cta	aat	tct	Gln	Leu	Asn	Ser
	25	agt	att	ttt	gtc	Ser	Ile	Phe	Val
	28	ggg	ccc	act	agt	Gly	Pro	Thr	Ser
LC CDR1	10	aag	ggt	gtt	agt	Lys	Gly	Val	Ser
	11	cat	ggg	gtg	gct	His	Gly	Val	Ala
	12a	cgt	acg	atg	gct	Arg	Thr	Met	Ala
	12b	cgt	ggt	gtt	aat	Arg	Gly	Val	Asn
	14	cgt	tcg	ctt	gcg	Arg	Ser	Leu	Ala
	16	cgg	ggt	gtt	gcg	Arg	Gly	Val	Ala
	18	agg	acg	gtg	tct	Arg	Thr	Val	Ser
	47	aag	ggg	gtg	gcg	Lys	Gly	Val	Ala
LC CDR2	1	aat	ccg	agg	ggt	Asn	Pro	Arg	Gly
	2	tcg	cct	cgg	agt	Ser	Pro	Arg	Ser
	3	tcg	cct	cgt	acg	Ser	Pro	Arg	Thr
	4	tcg	cct	cgg	cgt	Ser	Pro	Trp	Arg
	5	act	ccg	aat	tgg	Thr	Pro	Asn	Trp
	6	aat	cct	gcg	agg	Asn	Pro	Ala	Arg
	7	aat	ccg	tcg	ggg	Asn	Pro	Ser	Gly
	9	aat	cct	tat	tag	Asn	Pro	Tyr	Stop
	10	aat	ccg	cgg	tct	Asn	Pro	Arg	Ser
	11	aat	ccg	gat	gtg	Asn	Pro	Asp	Val
	12	tcg	ccg	tcg	cgg	Ser	Pro	Ser	Arg
	13	aat	cct	ctg	ttt	Asn	Pro	Leu	Phe
	14	aat	ctt	ggg	tat	Asn	Pro	Gly	Tyr
	15	aat	cct	att	agt	Asn	Pro	Ile	Ser
	16	aat	cct	cag	cgg	Asn	Pro	Gln	Arg
	18	aat	ccg	cgg	acg	Asn	Pro	Arg	Thr
	19	aat	ccg	cgt	ggg	Asn	Pro	Arg	Gly
	20	cat	ttg	aga	ctg	His	Leu	Arg	Leu
	21	aag	tag	att	tat	Lys	Stop	Ile	Tyr
	23	aat	cct	ggt	aag	Asn	Pro	Gly	Lys
	24	aat	cct	cgt	ggg	Asn	Pro	Arg	Gly
	26	aat	cct	aat	gtg	Asn	Pro	Asn	Val
	27	tct	ccg	cgg	gtt	Ser	Pro	Arg	Val
	29	acg	cct	cgg	ggt	Thr	Pro	Arg	Gly
	30	ct	tag	tgg	tgg	Pro	Stop	Trp	Trp

Samsung Bioepis Exhibit No. 1002
Page 2072



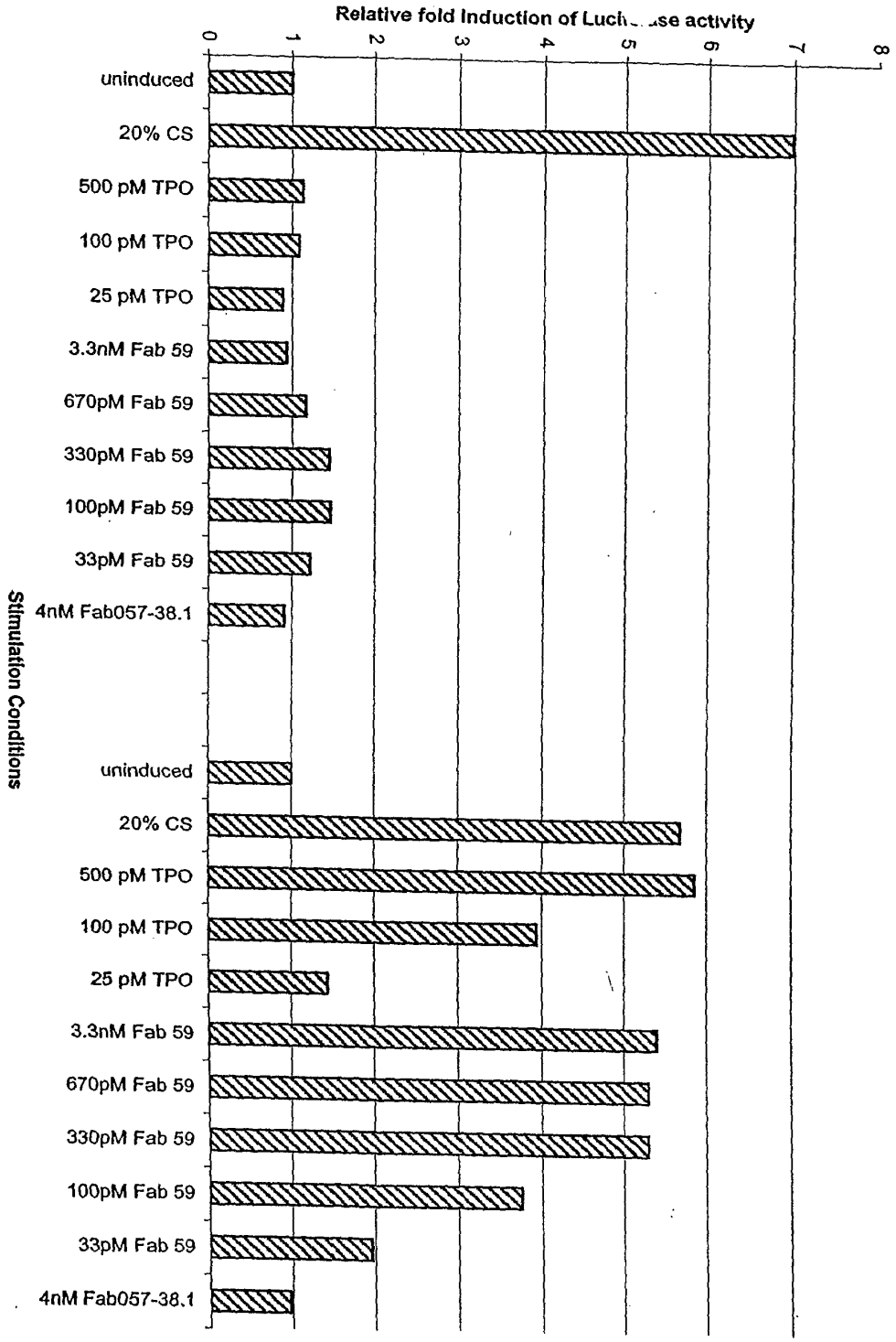
Activity of Fab clones containing 2 TPO mimetic peptides

Samsung Bioepis Exhibit No. 1002
Page 2073



Activity of Fab clones containing 2 or 3 TPO mimetic peptides

Samsung Bioepis Exhibit No. 1002
Page 2074



TPO mimetic Activity of semi-purified Fab clone 59

Samsung Bioepis Exhibit No. 1002
Page 2075

(SEQ ID NO: 67)

5G1.1 – TPO Heavy Chain (Bold denotes TPO mimetic) Amino acid sequence:

*MKIVSWVILFLLSVTAGVHS*QVQLVQSGAEVKKPGASVKVSCKASGYIFSNYWIQW
VRQAPGQGLEWMGEILPGSGSTEYTENFKDRVTMTRDTSTSTVYMELSSLRSED
TAVYYCARLPIEGPTLRQWLAARAPVWGQGTLVTVSSASTKGPSVFPLAPCSR
STSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTV
PSSNFGTQTYTCNVDHKPSNTKVDKTVERKCCVECPPCPAPPVAGPSVFLFPPKP
KDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTY
RVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQ
EEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRL
TVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK.

(SEQ ID NO: 68)

5G1.1 – TPO Heavy Chain (Bold denotes TPO mimetic) Nucleic acid sequence:

ATGAAGTGGAGCTGGGTTATTCTCTTCCTCCTGTCAGTAACTGCCGGCGTCCA
CTCCCAAGTCCAACTGGTGCAATCCGGCGCCGAGGTCAAGAAGCCAGGGGCC
TCAGTCAAAGTGTCCTGTAAAGCTAGCGGCTATATTTTTTCTAATTATTGGAT
TCAATGGGTGCGTCAGGCCCCCGGGCAGGGCCTGGAATGGATGGGTGAGATC
TTACCGGGCTCTGGTAGCACCGAATATACCGAAAATTTTAAAGACCGTGTTA
CTATGACGCGTGACACTTCGACTAGTACAGTATACATGGAGCTCTCCAGCCTG
CGATCGGAGGACACGGCCGTCTATTATTGCGCGCGTTTGCCAATTGAAGGG
CCGACGCTGCGGCAATGGCTGGCGGCGCGCGCGCCTGTTTGGGGTCAAG
GAACCCTGGTCACTGTCTCGAGCGCCTCCACCAAGGGCCCATCCGTCTTCCCC
CTGGCGCCCTGCTCCAGGAGCACCTCCGAGAGCACAGCCGCCCTGGGCTGCC
TGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGC
CCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCT
ACTCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAACTTCGGCACCCAGAC
CTACACCTGCAACGTAGATCACAAGCCCAGCAACACCAAGGTGGACAAGAC
AGTTGAGCGCAAATGTTGTGTCGAGTGCCCACCGTGCCCAGCACCACCTGTG
GCAGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGAT
CTCCCGGACCCCTGAGGTCACGTGCGTGGTGGTGGACGTGAGCCAGGAAGAC
CCCGAGGTCCAGTTCAACTGGTACGTGGATGGCGTGGAGGTGCATAATGCCA
AGACAAAGCCGCGGGAGGAGCAGTTCAACAGCACGTACCGTGTGGTCAGCG
TCCTCACCGTCCTGCACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGCAA
GGTCTCCAACAAAGGCCTCCCGTCCTCCATCGAGAAAACCATCTCCAAAGCC
AAAGGGCAGCCCCGAGAGCCACAGGTGTACACCCTGCCCCCATCCCAGGAG
GAGATGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTACC
CCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACT
ACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGC
AGGCTAACCGTGGACAAGAGCAGGTGGCAGGAGGGGAATGTCTTCTCATGCT
CCGTGATGCATGAGGCTCTGCACAACCACTACACACAGAAGAGCCTCTCCCT
GTCTCTGGGTAAATGA

Samsung Bioepis Exhibit No. 1002
Page 2076

(SEQ ID NO: 69)

5G1.1 Light Chain Amino Acid Sequence

*MDMRVPAQLLGLLLLWLRGARC*DIQMTQSPSSLSASVGDRVTITCGASENIYGALN
WYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
NVLNTPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK
VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTH
QGLSSPVTKSFNRGEC.

(SEQ ID NO: 70)

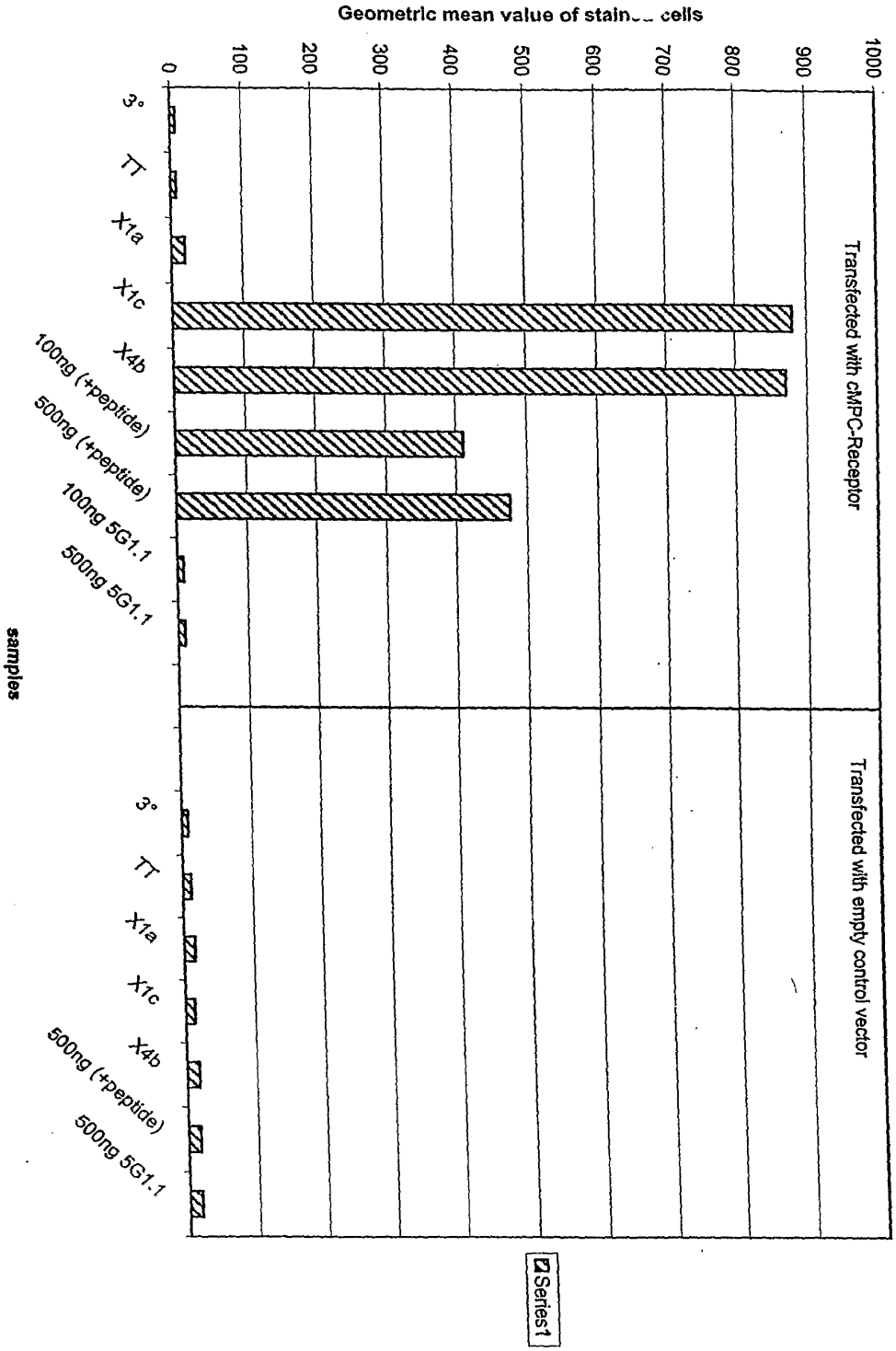
5G1.1 Light Chain Nucleic Acid Sequence

ATGGACATGAGGGTCCCCGCTCAGCTCCTGGGGCTCCTGCTACTCTGGCTCCG
AGGTGCCAGATGTGATATCCAGATGACCCAGTCCCCGTCCTCCCTGTCCGCCT
CTGTGGGCGATAGGGTCACCATCACCTGCGGCGCCAGCGAAAACATCTATGG
CGCGCTGAACTGGTATCAACAGAAACCCGGGAAAGCTCCGAAGCTTCTGATT
TACGGTGCGACGAACCTGGCAGATGGAGTCCCTTCTCGCTTCTCTGGATCCGG
CTCCGGAACGGATTTCACTCTGACCATCAGCAGTCTGCAGCCTGAAGACTTC
GCTACGTATTACTGTCAGAACGTTTTAAATACTCCGTTGACTTTCGGACAGGG
TACCAAGGTGGAAATAAAACGAACTGTGGCTGCACCATCTGTCTTCATCTTCC
CGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGCCTGCTG
AATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCC
TCCAATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACA
GCACCTACAGCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGACTACGAGA
AACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGT
CACAAAGAGCTTCAACAGGGGAGAGTGTTAG

Note: Italics denotes leader sequence

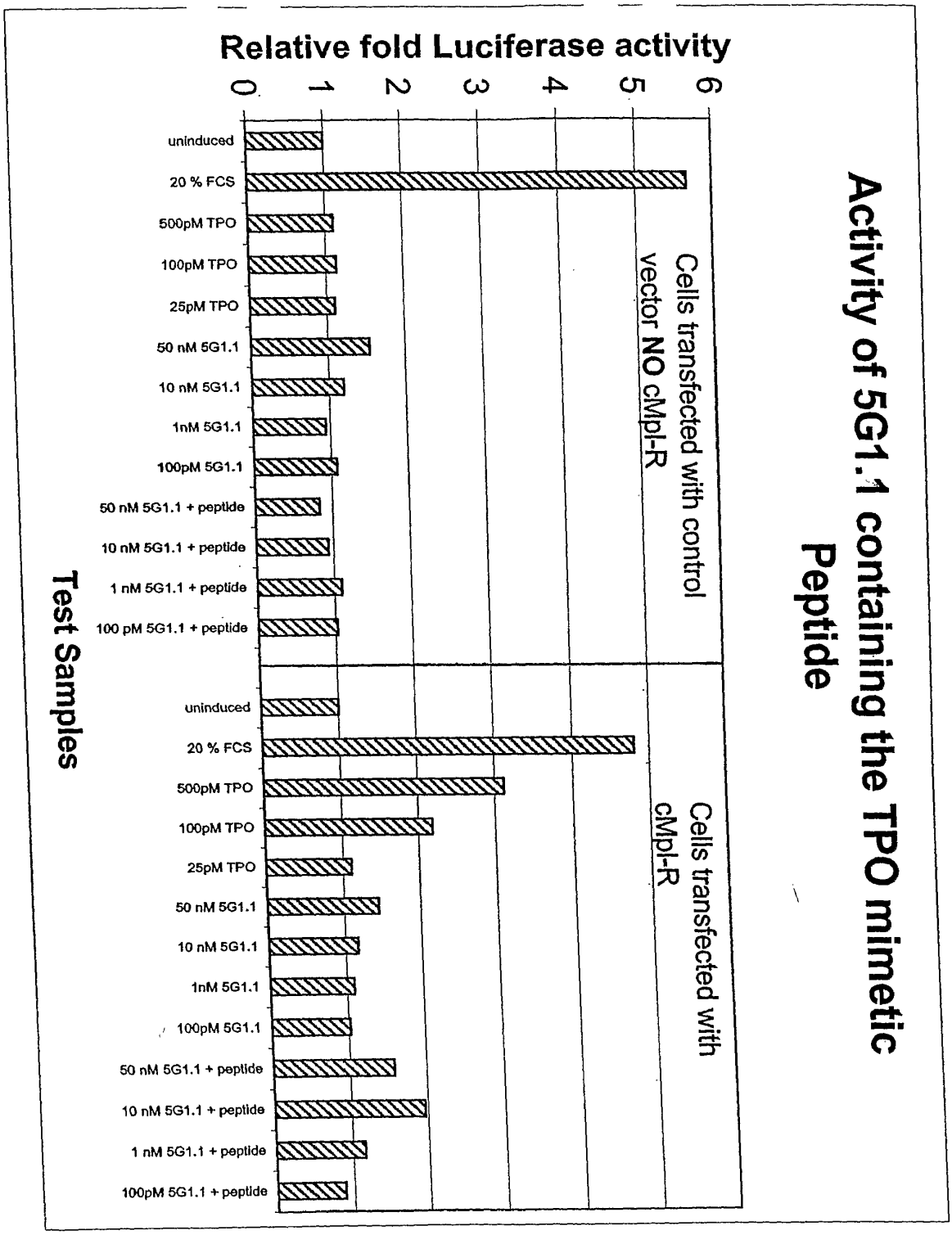
Fig. 13B

Samsung Bioepis Exhibit No. 1002
Page 2077



FACS staining on transfected 293 cells

Samsung Bioepis Exhibit No. 1002
Page 2078



Samsung Bioepis Exhibit No. 1002
Page 2079

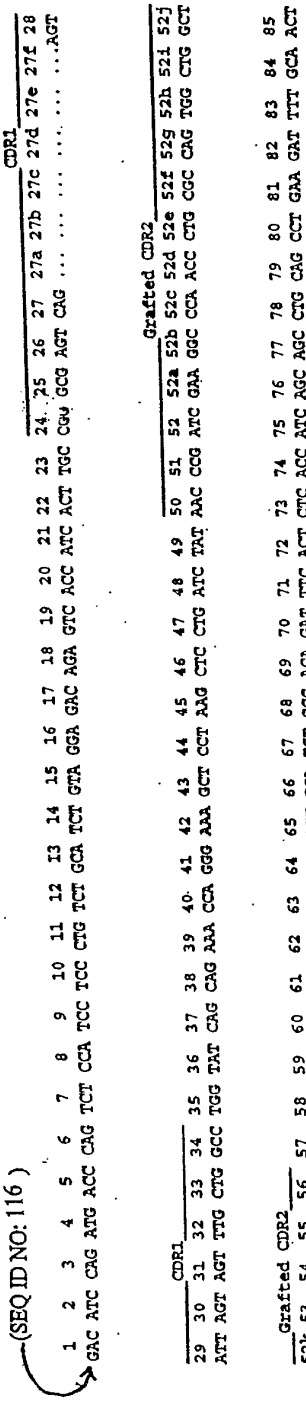


Fig. 16

Samsung Bioepis Exhibit No. 1002
Page 2080

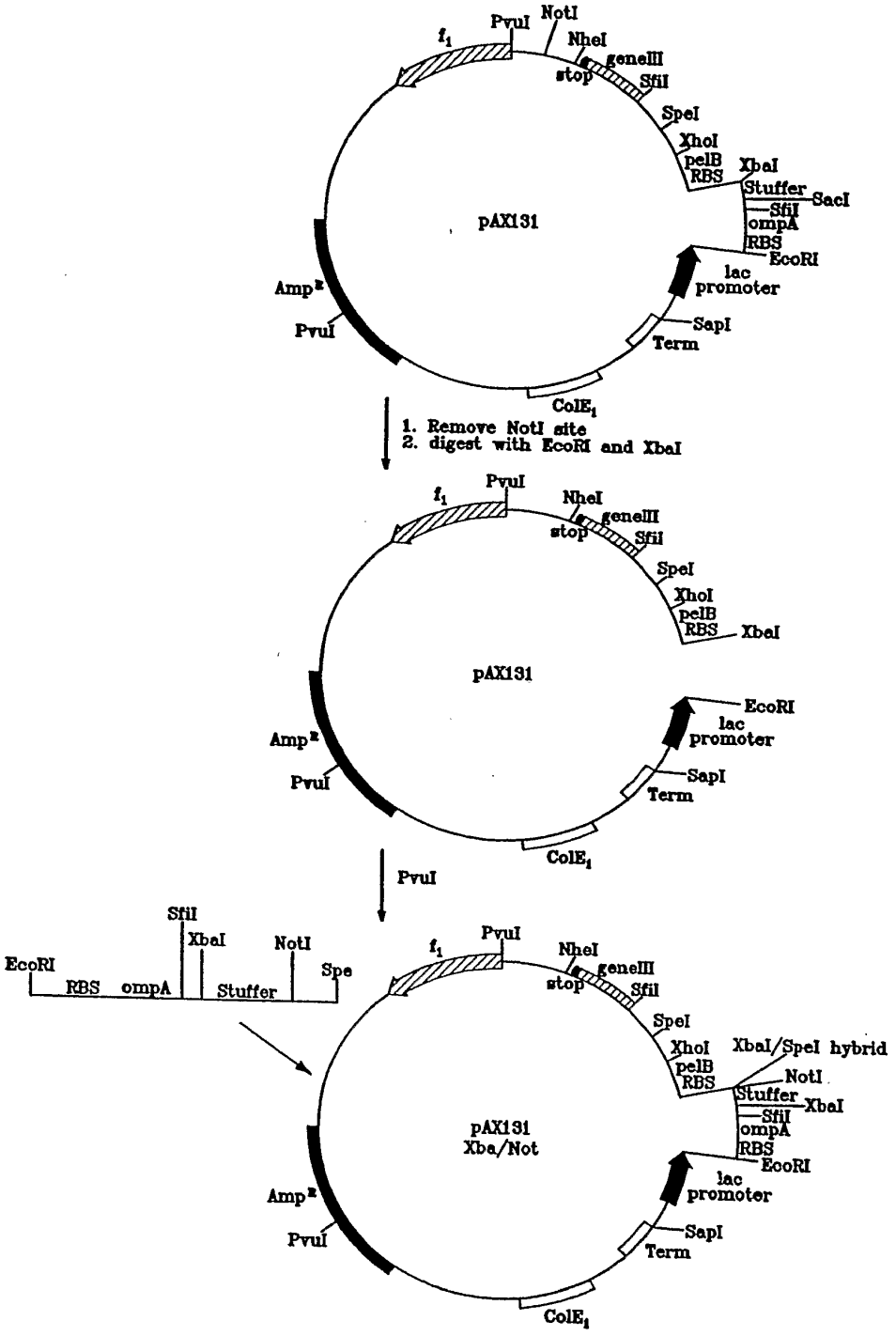


FIG. 17

Samsung Bioepis Exhibit No. 1002
Page 2081

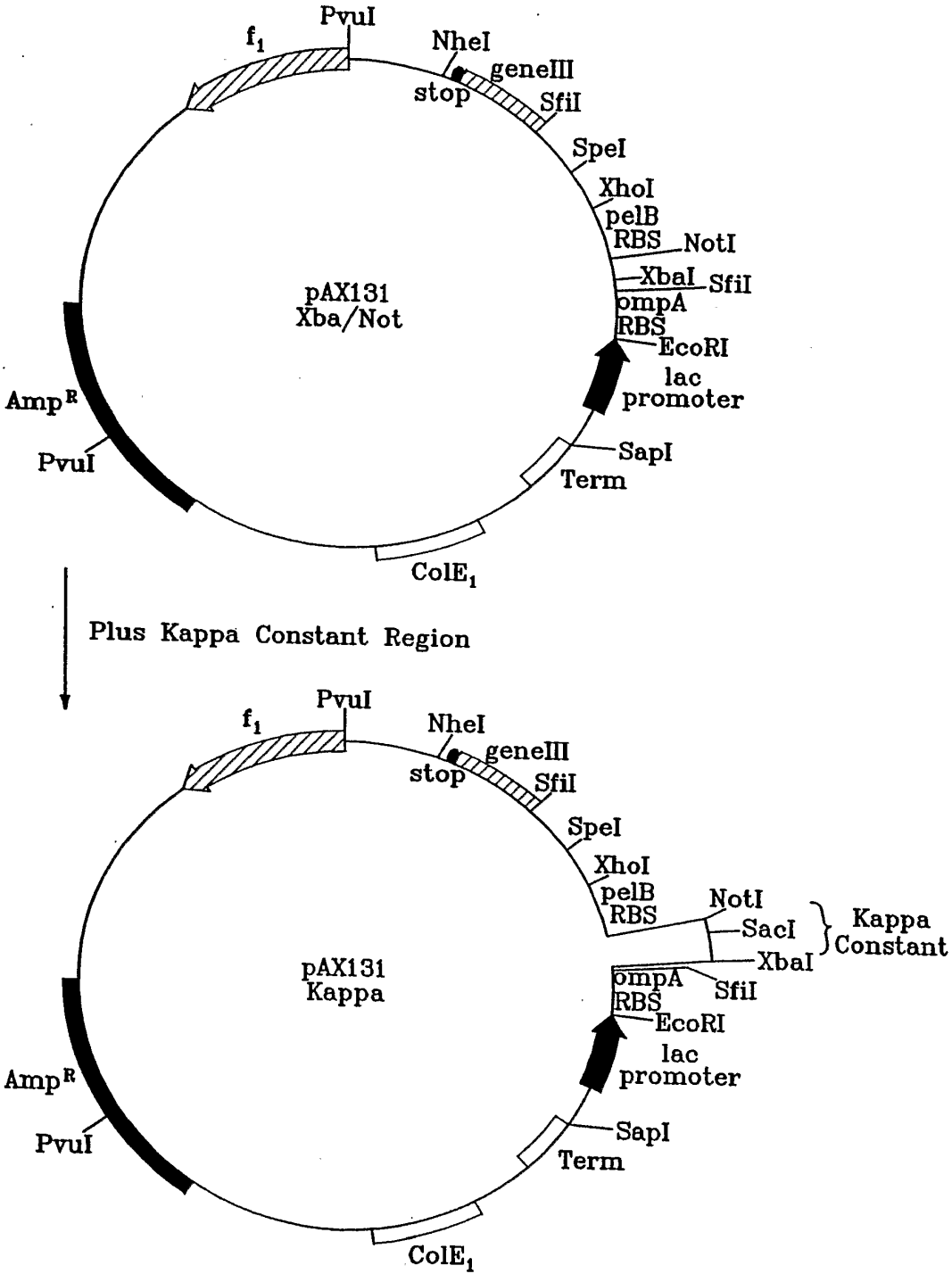


FIG. 18

Samsung Bioepis Exhibit No. 1002
Page 2082

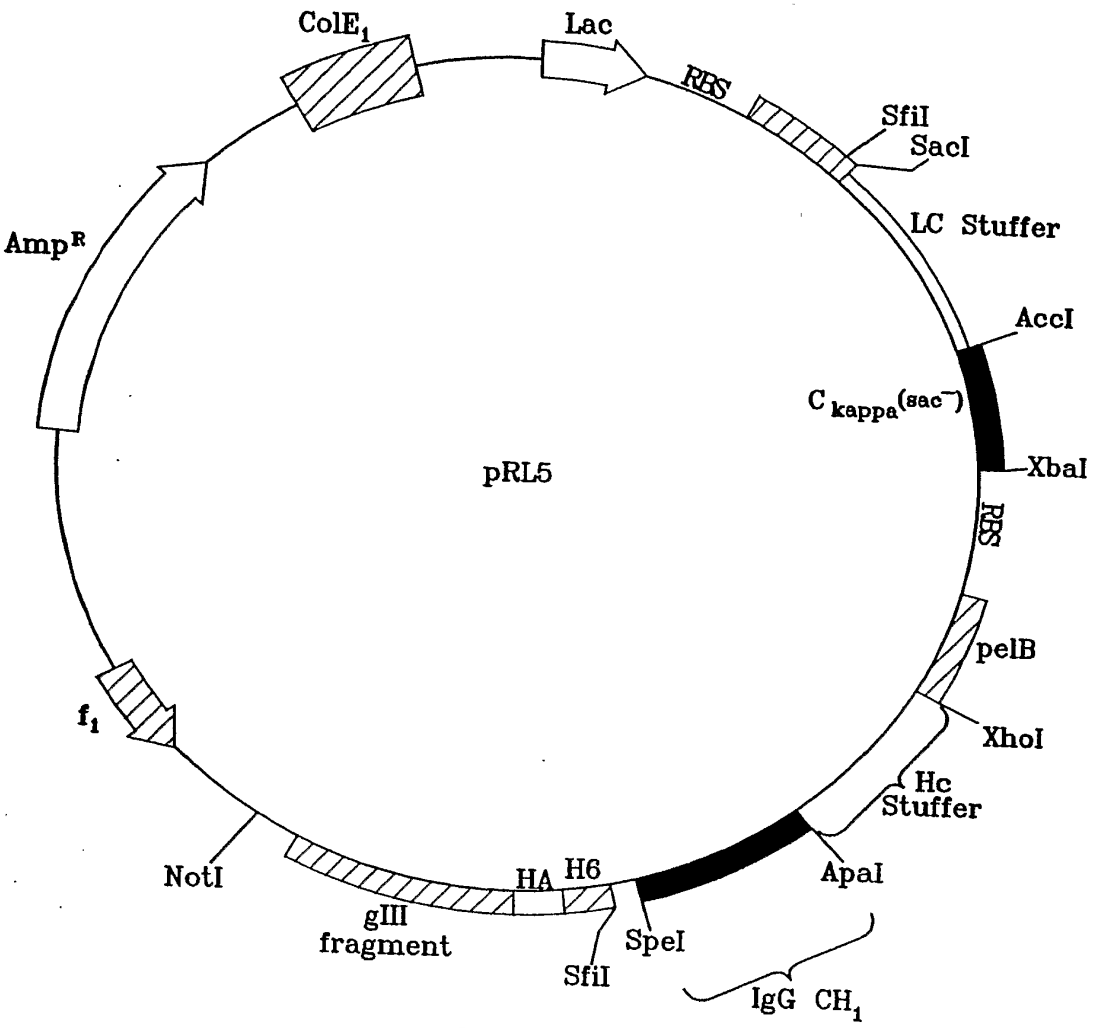


FIG. 19

Samsung Bioepis Exhibit No. 1002
Page 2083

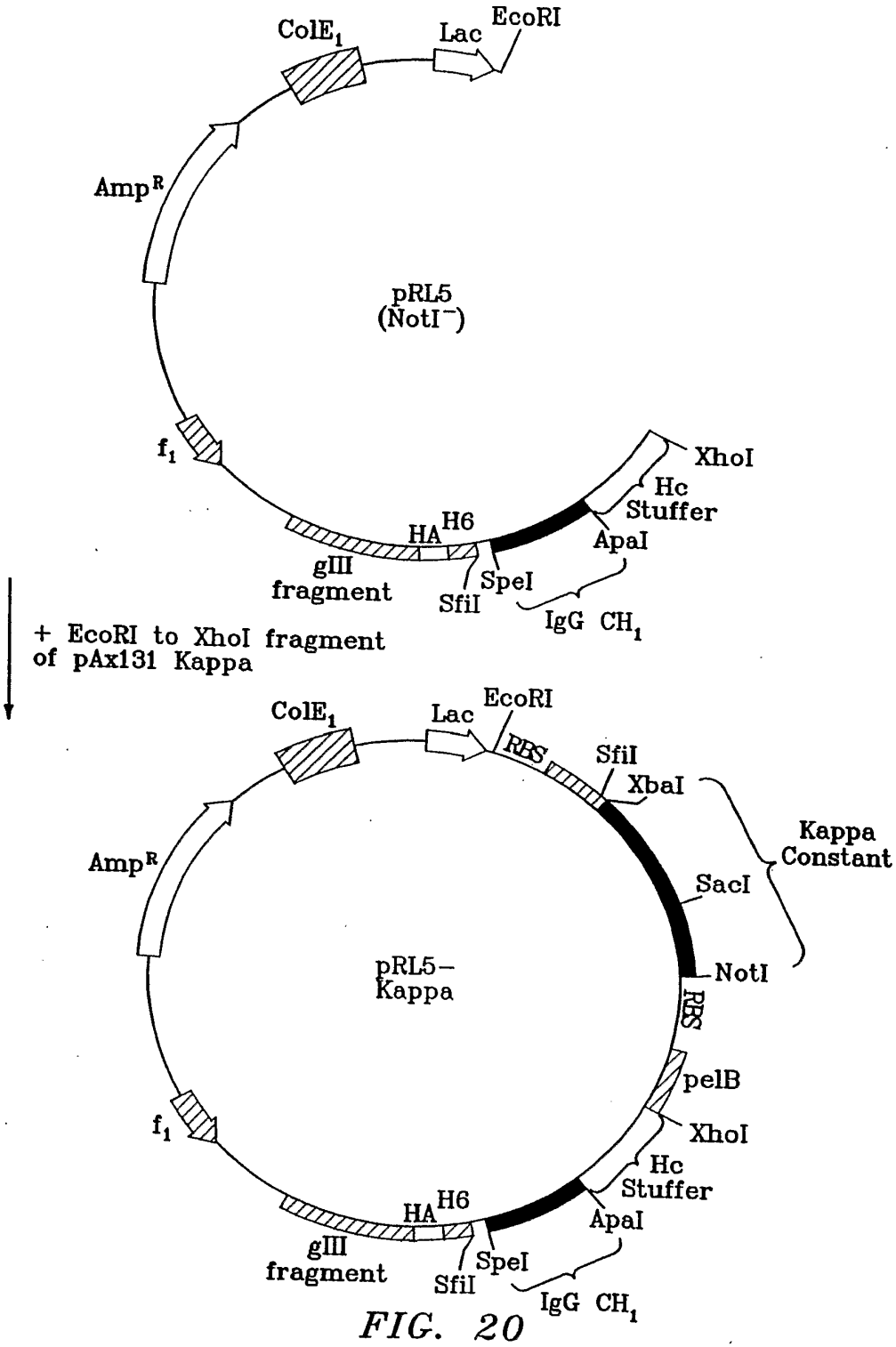


FIG. 20

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Page 2084

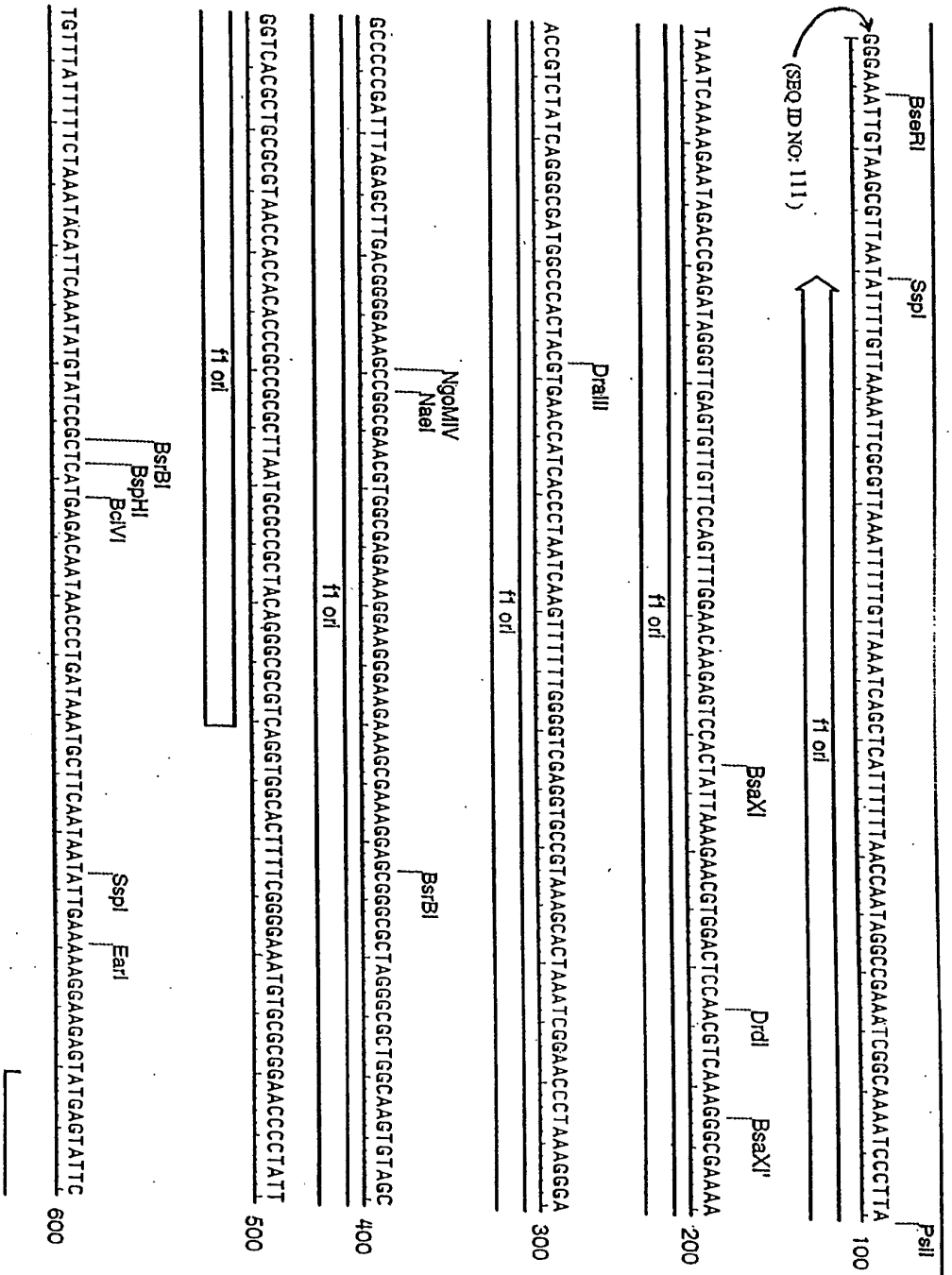


Fig. 21A

Samsung Bioepis Exhibit No. 1002
Page 2085

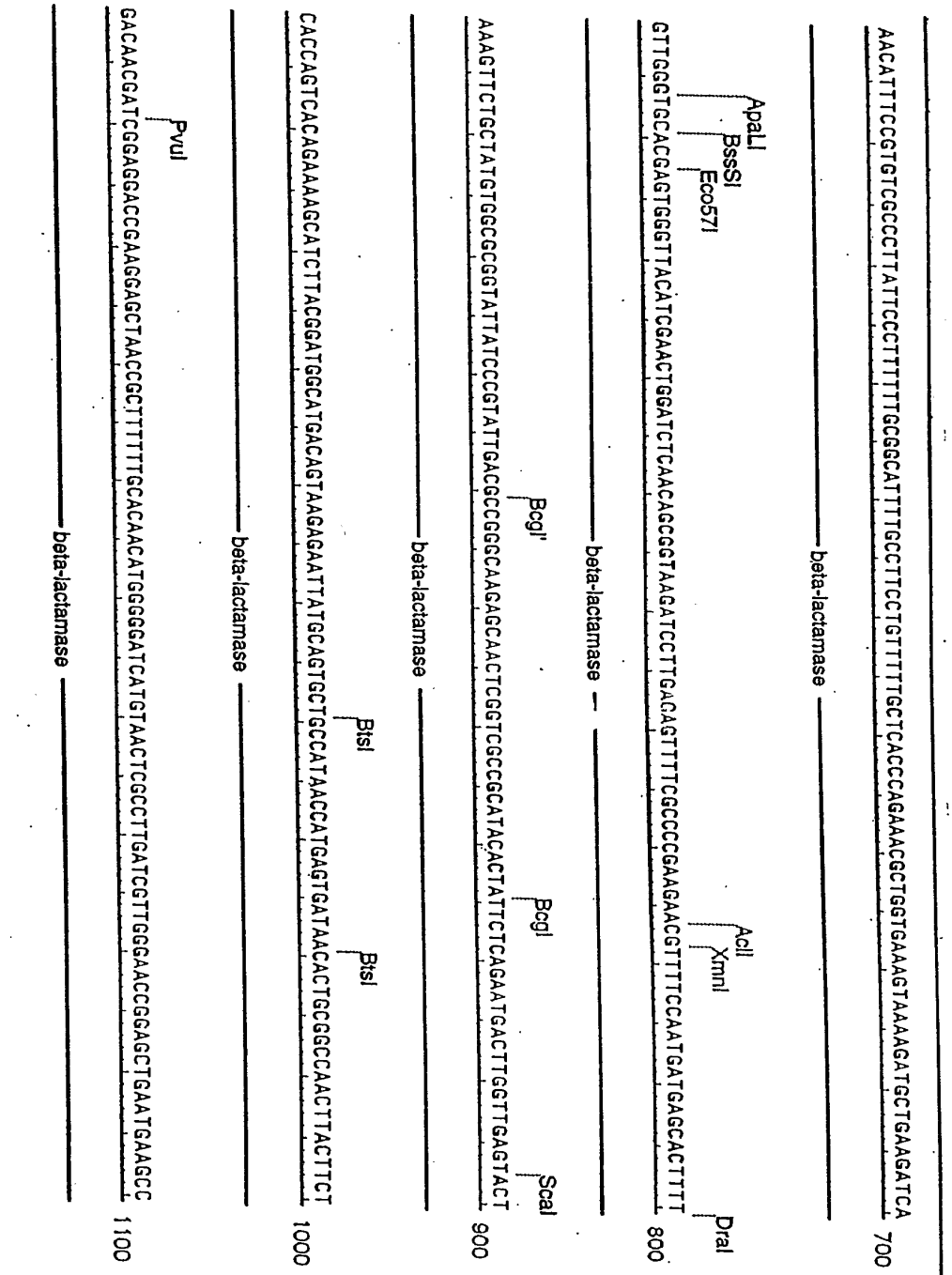


Fig. 21B

Samsung Bioepis Exhibit No. 1002
Page 2086

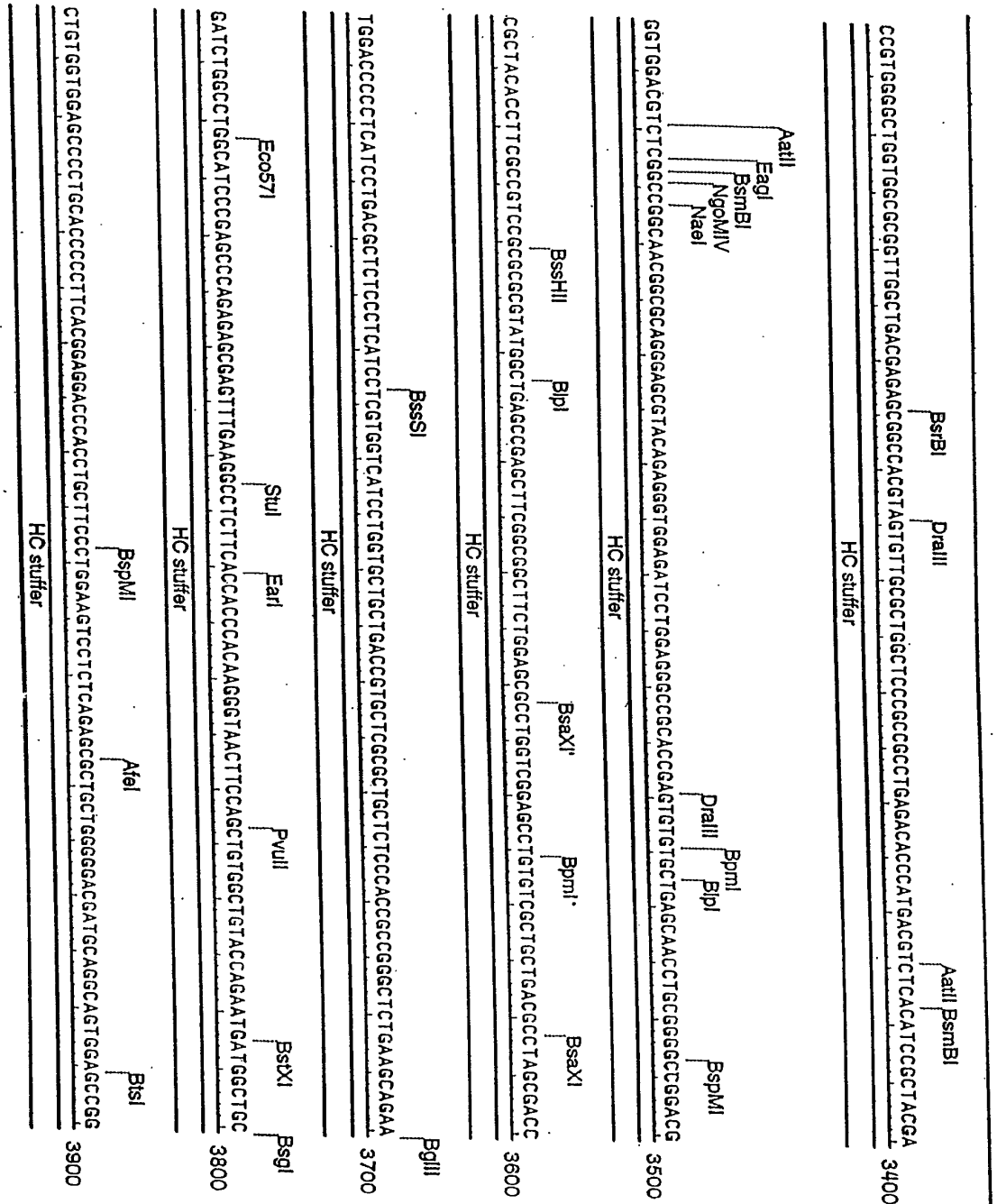


Fig. 21C

Samsung Bioepis Exhibit No. 1002
Page 2087

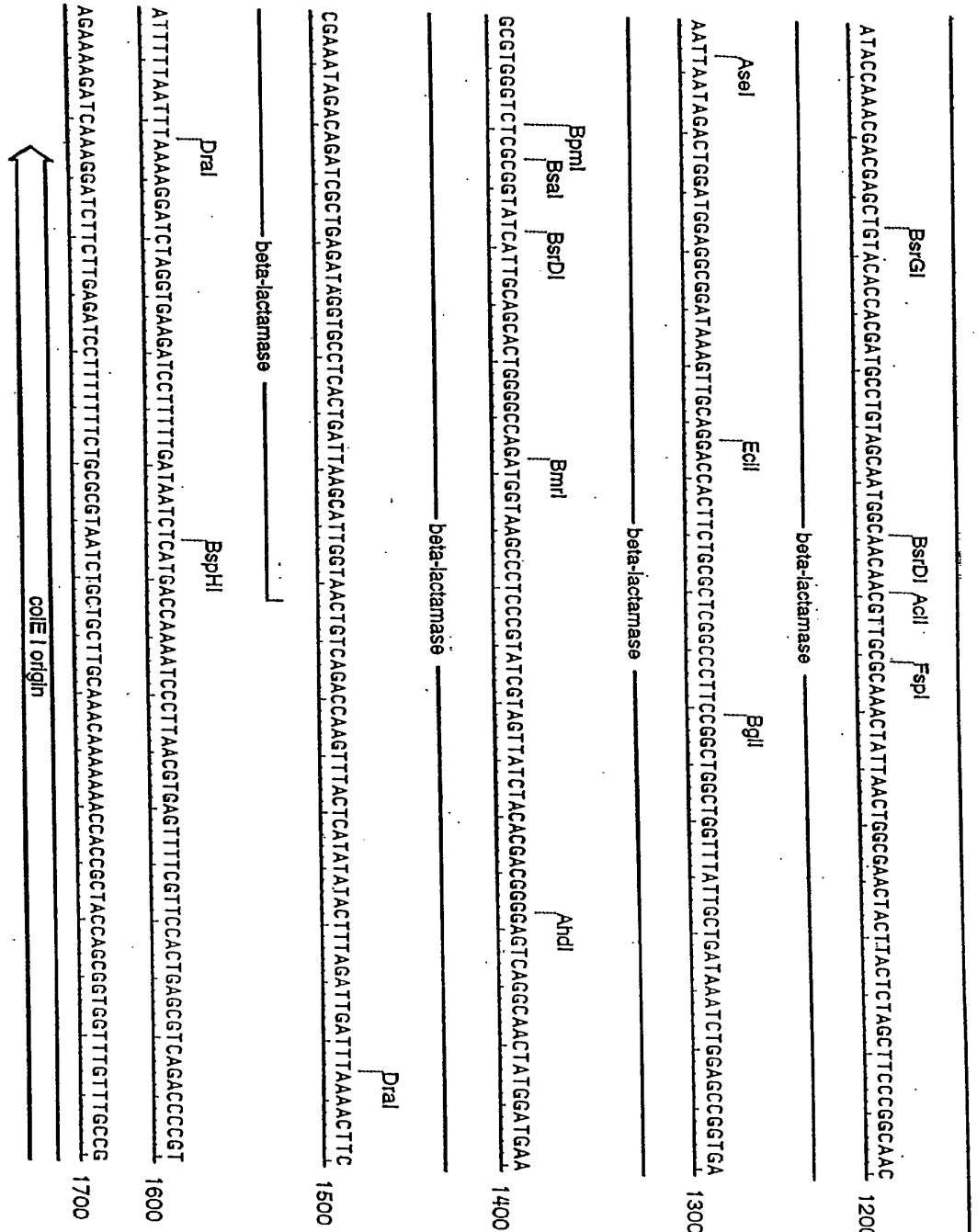


Fig. 21D

Samsung Bioepis Exhibit No. 1002
Page 2088

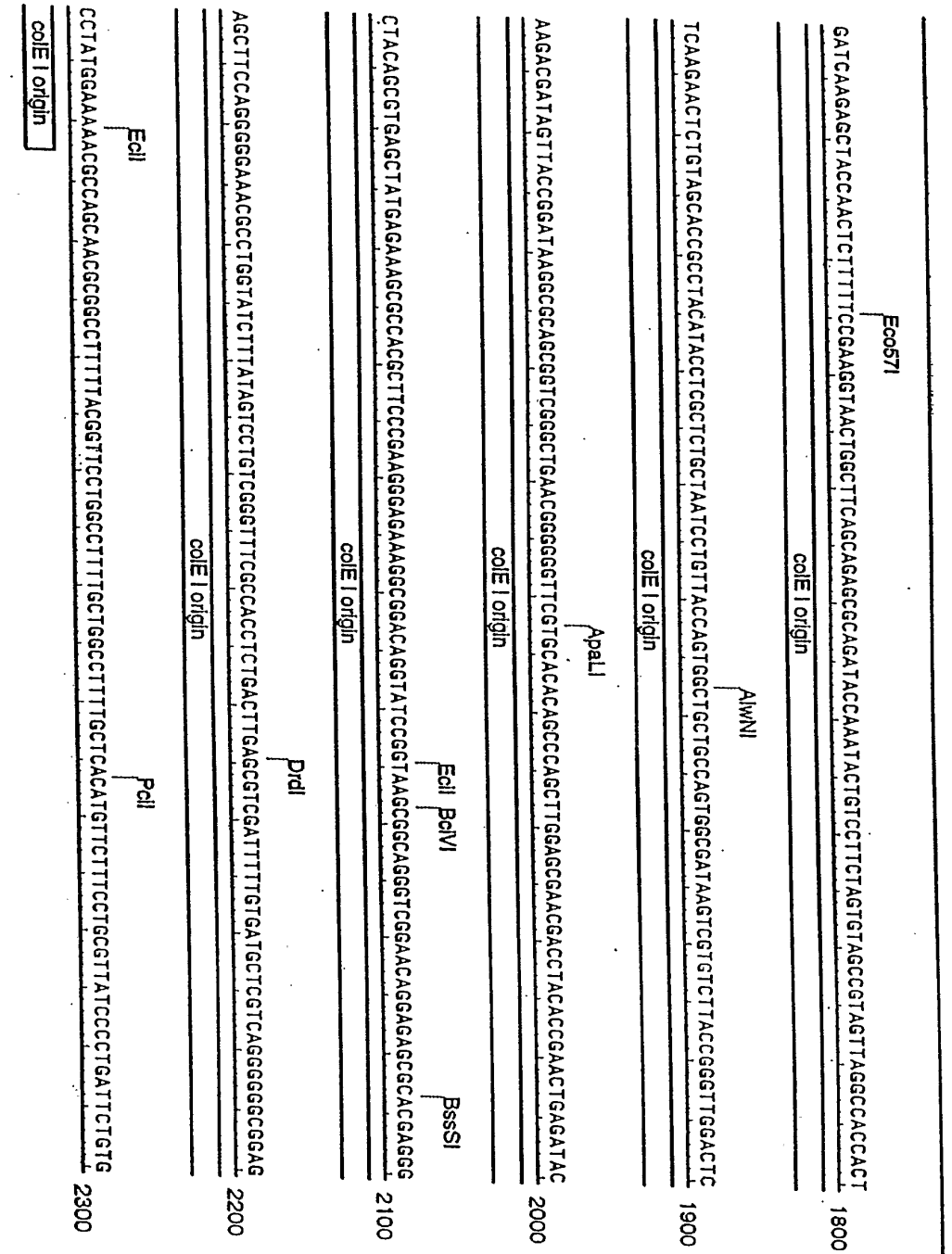


Fig. 21E

Samsung Bioepis Exhibit No. 1002
Page 2089

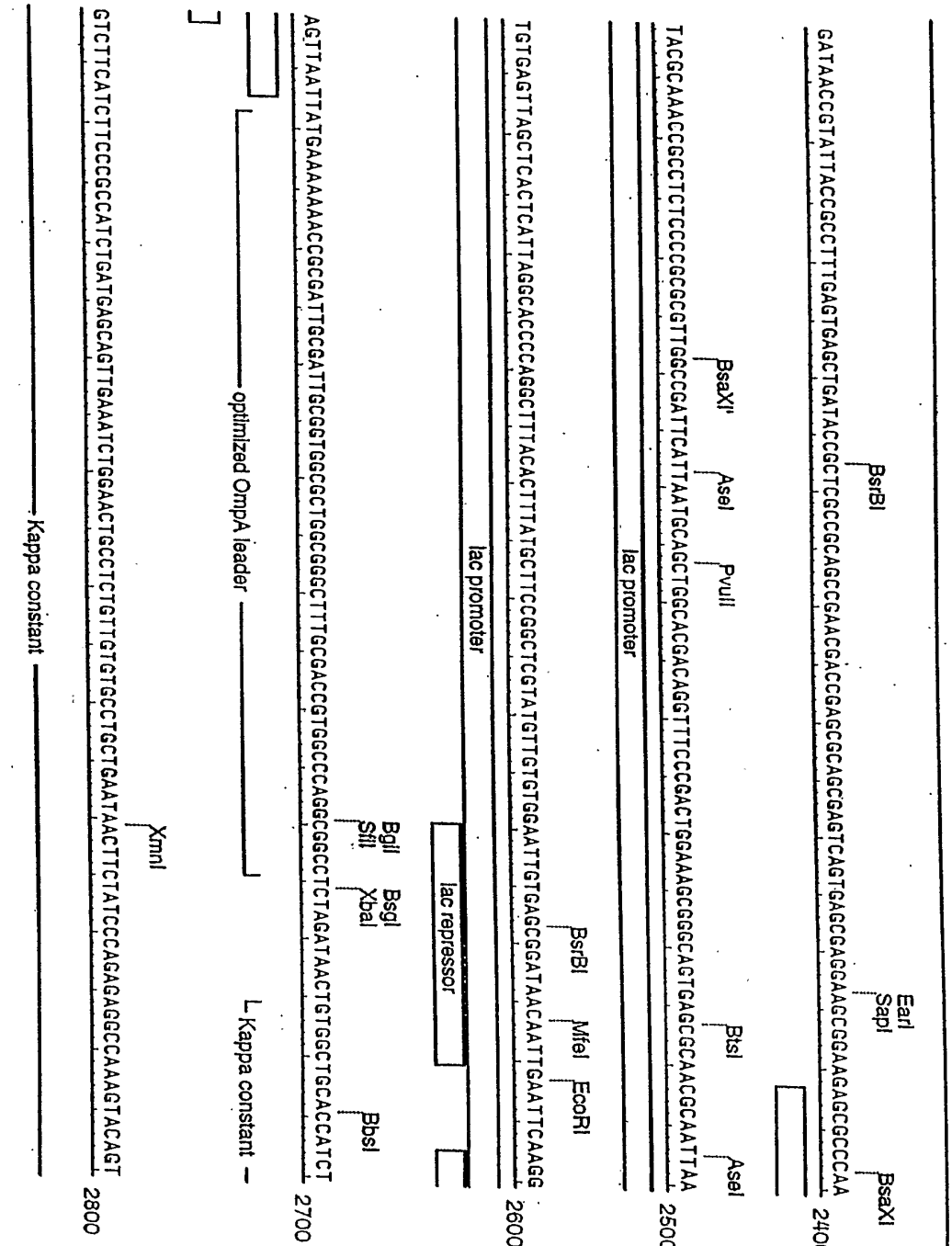


Fig. 21F

Samsung Bioepis Exhibit No. 1002
Page 2090

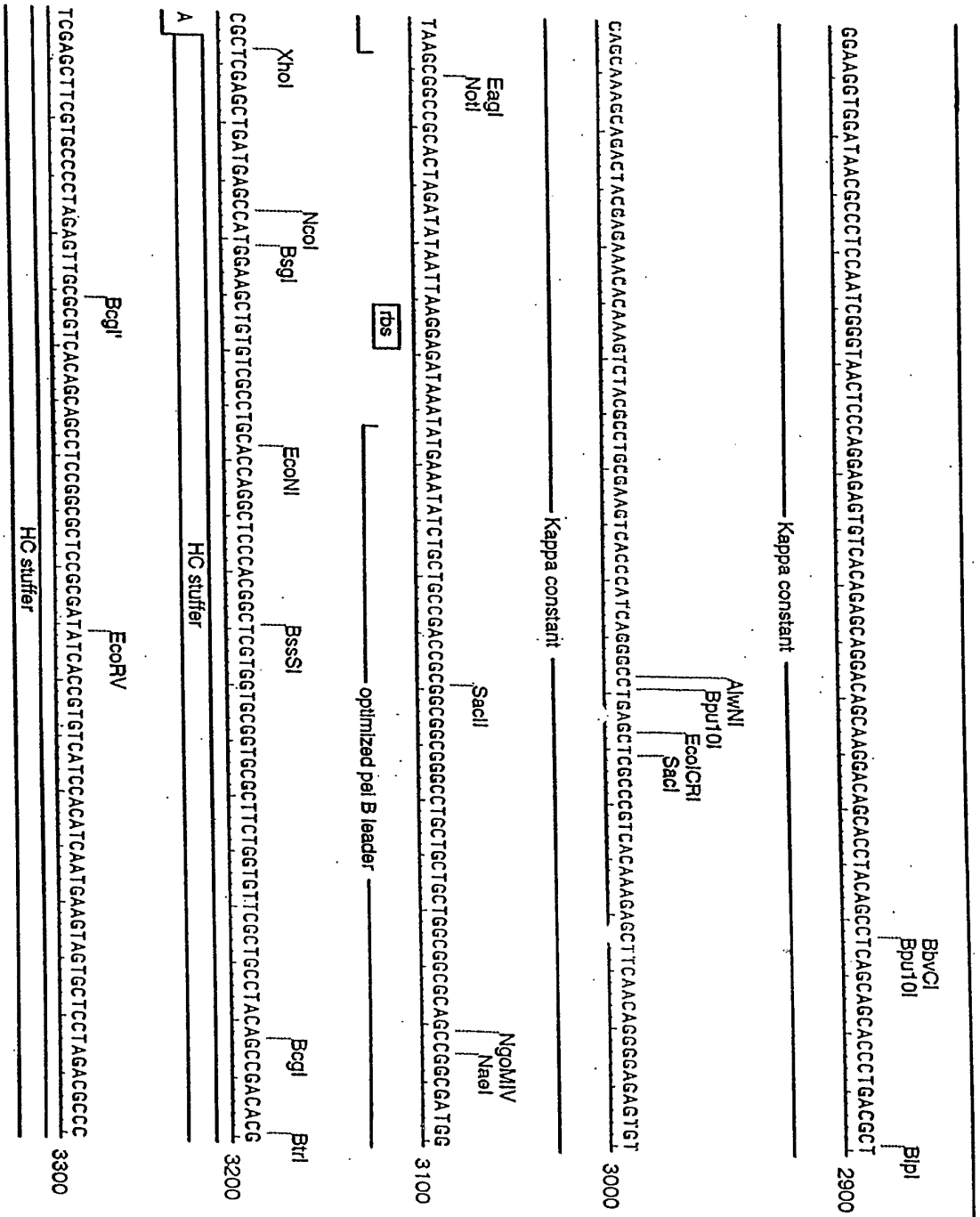


Fig. 21G

Samsung Bioepis Exhibit No. 1002
Page 2091

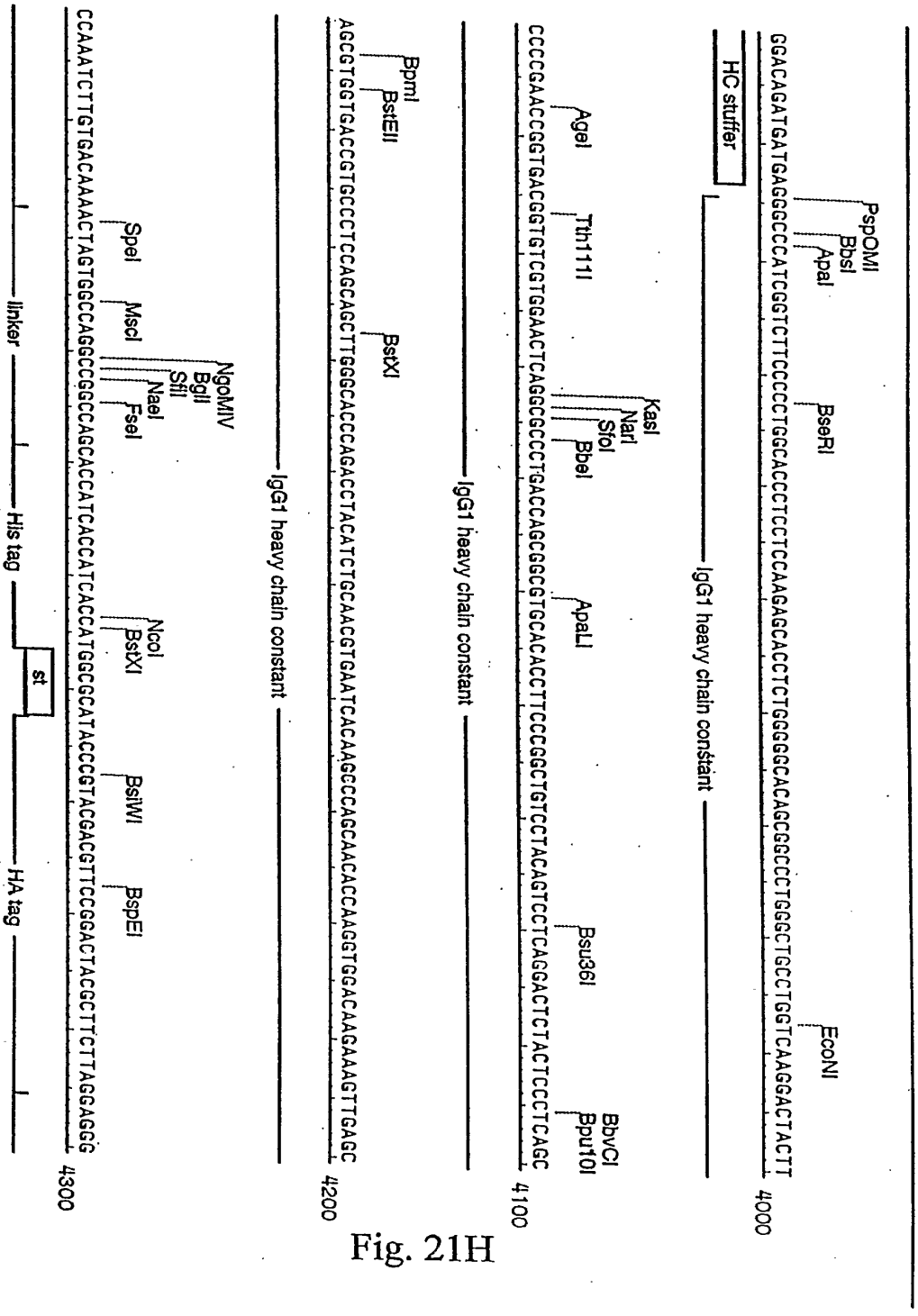


Fig. 21H

Samsung Bioepis Exhibit No. 1002
Page 2092

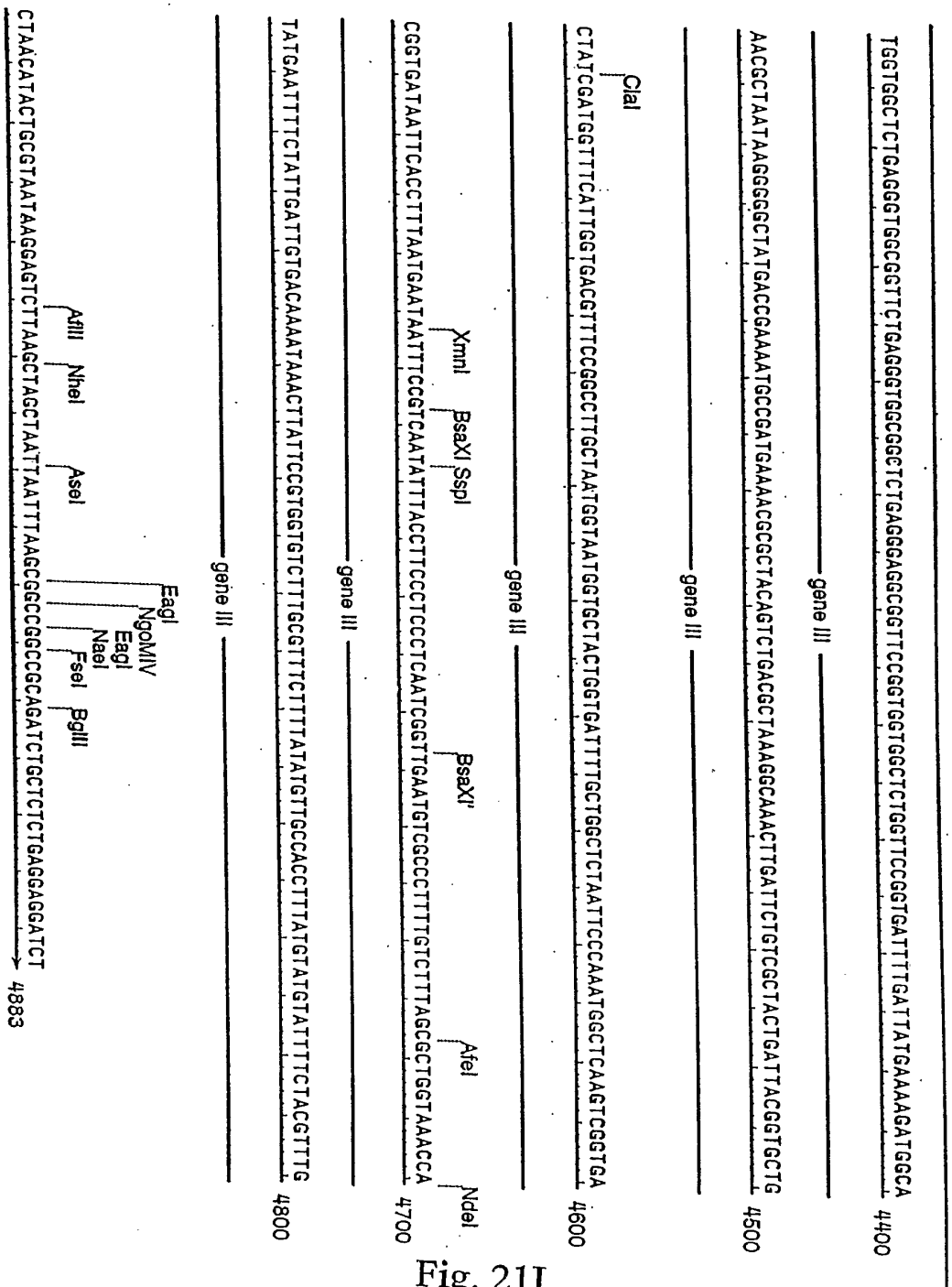


Fig. 21I

Samsung Bioepis Exhibit No. 1002
Page 2093

116 Light Chain (SEQ. ID NO. 122):

EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSG
SGSGTDFTLTISRLEPEDFAVYYCQQYGSSPWTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGT
ASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYA
CEVTHQGLSLPVTKSFNRGEC.

Variable Region of 116 Light Chain (SEQ. ID NO. 123):

EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSG
SGSGTDFTLTISRLEPEDFAVYYCQQYGSSPWTFGQGTKVEIK

116 Heavy Chain (SEQ. ID NO. 124):

QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGQLIEGPTLRQWLA
ARANSRVTITADESTSTAYMELSSLRSEDTAVYYCARLPIEGPTLRQWLAARAPVWGQGTTVTV
SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGL
YSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK **{ VEPKSCDKTHTCPPCP }** *APELLGGP*

end CH1 constant domain — hinge region — tail region

Variable Region of 116 Heavy Chain (SEQ. ID NO. 125):

QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGQLIEGPTLRQWLA
ARANSRVTITADESTSTAYMELSSLRSEDTAVYYCARLPIEGPTLRQWLAARAPVWGQGTTVTV
SS

Clone 116. The light chain sequence is as given. The heavy chain may have several forms depending on the final antibody form. For example: the minimum sequence ending with the CH1 constant domain may result in Fab assembly but the heavy chain/light chain interaction will not be stable. Commonly, a portion of the hinge region containing a cysteine (the underlined bold portion of hinge region) may be included for a covalent interaction between the heavy and light chains. Fab'_2 association would need further cysteines such as an entire IgG_1 hinge region (bold). In this example, clone 116 was cloned in a Xoma pING3302 modified vector which includes a transition tail region (italicized).

Figure 22

Samsung Bioepis Exhibit No. 1002
Page 2094

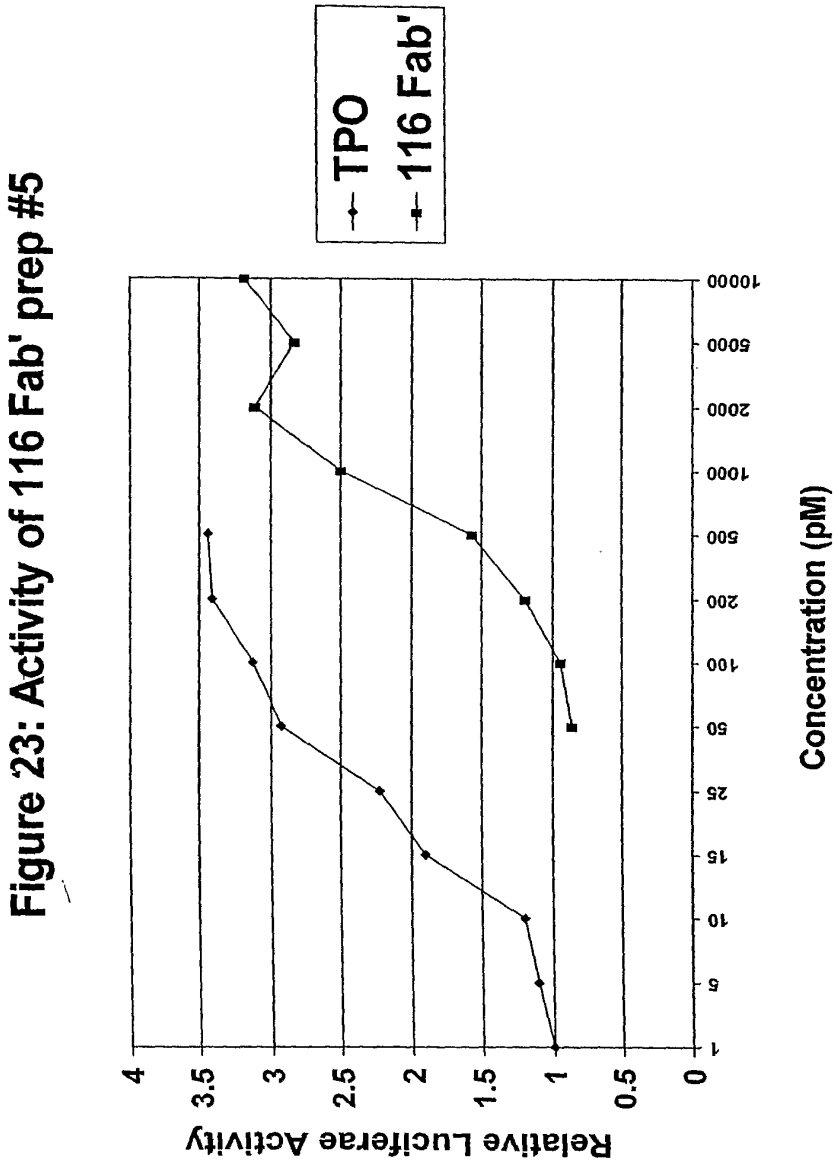


Figure 23: Activity of 116 Fab' prep #5

Samsung Bioepis Exhibit No. 1002
Page 2095

Figure 24

Sample Sequences of Heavy Chain CDR2 clones

Gly-Ile-Phe-**XXX-XXX**-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-**XXX- XXX**-Gly (SEQ. ID NO. 126)
TT backbone randomized TPO peptide randomized TT backbone

Clone	Amino Acid Sequence
HR2-14	Gly-Ile-Phe-**Ser-Pro**-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-**Ala-Gly**-Gly (SEQ. ID NO. 127)
HR2-20	Gly-Ile-Phe-**Pro-Gln**-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-**Lys-His**-Gly (SEQ. ID NO. 128)
HR2-23	Gly-Ile-Phe-**Pro-Asn**-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-**Thr-Gly**-Gly (SEQ. ID NO. 129)
HR2-28	Gly-Ile-Phe-**Lys-Gly**-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-**Pro-Gly**-Gly (SEQ. ID NO. 130)
HR2-43	Gly-Ile-Phe-**Pro-Pro**-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-**Ala-Val**-Gly (SEQ. ID NO. 131)
HR2-44	Gly-Ile-Phe-**Pro-Arg**-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-**Lys-Leu**-Gly (SEQ. ID NO. 132)
HR2-48	Gly-Ile-Phe-**Pro-Arg**-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-**Lys-Leu**-Gly (SEQ. ID NO. 133)
HR2-50	Gly-Ile-Phe-**Pro-Tyr**-Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala-**Lys-Arg**-Gly (SEQ. ID NO. 134)

Samsung Bioepis Exhibit No. 1002
Page 2096

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(71) Applicant *(for all designated States except US)*: **ELAN PHARMACEUTICALS, INC.** [US/US]; 800 Gateway Boulevard, South San Francisco, California 94080 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **BURKE, David, J.** [US/US]; 5957 Chabolyn Terrace, Oakland, California 94618 (US). **BUCKLEY, Shaun, E.** [US/US]; 427 Stockton St. #809, San Francisco, California 94108 (US). **LEHRMAN, Sherwood, Russ** [US/US]; 1731 Penny Way, Los Altos, California 94024 (US). **O'CONNOR, Barbara, Horsey** [US/US]; 150 Dale Avenue, San Carlos, California 94070 (US). **CALLOWAY, James** [US/US]; 4893 Drakewood Terrace, San Diego, California 92130 (US).

(74) Agents: **REA, Teresa, Stanek** et al.; BURNS, DOANE, SWECKER & MATHIS, LLP, PO BOX 1404, Alexandria, Virginia 22313-1404 (US).

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(54) Title: IMMUNOGLOBULIN FORMULATION AND METHOD OF PREPARATION THEREOF

(57) Abstract: A stable aqueous pharmaceutical formulation comprising a therapeutically effective amount of an antibody, polysorbate 80, a buffer which inhibits polysorbate oxidation is described along with methods of making the preparation. Also described are formulations with high antibody concentrations which maintain fixed volumes and which may be used on patients of variable weight.

WO 2004/071439 A2

IMMUNOGLOBULIN FORMULATION AND METHOD OF PREPARATION THEREOF

FIELD OF THE INVENTION

The invention is directed to stable, concentrated formulations of proteins or antibodies, such as natalizumab, wherein the activity of the antibody is retained and also can be administered in a small volume and can be administered to a subject of variable weight in need thereof.

BACKGROUND OF THE INVENTION

Antibody and protein formulations are known in the art. However, preparing protein formulations, such as antibody formulations, which are chemically and biologically stable, are fraught with challenges. Preparing formulations which are also not only stable but can maintain a small volume (*i.e.,* allowing for a small volume injection) even with an increased concentration of protein, such as antibody, also is problematic. The need for such formulations exist. For example, concentrated amounts of protein in a fixed volume that is also stable would be especially beneficial to patients of variable weight. Administration of fluids to patients of variable weights may, for example, have an adverse reaction. Development of such formulations has been hindered by the proteins or the antibodies themselves, which have a high tendency to aggregate and precipitate.

SUMMARY OF THE INVENTION

Therefore, notwithstanding what has previously been reported in the literature, there exists a need for improved methods of formulating proteins and/or antibodies. There is also a need for stable formulations with large concentrations of antibody or protein, where the activity of the antibody or protein is retained. Also needed are stable formulations of concentrated protein which maintain a fixed volume. Applicants disclose herein stable compositions which can be further utilized to prepare antibody formulations, especially formulations with high concentrations of antibody which do not precipitate out and are stable when stored at the recommended temperatures. The highly concentrated and stable antibody formulations will greatly aid physicians in treating subjects of variable weights.

One aspect of the invention provides for a stable, aqueous pharmaceutical formulation comprising an immunoglobulin (or other protein), a phosphate buffer, a polysorbate, and sodium chloride. Preferably the polysorbate is polysorbate 80, and preferably in the amount of about 0.001% to about 2.0% (w/v). Most preferably the polysorbate is present in the amount of about 0.02%. In another embodiment, the immunoglobulin or other protein is present in the formulation in an amount of about 0.1 mg/mL to about 200 mg/mL. Preferably the formulation is buffered to a pH between about 3.0 and about 7.0 and most preferably is about 6.0 ± 0.5. The formulation is preferably isotonic. The formulation may further comprise histidine. Preferably, the histidine is L-histidine.

In another aspect of the invention, the immunoglobulin of the above formulation is an anti-alpha-4 integrin antibody, such as natalizumab or another humanized antibody or monoclonal antibody. This antibody can be present in a standard amount or in a concentrated amount, *e.g.*, about 15 mg/mL or more. Preferably, the natalizumab is present in an amount from about 20 mg/mL to about 150 mg/mL. In instances wherein the formulation is present in a concentration of about 15 mg/mL or more, this formulation is maintained in a fixed volume, for example, of about 125 mL.

It is a further object of the invention to provide a method of treating a patient with variable weight for a condition with a therapeutic amount of an immunoglobulin comprising administering a formulation as described above and herein, wherein the condition is treated by administration of the formulation. It is a further aspect of the invention that the condition be one that is mediated by alpha-4 integrin, and in such conditions the immunoglobulin is one which recognizes and binds to alpha-4 integrin, such as natalizumab.

A further aspect of the invention provides for a composition comprising a sodium phosphate, a polysorbate, a protein and NaCl with a pH of 6.0±0.5, wherein the composition is stable when stored at 5°C to 8°C for a long period of time.

Another aspect of the invention provides for a method of preparing a stable protein containing formulation comprising admixing sodium phosphate, sodium chloride, a polysorbate and a protein and adjusting the pH of the mixture with phosphoric acid to about pH 6.0 ± 0.5.

Samsung Bioepis Exhibit No. 1002
Page 2099

The protein may be lyophilized in the formulation of the present invention. The polysorbate is preferably polysorbate 80k, present in an amount of about 0.02% (w/v), and the protein is preferably natalizumab. The formulation may further comprise histidine.

Preferably, the the protein is lyophilized in a solution comprising 5 mM histidine, 20 mg/mL sucrose and 0.02% polysorbate 80 at a pH 6, and the protein is natalizumab at a concentration of 20 mg/mL.

It is a further object of the invention to provide for an article of manufacture comprising a container holding the stable formulation described above and herein.

Another aspect of the invention provides for a method for treating a patient with variable weight for a condition, comprising simultaneously or sequentially administering to the patient a therapeutically effective combination of a formulation described above and herein and a compound or therapy effective against the condition.

It is a further aspect of the invention to provide a use of any of the stable formulations described herein for the preparation of a medicament for the treatment of a condition wherein the medicament is effective to treat said condition. This medicament may further comprise a second compound or therapy to treat the condition.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

1. Definitions

By "protein" is meant to include but is not limited to immunoglobulins, enzymes, receptor, and fragments thereof. Although discussion of the formulation is provided mainly in reference to an antibody or immunoglobulin, other proteins are contemplated as interchangeable in the formulations disclosed.

By "immunoglobulin" is meant to include but is not limited to an antibody and antibody fragment (such as scFv, Fab, Fc, $F(ab')_2$), and other genetically engineered portions of antibodies. Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM. Several of these may be further divided into subclasses (isotypes), *e.g.*, IgG1, IgG2, IgG3, and IgG4; IgA1 and IgA2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called alpha (α), delta (δ), epsilon (ε), gamma (γ), and mu (μ), respectively. The subunit structures and

Samsung Bioepis Exhibit No. 1002
Page 2100

three-dimensional configurations of different classes of immunoglobulins are well known. Preferably, the immunoglobulin recognizes and binds to alpha-4 integrin.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including agonist and antagonist antibodies), antibody compositions with polyepitopic specificity, and antibody fragments (*e.g.*, Fab, $F(ab')_2$, scFv and Fv), so long as they exhibit the desired biological activity. "Antibody" is meant to include polyclonal antibodies, monoclonal antibodies, humanized antibodies, human antibodies, primatized® antibodies and other antibodies produced via genetic engineering.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by mammalian cell expression systems or transgenic technology, uncontaminated by other immunoglobulins. For example, the monoclonal antibodies to be used in accordance with the present invention may be expressed in goats, as described by Behboodi, et al. (2002) *Transgenic cloned goats and the production of therapeutic proteins.* In *Principles of Cloning*. Elsevier Science (USA); and Meade *et al.* (1999). *Expression of recombinant proteins in the milk of transgenic animals* in *Gene expression systems: using nature for the art of expression*. J. M. Fernandez and J. P. Hoeffler ed., Academic Press. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the methods described by Shepherd *et al., Monoclonal Antibodies: A Practical Approach* (Oxford University Press, 2000).

The term "monoclonal antibodies" also includes "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or

Samsung Bioepis Exhibit No. 1002
Page 2101

belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity. For example, the ability to bind to alpha-4 integrin. The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described for example in Clackson *et al.*, 1991 *Nature* 352: 624-628 and Marks *et al.*, 1991 *J. Mol. Biol.*, 222: 581-597. "Humanized" forms of non-human (*e.g.*, murine, rabbit, bovine, equine, porcine, and the like) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies), which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin.

The expression "linear antibodies" are also included by the general term "antibody" and are a pair of tandem Fd segments (VH-CH1-VH -CH1), which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

A "variant antibody" (also included by the generic term "antibody") is a molecule which differs in amino acid sequence from a "parent" antibody's amino acid sequence by virtue of addition, deletion and/or substitution of one or more amino acid residue(s) in the parent antibody sequence. In the preferred embodiment, the variant comprises one or more amino acid substitution(s) in one or more hypervariable region(s) of the parent antibody. For

Samsung Bioepis Exhibit No. 1002
Page 2102

example, the variant may comprise at least one substitution, *e.g.*, from about one to about ten, and preferably from about two to about five, in one or more hypervariable regions of the parent antibody. Ordinarily, the variant will have an amino acid sequence having at least 75% amino acid sequence identity with the parent antibody heavy or light chain variable domain sequences, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%. Identity or homology with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the parent antibody residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. None of the N-terminal, C-terminal, or internal extensions, deletions, or insertions into the antibody sequence should be construed as affecting sequence identity or homology.

To analyze such properties, one should compare a Fab form of the variant to a Fab form of the parent antibody or a full length form of the variant to a full length form of the parent antibody, for example, since it has been found that the format of the antibody impacts its activity in the biological activity assays disclosed herein. The variant antibody of particular interest is one which displays at least about 10 fold, preferably at least about 20 fold, and most preferably at least about 50 fold, enhancement in biological activity when compared to the parent antibody. The "parent" antibody is one which is encoded by an amino acid sequence used for the preparation of the variant. Preferably, the parent antibody has a human framework region and has human antibody constant region(s). For example, the parent antibody may be a humanized or a human antibody.

An "isolated antibody" is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or non-reducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not

Samsung Bioepis Exhibit No. 1002
Page 2103

be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

"Antibody fragments" comprise a portion of an intact antibody, generally the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. "Single-chain Fv" or "sFv" antibody fragments comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the VH and VH domains which enables the sFv to form the desired structure for antigen binding.

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain (VH -VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. The route of antibody administration is in accord with known methods and are well known, and may include, for example, injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intralesional routes, or by sustained release systems. The antibody can be administered continuously by infusion or by bolus injection. Therapeutic antibody compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle. "Pharmaceutically acceptable" excipients (*e.g.*, vehicles, additives) are those which can reasonably be administered to a subject mammal to provide an effective dose of the active ingredient employed. A "stable" formulation is one in which the protein therein essentially retains its physical stability and/or chemical stability and/or biological activity upon storage. By "stable" is also meant a formulation which exhibits little or no signs of instability, including aggregation and/or deamidation. For example, the formulations provided by the present invention may remain stable for at least two years ,when stored as indicated at a temperature of 5-8°C.

Various analytical techniques for measuring protein stability are available in the art and are reviewed in *Peptide and Protein Drug Delivery*, 247-301 (Vincent Lee ed., New York, N.Y., 1991) and Jones, 1993 *Adv. Drug Delivery Rev.* 10: 29-90, for examples.

Samsung Bioepis Exhibit No. 1002
Page 2104

Stability can be measured at a selected temperature for a selected time period as exemplified by the provided examples. Storage of stable formulations if preferably for at least 6 months, more preferably 12 months, more preferably 12-18 months, and more preferably for 2 or more years.

A protein, such as an antibody or fragment thereof, "retains its physical stability" in a pharmaceutical formulation if it shows no signs of aggregation, precipitation, deamidation and/or denaturation upon visual examination of color and/or clarity, or as measured by UV light scattering or by size exclusion chromatography.

A protein "retains its chemical stability" in a pharmaceutical formulation, if the chemical stability at a given time is such that the protein is considered to still retain its biological activity. Chemical stability can be assessed by detecting and quantifying chemically altered forms of the protein. Chemical alteration may involve size modification (*e.g.*, clipping), which can be evaluated using size exclusion chromatography, SDS-PAGE and/or matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI/TOF MS), for examples. Other types of chemical alteration include charge alteration (*e.g.*, occurring as a result of deamidation), which can be evaluated by ion-exchange chromatography, for example. An antibody "retains its biological activity" in a pharmaceutical formulation, if the biological activity of the antibody at a given time is within about 10% (within the errors of the assay) of the biological activity exhibited at the time the pharmaceutical formulation was prepared as determined in an antigen binding assay, for example.

By "isotonic" is meant that the formulation of interest has essentially the same osmotic pressure as human blood. Isotonic formulations will generally have an osmotic pressure from about 250 to 350 mOsm. Isotonicity can be measured using a vapor pressure or ice-freezing type osmometer, for example.

As used herein, "buffer" refers to a buffered solution that resists changes in pH by the action of its acid-base conjugate components. The buffer of this invention has a pH in the range from about 3.0 to about 7.5; preferably from about pH 4.0 to about 7.0; more preferably from about pH 5.0 to about 6.5; and most preferably has a pH of about 6.0 ± 0.5. A pH of any point in between the above ranges is also contemplated.

In a pharmacological sense, in the context of the present invention, a "therapeutically effective amount" of an antibody refers to an amount effective in the prevention or treatment

Samsung Bioepis Exhibit No. 1002
Page 2105

of a disorder for the treatment of which the antibody is effective. A "disorder" is any condition that would benefit from treatment with the antibody or protein. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

A "preservative" is a compound which can be included in the formulation to essentially reduce bacterial action therein, thus facilitating the production of a multi-use formulation, for example. Examples of potential preservatives include octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride (a mixture of alkylbenzyldimethylammonium chlorides in which the alkyl groups are long-chain compounds), and benzethonium chloride. Other types of preservatives include aromatic alcohols such as phenol, butyl and benzyl alcohol, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol.

By "patient" or "subject" is meant to include any mammal. A "mammal," for purposes of treatment, refers to any animal classified as a mammal, including but not limited to humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, and the like. Preferably, the mammal is human.

By "Antegren®" and is meant to include the antibody also known as AN100226 (antibody code number) or natalizumab (USAN name). Natalizumab is a recombinant, humanized anti-alpha-4 integrin antibody. Preferably the disease or condition being treated in the mammal is one which is modulated when a therapeutically effective dose of natalizumab is administered.

By "stable" is meant a formulation which exhibits little or no signs of instability, including aggregation and/or deamidation. In addition, "stable" may also refer to a formulation which does not exhibit any signs of instability for greater than or equal to two years ,when stored as indicated at

2. General Description

In the discussion below and the examples to follow, formulations for stable antibody formulations are disclosed. Certain stable formulations disclosed have high concentrations of

Samsung Bioepis Exhibit No. 1002
Page 2106

antibody but maintain a fixed volume, wherein the antibodies in these formulations are stable and the antibody does not precipitate out of solution or aggregate. Proteins other than antibodies are also contemplated for the high concentration formulations.

Antibodies are typically administered to a subject (*e.g.*, a human) at a concentration of about 0.01 mg/mL to about 200 mg/mL. More typically, antibodies range in concentration from about 0.1 mg/mL to about 150 mg/mL. However, instances exist when greater concentrations are required to be administered to a patient, *e.g.*, about 15 to about 200 mg/mL, more preferably about 15 mg/mL to 150 mg/mL, more preferably about 20 to about 50 mg/mL, and most preferably about 20 mg/mL and any integer value in between.

The antibody formulation may be administered to a mammal in need of treatment with the protein, in accordance with known methods. These methods may include, but are not limited to intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. In preferred embodiments, the antibody formulation is administered to the mammal by intravenous administration.

The appropriate dosage of the protein will depend, for example, on the condition to be treated, the severity and course of the condition, whether the protein is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the protein, the type of protein used, and the discretion of the attending physician. The protein is suitably administered to the patient at one time or over a series of treatments and may be administered to the patient at any time from diagnosis onwards. The protein may be administered as the sole treatment or in conjunction with other drugs or therapies useful in treating the condition in question. As used herein, two (or more) agents are said to be administered in combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act contemporaneously.

In practicing the methods of this invention, the compounds of this invention may be used alone or in combination, or in combination with other therapeutic agents. In certain preferred embodiments, the compounds of this invention may be co-administered along with other compounds typically prescribed for these conditions according to generally accepted medical practice. For example, the formulations of this invention can be administered in combination with other therapeutic agents or physical therapies for the treatment of rheumatoid arthritis, multiple schlerosis and Crohn's Disease.

Samsung Bioepis Exhibit No. 1002
Page 2107

2.1 Method of Making The Antibody Formulation

The process can be altered as would be known to the skilled artisan, but generally would follow a procedure such as the following. Obtain an ampoule from a working cell bank which contains cells which make the antibody or protein of interest. Prepare the inoculum. Culture or ferment the cells with additional feedings as is necessary. Harvest/clarify the cells by centrifugation and/or filtration. This can be done for example by concentrating the cells 10 fold by spiral wound filtration. Filter by a 0.2 μm intermediate filtration followed by purification by protein A Sepharose Fast Flow® (*i.e.*, affinity chromatography) and reverse elution. The antibody containing composition then receives a treatment at pH 3.6-3.7. The mixture then receives a viral filtration followed by a concentration/diafiltration step. The composition can then be purified by DEAE Sepharose Fast Flow® (anion exchange). This step can be performed multiple times. From this point, the composition is then further concentrated followed by a purification step using Sephacryl S300HR® (*i.e.*, gel filtration chromatography) system, wherein the running buffer used is phosphate/NaCl. The antibody containing composition can be further concentrated for the high concentration formulations (*e.g.*, 20 mg/mL or more) if so desired. The antibody containing composition is then further buffered and the concentration adjusted by adding 0.02% (w/v) polysorbate 80. This composition then receives a final filtration using a 0.2 μm filter and can be dispensed at this point into 100 mL to 10 L polypropylene bottles. The antibody or immunoglobulin so obtained can then be QC tested and QA released.

The above can be done, for example for natalizumab, as diagramed below:

200L Material (5.0 mg/mL)	2000L Material (5.0, 20 mg/mL)
Ampoule from Working Cell Bank	Ampoule from Working Cell Bank
↓	↓
Inoculum Preparation	Inoculum Preparation
↓	↓
Fermentation	Fermentation (Additional Media Feed)
↓	↓
Harvest/Clarification by filtration: Concentration 10x by spiral wound filtration	Harvest/Clarification by centrifugation & filtration: Concentration 10x by spiral would

Samsung Bioepis Exhibit No. 1002
Page 2108

200L Material (5.0 mg/mL)	2000L Material (5.0, 20 mg/mL)
	filtration
↓	↓
0.2 μm Intermediate Filtration	0.2 μm Intermediate Filtration
↓	↓
Purification by Protein A Sepharose Fast Flow® (Affinity Chromatography) (Forward Elution)	Purification by Protein A Sepharose Fast Flow® (Affinity Chromatography) (Reverse Elution)
↓	↓
pH 3.7-3.8 treatment	pH 3.6-3.7 treatment
↓	↓
--	Viral filtration
↓	↓
Concentration/Diafiltration	Concentration/Diafiltration
↓	↓
Purification by DEAE Sepharose Fast Flow® (Anion Exchange Chromatography) (Single Cycle)	Purification by DEAE Sepharose Fast Flow® (Anion Exchange Chromatography) (Multiple Cycles)
↓	↓
Concentration	Concentration
↓	↓
Purification by Sephacryl S300HR® (Gel Filtration Chromatography) (Running Buffer: histidine/NaCl)	Purification by Sephacryl S300HR® (Gel Filtration Chromatography) (Running Buffer: phosphate/NaCl)
↓	↓
--	(Additional concentration for 20 mg/mL)
↓	↓
Buffer & Concentration Adjustment Phosphate/NaCl, 0.02% (w/v) polysorbate 80	Buffer & Concentration Adjustment (add 0.02% (w/v) polysorbate 80)
↓	↓
0.2 μm Final Filtration and Dispensing (100 mL- 1 L polypropylene bottles)	0.2 μm Final Filtration and Dispensing (100 mL - 10L polypropylene bottles)
↓	↓
QC Testing and QA Release of Bulk Purified AN100226	QC Testing and QA Release of Bulk Purified AN100226

Samsung Bioepis Exhibit No. 1002
Page 2109

2.2 The Antibody Formulation

In one aspect of the invention, an immunoglobulin is formulated in concentrations of about 1.7, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 40.0 or 50.0 mg/mL in 10 mM sodium phosphate, 140 mM NaCl (pH 6.0 ± 0.5) and 0.02% polysorbate 80. If necessary, the pH is adjusted to 6.0 ± 0.5 with phosphoric acid.

One example of the different formulations is displayed below.

QUANTITATIVE COMPOSITION: PHOSPHATE BUFFER FORMULATION

Component	Function	Unit Formula (per mL) 1.7 mg/mL	Unit Formula (per mL) 5.0 mg/mL	Unit Formula (per mL) 20 mg/mL
Active Ingredient:				
Anti-α4 integrin humanized monoclonal antibody	Active	1.7 mg	5.0 mg	20 mg
Other Ingredients:				
Sodium Phosphate, USP	Buffer and tonicity	1.4 mg	1.4 mg	1.4 mg
Sodium chloride, USP	Buffer and tonicity	8.2 mg	8.2 mg	8.2 mg
phosphoric acid, NF	Adjust pH to 6.0 ± 0.5	QS	QS	QS
Polysorbate 80, NF	Inhibit protein aggregation	0.2 mg	0.2 mg	0.2 mg
Water for Injection, USP	Diluent	QS to 1 mL	QS to 1 mL	QS to 1 mL

Any of the above formulations are optimally vialed in an aseptic vial. These vials can be, for example, Type I EP neutral glass vials (*e.g.*, 5.0 or 20 mL fill vials) with Helvoet Pharma V9145/FM 157/1 gray butyl rubber stoppers with aluminum seals. However other suitable aseptic vials are also contemplated. For example, the formulations can be bottled as follows:

Samsung Bioepis Exhibit No. 1002
Page 2110

BOTTLING
Compounding of phosphate/sodium chloride/polysorbate 80 buffer, if needed
↓
Sterilization of vials, stoppers and manufacturing equipment
↓
Dilution with phosphate/sodium chloride/polysorbate 80 buffer antibody to 1.7 or 50 mg/mL, as required
↓
Sterile filtration
↓
Aseptic filling and stoppering of vials
Capping
↓
Release Testing

More specifically, natalizumab obtained for example by the procedures discussed above, can be bottled as follows. Natalizumab 200 L and 2000 L drug product can be filled on a fully automated filling line equipped with a vial washing, sterilization and depyrogenation tunnel. Stoppers, seals and filling equipment are washed and sterilized prior to use. This process allows for large scale tilling operations consistent with the volumes produced from the 2000 L fermentation.

When necessary, the formulation buffer, about 10 mM phosphate, about 140 mM NaCl, pH 6.0 ± 0.5, about 0.02% polysorbate 80, can be compounded in a Class 10,000 suite and used to dilute the bulk drug product to the final concentration. In-process specifications of concentration, pH and density are preferably reached prior to filtration.

The formulated natalizumab or other immunoglobulin can be sterile filtered through a 0.2 μm Millipak filter into a stainless steel surge tank inside the sterile core. Filling, stoppering and capping of natalizumab is fully automated. In-process samples for bulk sterility are collected; fill weight and headspace testing is conducted throughout the filling operation. Filled drug product is stored under refrigeration at about 2-8°C.

Filling occurs within a fully validated Class 100 sterile core. The filling line is validated to provide fill volumes within expected tolerances for the 5.0 mL and 20 mL fill volumes. Comprehensive environmental monitoring is conducted throughout the filling operation and reviewed to verify continual compliance with this standard. Media fills are conducted on a quarterly basis to support aseptic filling operations.

Samsung Bioepis Exhibit No. 1002
Page 2111

Alternatively, the 200 L drug substance material produced can be bottled pursuant to the following example. Vials, filling needles, the filtration assembly and tubing are prepared and sterilized prior to use. Stoppers can be prepared by the supplier. The stoppers are then sterilized prior to filling.

Natalizumab drug product or other protein is filled on a semi-automatic filling line, with batch preparation of components, automated filling, immediate stoppering, and a subsequent capping operation. This operation is appropriate for small batch scale operations.

The final bulk solution is then sterile filtered through a 0.2 μm Millipak filter into a sterile glass receiving vessel within a Class 100 environment. Regular calibration and in-process checks ensure that the filling tolerance remains within ± 2%. Vials are stoppered and capped immediately. Filled drug product is preferably stored refrigerated at 2-8°C.

The glass receiving vessel can be any number of vials, but can for example be a 5.0 or 20 mL neutral glass vial Type I (EP) supplied by, for example, Epsom Glass or AMILCO or a 5.0 mL or 20 mL USP Type I borosilicate glass vial, supplied by, for example Kimble or Wheaton. These vials can use any means of closure suitable. Vial closures include, but are not limited to a 13 mm Helvoet Pharma V9145/FM 157/1 grey butyl rubber stopper or a 13 mm and 20 mm Helvoet Pharma V9145/FM 157/1 grey butyl rubber stopper or a 13 mm or 20 mm West 4432/50 gray butyl rubber stopper. The rubber-stoppered bottled is then sealed most typically using an aluminum seal, such as that manufactured by West.

Although the present invention has been described in detail with reference to examples below, it is understood that various modifications can be made without departing from the spirit of the invention, and would be readily known to the skilled artisan.

EXAMPLES

Example 1

Selection of Polysorbate 80

The typical method of administering natalizumab is intravenous. Intravenous administration requires the final formulation to be isotonic. A formulation of AN100226 (natalizumab), 5 mg/mL in 50 mM L-histidine, 150 mM NaCl, pH 6.0 was initially chosen (Formulation #1). During a Phase II study, protein precipitation of the antibody was

Samsung Bioepis Exhibit No. 1002
Page 2112

observed during the dilution and introduction of natalizumab into the clinical dosing apparatus. Polysorbate 80 was introduced into the formulation (Formulation #2) to resolve the observed protein precipitation. Preferably, the polysorbate for use with the present invention is low in peroxide, *i.e.*, polysorbate from Sigma, Product number P6479, Lot Number 071K7283.

The two factors that have been shown to accelerate the precipitation of the AN100226 antibody are the presence of trace levels of silicone oil and denaturation at the air-liquid interface. The silicone oil was introduced into the product upon use of standard lubricated polypropylene syringes equipped with siliconized rubber stoppers. The introduction of the silicone oil is sufficient to cause discernible antibody precipitation in Formulation # 1 upon gentle agitation and room temperature storage. The aggregation, deamidation and subsequent precipitation caused by denaturation at the air-liquid interface has become more discernable problematic with the drug being shipped to more clinical sites. Both causes of protein precipitation have been resolved by the addition of polysorbate 80 at a concentration of 0.02% (w/v).

Formulation #2 shows comparable stability to the histidine/NaCl formulation (Formulation #1) in all protein characterization assays, while providing increased stability during product shipping and handling in the clinical setting.

The addition of polysorbate 80 to the formulation also overcomes the problem of precipitating or aggregating antibody when preparing formulations with higher protein content. Initial work focused on agitation-induced aggregation at high protein concentrations, including 50 mg/mL. By subjecting the material to agitation using a vortex-type mixer, aggregated species were detected by size exclusion-high performance liquid chromatography (SEC-HPLC). This model identified polysorbate 80 as an effective inhibitor of aggregation, while sucrose and other buffering components had little beneficial effect.

The effectiveness of the addition of 0.02% (w/v) polysorbate 80 in preventing agitation-induced precipitation at a protein concentration of 5 mg/mL was assessed following addition of 10 μL of a 10% polysorbate 80 solution to vials of natalizumab (Lot No. AN100226-0003). The vials were shaken on their sides along with several vials of natalizumab in Formulation #1 at 150 rotations per minute in a horizontal plane. Within 3 hours of this treatment at room temperature, the vials of Formulation #1 were laden with

Samsung Bioepis Exhibit No. 1002
Page 2113

particles and appeared turbid while the vials with 0.02% (w/v) polysorbate 80 remained clear and free of particles.

The observed aggregation is presumed to be caused by the air-surface interface, as vials completely filled with AN100226 in the absence of polysorbate 80 were shaken for extended periods of time without additional particle formation being induced.

An evaluation of the ability of 0.02% (w/v) polysorbate 80 to inhibit the protein precipitation facilitated by trace levels of silicone was conducted. A vial of natalizumab (Lot No. AN100226-0003) was adjusted to 0.02% (w/v) polysorbate 80 and drawn into a commercially available, lubricated 60 mL polypropylene syringe. The material was allowed to stand for several hours at room temperature. Visual inspection confirmed that no precipitation was occurring. The material was then filtered through a 0.2 μm filter into a 5-mL vial and inspected and found to be substantially free of particles after several days, while vials treated in the same manner in the absence of polysorbate 80 (Formulation #1) were laden with particles.

Example 2

Selection of The Phosphate Buffer

During release testing of the histidine placebo (containing 0.02% w/v polysorbate 80), new trace impurities were detected. These impurities arose from degradation of polysorbate 80, apparently through an oxidation reaction involving metal ions and histidine. For active natalizumab drug product, these trace impurities have been detected only after storage at elevated temperatures (*e.g.*, 25°C and 40°C). Thus, the decision was made to modify the placebo and use phosphate to replace histidine in the buffer. The lot of histidine used in the product placebo was different than that used for the active natalizumab drug product lots AN100226-004 and AN100226-005. The impact of the source of histidine on polysorbate 80 degradation is discussed in greater detail infra.

During testing of the placebo, trace impurities were detected in the histidine/NaCl/0.02% (w/v) polysorbate 80 placebo (Formulation #2). These impurities were detected by their absorbency in the low wavelength ultraviolet region. The absorbency profiles from 200 to 400 nm for placebo stored at 5°C, 25°C, and 40°C for one month were

Samsung Bioepis Exhibit No. 1002
Page 2114

determined. These data indicate that the impurities are increasing as a function of temperature.

The size exclusion HPLC method used to monitor antibody aggregation was modified to increase sensitivity for detection of the trace impurities by increasing the column load 5-fold to 100 μL, and the sample is applied undiluted rather than 10-fold diluted. In addition, the absorbency is monitored at 260 nm to reflect the absorbency maximum of the impurities. The method provides a tool to evaluate the presence of these trace impurities in both placebo and product, as the antibody emerges much earlier in the elution profile.

Placebo and natalizumab final drug product formulated in Formulations #1 and #2 were analyzed by the SEC-HPLC method described above. Analysis of the placebo in Formulation #1 spiked with polysorbate 80 to 0.02% (w/v) was performed just prior to chromatography. This shows the UV absorbency of polysorbate 80, histidine, and salt in the absence of the trace impurities. The broad peak at 16 minutes is associated with polysorbate 80 while the peaks at approximately 26-27 minutes are attributed to histidine and salt. The histidine/NaCl/0.02% (w/v) polysorbate 80 placebo (Formulation #2) stored at 5°C for two months shows a marked increase in the late eluting peaks which implicates polysorbate 80 in the production of these impurities. In addition, the eluting at 16 minutes disappeared, indicating that the polysorbate 80 has been degraded.

AN100226 stored as bulk drug substance for approximately 5 months at 5°C in Formulation #1 was analyzed after spiking with polysorbate 80 to 0.02% (w/v) just prior to chromatography. The antibody elutes at 16-18 minutes using these overload conditions. The remainder of the profile resembles the placebo with polysorbate 80 spike. The two-month stability samples for natalizumab lot #AN100226-0004 were also analyzed by this method. The elution profile for the 5°C natalizumab sample indicates the absence of any additional peaks and comparable levels of the histidine/salt peaks at 26-27 minutes. The trace impurities are detected at two months for the 25°C sample, and elevated levels are present for the two month 40°C sample. Thus, these impurities have not been detected in the clinical supplies, which are stored at 5°C. The appearance of these trace impurities is occurring much more rapidly in placebo than in natalizumab.

To avoid the formation of these impurities in the placebo, histidine was replaced with inorganic buffering components in the placebo formulation. The placebo product for the

Samsung Bioepis Exhibit No. 1002
Page 2115

clinical trials is a sterile isotonic phosphate buffered solution with 0.02% (w/v) polysorbate 80 at pH 6.0. The substitution of histidine with phosphate was demonstrated to significantly reduce the rate of polysorbate 80 degradation. 100 µL of the phosphate/NaCl/0.02% (w/v) polysorbate 80 placebo formulation was analyzed by size exclusion HPLC monitored at 260 nm both at time zero and after 3 days at 60°C. Little change is seen in the SEC-HPLC profile as a result of this incubation. The SEC-HPLC profile for the histidine/NaCl/0.02% (w/v) polysorbate 80 formulation following only two days at 60°C shows significant levels of trace impurities related to the degradation of polysorbate 80. These data demonstrate that the polysorbate 80 degradation is significantly impeded by replacing histidine with phosphate in the placebo formulation.

Example 3

Natalizumab Formulation with Polysorbate 80 and Histidine Combined

The mechanism by which these trace impurities are produced is thought to be through a metal catalyzed oxidation of polysorbate (*see* Donbrow *et al.*, 1978 *J. Pharmaceutical Sciences*, 67(12): 28). Donbrow describes that autoxidation during storage occurs for different types of polysorbate (*e.g.*, polysorbate 20). Light, temperatures and metal ions also impact autoxidation. Donbrow *et al.* (1978). It has been confirmed that both histidine and polysorbate 80 are required for this reaction to proceed at a significant rate. Ajinomoto is the single histidine source used for formulation. However significant differences in the rate of reaction between lots supplied by the Ajinomoto have been observed.

An additional factor that plays a role in accelerating the reaction is the presence of metal. This was demonstrated by running a reaction at 60°C for five days in glass vessel with 50 mM histidine (Lot No. R016A008) and 2% (w/v) polysorbate 80. Under these conditions, minimal levels of the trace impurities are produced for this histidine lot. The reaction was then split into three vessels: (1) the first vessel remained as the control; (2) a gray butyl container closure (stopper) was added to the second vessel; and (3) a stainless steel needle was added to the third vessel. These reactions were then run for four days at 60°C and analyzed by a UV scan from 200 to 400 nm. The reaction progressed further over this time period in the presence of the needle.

Samsung Bioepis Exhibit No. 1002
Page 2116

Example 4

Evaluation of Impurities — Mouse Single-Dose Limit Test

The potential toxicity of these impurities was evaluated in a mouse single-dose limit test. Histidine placebo and natalizumab samples in Formulation #2 stored at 40°C for six weeks were used because these samples provide the largest amount of the impurities. There were no signs of toxicity due to the impurities. Preliminary data are provided in the non-clinical section of the submission.

The SEC-HPLC profiles for 100 μL injections of samples used in the mouse single-dose limit test were determined. The natalizumab sample in Formulation #1 spiked with polysorbate 80 prior to analysis has little 260 nm absorbing material eluting after 20 minutes. The peak eluting at 34 minutes was found in this lot of natalizumab without polysorbate 80 addition and therefore does not indicate polysorbate 80 degradation. By contrast, the placebo in Formulation #2 stored at 40°C for six weeks, showed complete degradation, as the total area under the curve was 12.8 million μV•seconds. The natalizumab sample in Formulation #2 and stored at 40°C for six weeks had less complete degradation. The analysis confirms that the samples stored at 40°C and tested in the mouse single-dose limit test are laden with degraded polysorbate 80.

Example 5

Establishing Impurity Specifications

To assess if partially degraded polysorbate 80 is still capable of preventing antibody aggregation, Formulation #2 was heated to 60°C for 3 days in the presence of a needle to convert all the polysorbate 80 to the maximum levels of impurities. The reaction was confirmed to have degraded all the polysorbate 80 by both SEC and reverse phase HPLC. This material was diluted one-to-one with a 10 mg/mL solution of AN100226 in Formulation #2 resulting in a solution containing 0.01% (w/v) polysorbate 80 and 50% of the maximum level of degraded polysorbate 80. These antibody solutions were exposed to both shaking and siliconized syringes for several hours; aggregation of the antibody was prevented. The control samples exposed to the same conditions but without polysorbate 80 showed significant precipitation.

Samsung Bioepis Exhibit No. 1002
Page 2117

It is concluded from this work that as long as no more than fifty percent of the polysorbate 80 is degraded, this material can provide the appropriate environment to prevent antibody aggregation.

Although very little polysorbate degradation is occurring in the active drug product, monitoring the isoelectric focusing (IEF) pattern is done to confirm that this formulation is not compromising the antibody. IEF profiles for the six week stability time points for 5°C, 25°C and 40°C storage conditions were determined. Both the 5°C and 25°C samples are comparable to the reference standard, with no evidence of any change in overall charge of the protein. The 40°C sample showed the shift to a more acidic species typical of this product in a liquid formulation either in the presence or absence of polysorbate 80.

To establish a relationship between the extent of polysorbate 80 degradation and the peak area from the SEC-HPLC, a dilution series for six concentrations of degraded polysorbate 80 from 0 to 50% was evaluated. Formulation #2 was heated to 60°C for 3 days with a needle and confirmed to be 100% degraded by SEC and reverse phase HPLC. The reaction mixture was then diluted with AN100226 purified bulk in Formulation #1, freshly spiked with 0.02% (w/v) polysorbate 80 and analyzed by SEC-HPLC. The SEC profiles were monitored at 260 nm.

The data from this dilution series were plotted by the total peak area integrated (μV•seconds) for the absorbency profile after approximately 24 minutes as a function of the ratio of degraded polysorbate 80. This plot indicates that an area of less than 5 million μV•seconds represents about a 50% loss of polysorbate 80 to degradation products. There is a direct relationship between the amount of oxidized polysorbate 80 added and the peak area for the peaks eluting late in the chromatogram.

A preliminary limit for these trace impurities in natalizumab has been established based on the mouse single-dose limit test, the antibody solubility data with 50% degraded polysorbate 80, and estimates of the extent of the polysorbate 80 degradation. The method has been included in the ongoing stability program and a limit has been established. If additional lots are manufactured with histidine buffer, these limits will be applied at time of release as well.

Limit: total area under the peaks after approximately 24 minutes is not to exceed 4 x 106 μV•seconds.

Samsung Bioepis Exhibit No. 1002
Page 2118

Example 6

1.7 mg/mL Natalizumab Formulation

1.7 mg Natalizumab
1.4 mg Sodium phosphate, USP
8.2 mg Sodium chloride, USP
0.2 mg Polysorbate 80, NF

Adjust pH to 6.0 ± 0.5 with phosphoric acid, NF. QS to 1 mL. With preferred storage at 5-8°C.

Example 7

5.0 mg/mL Natalizumab Formulation

5.0 mg Natalizumab
1.4 mg Sodium phosphate, USP
8.2 mg Sodium chloride, USP
0.2 mg Polysorbate 80, NF

Adjust pH to 6.0 ± 0.5 with phosphoric acid, NF. QS to 1 mL. With preferred storage at 5-8°C.

Example 8

20 mg/mL Natalizumab Formulation

20.0 mg Natalizumab
1.4 mg Sodium phosphate, USP
8.2 mg Sodium chloride, USP
0.2 mg Polysorbate 80, NF

Adjust pH to 6.0 ± 0.5 with phosphoric acid, NF. QS to 1 mL. With preferred storage at 5-8°C.

Samsung Bioepis Exhibit No. 1002
Page 2119

Example 9

50.0 mg/mL Natalizumab Formulation

50.0 mg Natalizumab
1.4 mg Sodium phosphate, USP
8.2 mg Sodium chloride, USP
0.2 mg Polysorbate 80, NF

Adjust pH to 6.0 ± 0.5 with phosphoric acid, NF. QS to 1 mL. With preferred storage at 5-8°C.

Example 10

5.0 mg/mL Natalizumab Formulation

5.0 mg/mL natalizumab
140 mM NaCl
0.02% Polysorbate 80 (w/v)
10 mM sodium phosphate

Adjust pH to 6.0 ± 0.5 with phosphoric acid. Optimally store formulation at about 5°C to about 8°C.

Example 11

10 mg/mL Natalizumab Formulation

10.0 mg Natalizumab
1.4 mg Sodium phosphate, USP
8.2 mg Sodium chloride, USP
0.2 mg Polysorbate 80, NF

Adjust pH to 6.0 ± 0.5 with phosphoric acid, NF. QS to 1 mL. With preferred storage at 5-8°C.

Samsung Bioepis Exhibit No. 1002
Page 2120

Example 12

10 mg/mL Natalizumab Formulation

10.0 mg Natalizumab
1.4 mg Sodium phosphate, USP
8.2 mg Sodium chloride, USP
0.1 mg Polysorbate 80, NF

Adjust pH to 6.0 ± 0.5 with phosphoric acid, NF. QS to 1 mL. With preferred storage at 5-8°C.

Example 13

20 mg/mL Natalizumab Formulation

20.0 mg/mL Natalizumab
140 mM NaCl
0.02% Polysorbate 80 (w/v)
10 mM sodium phosphate

Adjust pH to 6.0 ± 0.5 with phosphoric acid and bring volume to 125 mL. Optimally store formulation at about 5°C to about 8°C.

Example 15

Lyopholized Natalizumab Formulation

Additional liquid formulations of antibody at high concentration, from 20 –200 mg/mL may consist of phosphate or other suitable buffer (such as histidine, citrate, acetate or succinate) in the concentration range of 2 to 50 mM, to provide buffering in the pH range of 3.0 to 7.0. Most preferably, the pH is 6.0, +/- 0.5. The addition of polyols (such as sorbitol and mannitol), disaccharides (such as sucrose or trehalose) and amino acids (such as glycine) may be added in varying amounts with sodium chloride to maintain stability and provide an isotonic solution. The use of surfactants, such but not limited to the polysorbates, add stability when used in the range of 0.001 to 2%. To prepare a liquid formulation,

Samsung Bioepis Exhibit No. 1002
Page 2121

natalizumab was concentrated to 65 mg/mL in 10 mM sodium phosphate, 140 mM sodium chloride, pH 6, with about 0.06% polysorbate 80. The resulting solution was slightly opalescent but without particulates. The sample contained greater than 99% monomer with no high molecular weight aggregate or low molecular weight species by SEC.

A stable lyophilized pharmaceutical formulation is provided. Because phosphate buffer undergoes a pH change during freezing, it is necessary to replace the phosphate with a different buffer. This buffer may be comprised of histidine, citrate or succinate,with the ability to buffer effectively in the pH range of 3.0 to 7.0, most preferably in the range of 6.0 +/- 0.5.

The use of polyols (such as mannitol) and sugars (such as sucrose) are necessary to provide cryo- and lyo-protection. These polyols may be used alone or in a combination to provide for stability and adjustment of the tonicity. Additionally, the use of amino acids (such as glycine), at levels of 10-1000 mM may used to prevent aggregation.

Surfactants, such as polysorbates or poloxamers, may be used at levels from 0.001% to 2.0% to provide for stability before lyophilization and after reconstitution and to provide for more rapid reconstitution times.

The protein, following the final purification step, may be formulated using ultrafiltration for concentration and diafiltration for buffer exchange. The protein may also be formulated using column chromatography for buffer exchange. Some combination of these techniques may also be used.

In addition, the final desired protein concentration may be obtained by filling at a protein and excipient concentration lower than desired and reconstitution at a smaller volume. For example, a 2.5 mL fill volume of a 40 mg/mL solution may be used, followed by reconstitution with 1 mL to obtain a 100 mg/mL solution.

For example, natalizumab, at a concentration of 20 mg/mL, was lyophilized in a solution containing 5 mM histidine, 20 mg/mL sucrose and 0.02% polysorbate 80, pH 6. The solution was filled at 5mL per vial into 10 mL borosilicate glass vials and fitted with gray butyl rubber lyophilization stoppers. Lyophilization was done using a Virtis Gensis model lyophilizer. The product was frozen at a shelf temperature of -60° C for 10 hours and then the shelf temperature was raised to -40°. Primary drying was performed at a shelf temperature of -10° C and a chamber pressure of 100 mTorr for 20 hours. Secondary drying

Samsung Bioepis Exhibit No. 1002
Page 2122

was achieved at a shelf temperature of 25°C with a chamber pressure of 100 mTorr for 10 hours. The vials were stoppered under vacuum.

The vials were then reconstituted using 1 mL of sterile WFI to give a formulation containing 100 mg/mL natalizumab. The samples were analyzed immediately after lyophilization and after 2 wks of storage in the lyophilized form at 40 deg. In both cases reconstitution times were immediate. The reconstituted solutions were clear and colorless with an absence of particulate material. The samples contained greater than 99% monomer by SEC, with no high molecular weight aggregate, or low molecular weight species. After 2 weeks storage at 40 deg, the sample showed 94% potency relative to reference (specification 80-125%).

All cited patents and publications referred to in this application are herein incorporated by reference in their entirety for all purposes.

Samsung Bioepis Exhibit No. 1002
Page 2123

Claims:

We claim:

1. A stable, aqueous pharmaceutical formulation comprising an immunoglobulin, a phosphate buffer, a polysorbate, and sodium chloride.

2. The formulation of claim 1, wherein the polysorbate is polysorbate 80.

3. The formulation of claim 2, wherein the polysorbate 80 is present in an amount of about 0.001% to about 2.0% (w/v).

4. The formulation of claim 3, wherein the polysorbate 80 is present in the amount of about 0.02% (w/v).

5. The formulation of claim 1, wherein the immunoglobulin is present in the amount of about 0.1 mg/mL to about 200 mg/mL.

6. The formulation of claim 5, wherein the immunoglobulin is present in the amount of about 1.7 mg/mL.

7. The formulation of claim 5, wherein the immunoglobulin is present in the amount of about 5 mg/mL.

8. The formulation of claim 5, wherein the immunoglobulin is present in the amount of about 20 mg/mL.

9. The formulation of any one of claims 1 to 8, wherein the formulation has a pH of about 3.0 to about 7.0.

10. The formulation of claim 9, wherein the pH is about 5.5 to about 6.5.

11. The formulation of claim 10, wherein the pH is about 6.0±0.5.

Samsung Bioepis Exhibit No. 1002
Page 2124

12. The formulation of claim 1, wherein the formulation is in a fixed volume and the immunoglobulin is present in the amount of about 50 mg/mL.

13. The formulation of claim 12, wherein the immunoglobulin binds alpha-4 integrin.

14. The formulation of claim 13, wherein the immunoglobulin is natalizumab.

15. The formulation of claim 1, wherein the phosphate buffer is at pH 6.0±0.5, the polysorbate is polysorbate 80 and is present in an amount of about 0.02% (w/v), the immunoglobulin is natalizumab, and wherein the formulation is stable at a temperature of about 2°C to about 8°C for at least 6 months.

16. The formulation of claim 15, wherein natalizumab is present in an amount of about 20 mg/mL to about 150 mg/mL.

17. The formulation of claim 1, wherein the formulation is isotonic.

18. The formulation of any one of claims 1-14 or 17, wherein the immunoglobulin is a monoclonal antibody.

19. The formulation of claim 18, wherein the monoclonal antibody is natalizumab.

20. The formulation of claim 18-19, wherein the antibody is present in an amount of about 0.1 mg/mL to about 200 mg/mL.

21. The formulation of claim 19, wherein the antibody is present in an amount of about 1 mg/mL to about 150 mg/mL.

22. The formulation of claim 21, wherein the antibody is present in an amount of about 1.7 mg/mL or about 50 mg/mL.

Samsung Bioepis Exhibit No. 1002
Page 2125

23. The formulation of claim 18, wherein the antibody is present in the amount of about 15 mg/mL to about 50 mg/mL.

24. The formulation of claim 18, wherein the antibody is present in the amount of about 20 mg/mL.

25. The formulation of claim 1, wherein the formulation further comprises histidine.

26. The formulation of claim 25, wherein the polysorbate is polysorbate 80.

27. A method of treating a patient with variable weight for a condition with a therapeutic amount of an immunoglobulin comprising administering a formulation of claim 1 to said patient wherein the condition is treated by administration of the formulation.

28. The method of claim 27, wherein the immunoglobulin is natalizumab.

29. A composition comprising a sodium phosphate, a polysorbate, a protein and NaCl with a pH of 6.0±0.5, wherein the composition is stable when stored at about 5°C to about 8°C for greater than 6 months.

30. The composition of claim 29, wherein the polysorbate is polysorbate 80 and is present in an amount of about 0.001% to about 2.0% (w/v).

31. The composition of claim 29, wherein the protein is an immunoglobulin which is present in an amount of about 0.01 mg/mL to about 200 mg/mL.

32. The composition of claim 29, wherein the polysorbate is polysorbate 80 and is present in the amount of about 0.02% (w/v), the NaCl is present in the amount of 150 mM, the phosphate buffer is present in the amount of 10 mM, and the immunoglobulin is natalizumab and is present in the amount of 1.7 mg/mL, 5 mg/mL, 20 mg/mL or 50 mg/mL.

Samsung Bioepis Exhibit No. 1002
Page 2126

33. A method of preparing a stable protein containing formulation comprising admixing sodium phosphate, sodium chloride, a polysorbate and a protein and adjusting the pH of the mixture with phosphoric acid to about pH 6.0 ± 0.5.

34. The method of preparing a stable protein containing formulation of claim 33, wherein the sodium phosphate is present in an amount of about 10 mM, the sodium chloride is present in an amount of about 150 mM, the polysorbate is polysorbate 80 and is present in an amount of about 0.02% (w/v) and the protein is natalizumab.

35. The method of preparing a stable protein containing formulation of claim 33, wherein natalizumab is present in an amount of about 20 mg/mL to about 200 mg/mL.

36. The method of preparing a stable protein containing formulation of claim 35, wherein natalizumab is present in an amount of about 150 mg/mL.

37. The method of preparing a stable protein containing formulation of claim 33, wherein the protein is lyophilized in the formulation of claim 1.

38. The method of preparing a stable protein containing formulation of claim 37, wherein the polysorbate is polysorbate 80 and is present in an amount of about 0.02% (w/v) and the protein is natalizumab.

39. The method of preparing a stable protein containing formulation of claim 37, wherein the formulation further comprises histidine.

40. The method of preparing a stable protein containing formulation of claim 33, wherein the protein is lyophilized in a solution comprising 5 mM histidine, 20 mg/mL sucrose and 0.02% polysorbate 80 at a pH 6, and wherein the protein is natalizumab at a concentration of 20 mg/mL.

41. An article of manufacture comprising a container holding the stable formulation of any one of claims 1-29.

Samsung Bioepis Exhibit No. 1002
Page 2127

42. A method for treating a patient with variable weight for a condition, comprising simultaneously or sequentially administering to the patient a therapeutically effective combination of a formulation of any one of claims 1-24 and a compound or therapy effective against the condition.

43. The use of the stable formulation of any one of claims 1-24 for the preparation of a medicament for the treatment of a condition in a patient with a variable weight condition wherein the medicament is effective to treat said condition.

44. The use of the stable formulation of any one of claims 1-24 for the preparation of a medicament to treat a condition in a patient with a variable weight condition, wherein the medicament is a combination of a formulation of any one of claims 1-24 and a compound or therapy, and wherein said medicament is effective to treat the condition.

Samsung Bioepis Exhibit No. 1002
Page 2128

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(71) Applicant *(for all designated States except US)*: **ALEXION PHARMACEUTICALS, INC.** [US/US]; 352 Knotter Drive, Cheshire, CT 06410 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **ROTHER, Russell, P.** [US/US]; 25 Blue Trail Drive, Prospect, CT 06712 (US). **FAAS-KNIGHT, Susan** [US/US]; 14 Stonewood Drive, Old Lyme, CT 06371 (US). **WU, Dayang** [CN/US]; 755 Devonwood Drive, Cheshire, CT 06410 (US). **CARR, Francis, J.** [GB/GB]; Aberdeen AB228GU (GB). **HAMILTON, Anita** [GB/GB]; Aberdeen AB228GU (GB).

(74) Agents: **FARBER, Mark** et al.; Alexion Pharmaceuticals, Inc., 352 Knotter Drive, Cheshire, CT 06410 (US).

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(54) Title: DE-IMMUNIZED ANTI-CD3 ANTIBODY

(57) Abstract: Antibodies or functional antibody fragments that recognize or interfere with the production of a component of the CD3 antigen complex are de-immunized.

106 PCT (1087-59PCT)

DE-IMMUNIZED ANTI-CD3 ANTIBODY

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application 60/475,155 filed June 2, 2003, the entire disclosure of which is incorporated herein by this reference.

BACKGROUND

1. Technical Field

The present disclosure relates to the field of genetically engineered antibodies. More specifically this disclosure relates to anti-CD3 antibodies which have been structurally altered to eliminate binding to HLA proteins, thereby potentially reducing immunogenicity.

2. Background of Related Art

Antibodies are produced by B lymphocytes and defend against infections. The basic structure of an antibody consists of two identical light polypeptide chains and two identical heavy polypeptide chains linked together by disulphide bonds. The first domain located at the amino terminus of each chain is highly variable in amino acid sequence, providing the vast spectrum of antibody binding specificities found in each individual. These are known as variable heavy (VH) and variable light (VL) regions. The other domains of each chain are relatively invariant in amino acid sequence and are known as constant heavy (CH) and constant light (CL) regions.

The interaction between the antigen and the antibody takes place by the

formation of multiple bonds and attractive forces such as hydrogen bonds, electrostatic forces and Van der Waals forces. Together these form considerable binding energy which allows the antibody to bind the antigen. Antibody binding affinity and avidity have been found to affect the physiological and pathological properties of antibodies.

The advent of genetic engineering technology has led to various means of producing unlimited quantities of uniform antibodies (monoclonal antibodies) which, depending upon the isotype, exhibit varying degrees of effector function. For example, certain murine isotypes (IgG1, IgG2) as well as human isotypes (particularly IgG1) can effectively bind to Fc receptors on cells such as monocytes, B cells and NK cells, thereby activating the cells to release cytokines. Such antibody isotypes are also potent in activating complement, with local or systemic inflammatory consequences. The anti-CD3 murine antibody OKT3 is one antibody that has been observed to cause significant cytokine release leading to cytokine release syndrome (CRS). The human CD3 antigen consists of at least four invariant polypeptide chains, which are non-covalently associated with the T cell receptors (TCR) on the surface of T-cells, typically referred to as the CD3 antigen complex. The CD3 antigen complex plays an important role in the T-cell activation upon antigen binding to the T cell receptor. Some anti-CD3 antibodies can activate T-cells in the absence of antigen-TCR ligation, but such activation also depends on the interaction of the Fc portion of the mAb and the Fc receptors on accessory cells to crosslink CD3 complexes on the T-cells. The importance of Fc interactions in anti-CD3 mediated T-cell activation is illustrated by the observation that "mitogenic" anti-CD3 antibodies such as OKT3 do not stimulate T-cells to proliferate in vitro unless they are bound to plastic (which permits CD3 cross-leaking) or bound to Fc

receptor bearing cells.

Antibodies to the CD3 ε signaling molecule of the T-cell receptor complex have proven to be powerful immunosuppressants. For example, OKT3 is a mouse IgG2a/k MAb which recognizes an epitope on the T-cell receptor-CD3 epsilon chain and has been approved for use in many countries throughout the world as an immunosuppressant in the treatment of acute allograft rejection. The binding of OKT3 to CD3 results in a coating and/or modulation of the entire TcR complex, which mediates TcR blockade and may be one mechanism by which alloantigen and cell-mediated cytotoxicity are inhibited.

The murine OKT3 antibody has been in use therapeutically since its approval in 1985. However, in view of the murine nature of this MAb, a significant human anti-mouse antibody (HAMA) response, with a major anti-idiotype component occurs, which severely limits the dosing potential of this antibody. A HAMA response is initiated when T cells from an individual make an immune response to the administered antibody. The T cells then recruit B cells to generate specific "anti-antibody" antibodies. Thus the HAMA response, though mediated by B cell-generated antibodies directed against mouse antibodies, depends upon an initial T cell response to occur. Clearly, it would be highly desirable to diminish or abolish this HAMA response by suitable humanization or other recombinant DNA manipulation of this very useful antibody and thus enlarge its area of use.

Several techniques have been employed to address the HAMA problem and thus enable the use of therapeutic murine-derived monoclonal antibodies in humans. A common aspect of these methodologies has been the introduction into the therapeutic

antibody, which in general is of rodent origin, of significant tracts of sequence identical to that present in human antibody proteins. Such alterations are also usually coupled to alteration of particular single amino acid residues at positions considered critical to maintaining the antibody-antigen binding interaction. For antibodies, this process is possible due to the very high degree of structural (and functional) conservation between antibody molecules of different species. However for potentially therapeutic proteins where no structural homologue may exist in the host species (e.g. human) for the therapeutic protein, such processes are not applicable.

The term humanized antibody describes a molecule having certain components of the antigen binding site called complementarity determining regions (CDRs) derived from an antibody from a non-human species, while the remaining regions of the antigen binding site (called framework regions) are derived from human antibodies. The antigen binding site may also comprise complete non-human variable regions fused onto human constant domains (a "chimeric" antibody). Since a primary function of an antibody is to bind its target antigen, it is important that the original features of the antibody are preserved in such a way that the antigen specificity and affinity are maintained. Unfortunately, however, humanization of non-human antibodies has unpredictable effects on antibody-antigen interactions, e.g., antigen binding properties. This means that in therapeutic applications, more of the humanized antibody may be required per dose resulting in a higher cost of treatment and potentially greater risk of adverse events. In addition, both fully human and humanized antibodies can provoke an immune response or be immunogenic when administered to certain individuals.

According to another method, an antibody is rendered non-immunogenic, or less

immunogenic, to a given species, by first determining at least part of the amino acid sequence of the protein and then identifying in that amino acid sequence one or more potential epitopes to which T cells from the given species can react. Next, the amino acid sequence of the antibody is modified to eliminate at least one of the T cell epitopes identified to reduce the immunogenicity of the protein or part thereof when exposed to the immune system of the given species. Unlike antibodies, which can recognize and bind to soluble antigens, T cells must encounter their antigen targets through the activity of specialized antigen-presenting cells (APC's) such as dendritic cells and macrophages. APC's ingest foreign antigens and process them into peptides, which are then complexed to the HLA proteins and expressed on the surface of the APC. T cells can only recognize antigen fragments "presented" in the context of HLA. In the method which has been termed "de-immunization" and is described herein, amino acids within the antibody sequence that are predicted to bind effectively to HLA molecules are changed such that they no longer bind HLA and thus can no longer stimulate a T cell response. The lack of a T cell response to antigen translates into a reduction or elimination of a HAMA response.

SUMMARY

Antibodies in accordance with this disclosure recognize the CD3 antigen complex or interfere with the cell –surface expression of a component of the CD3 antigen complex. The anti-CD3 antibodies are also de-immunized (that is, rendered non-immunogenic, or less immunogenic, to a given species). In a particularly useful embodiment, de-immunization is achieved by first determining at least part of the amino acid sequence of the protein and then identifying in the amino acid sequence one or

more potential epitopes for T cells ("T cell epitopes") which are able to bind to HLA proteins of the given species. Next, the amino acid sequence of the antibody is modified to eliminate at least one of the T cell epitopes identified in order to reduce the immunogenicity of the protein or part thereof when exposed to the immune system of the given species.

In another aspect, this disclosure relates to a process for producing an antibody which includes the steps of: (a) producing an expression vector having a DNA sequence which includes a sequence that encodes an anti-CD3 antibody, at least a portion of which has been de-immunized; (b) transfecting a host cell with the vector; and (c) culturing the transfected cell line to produce the engineered antibody molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show the complete nucleotide and amino acid sequences of the OKT3 heavy chain variable region (GenBank Accession number A22261) and light chain variable region (GenBank Accession number A22259), respectively.

Fig. 2 schematically shows expression cassettes for the heavy and light chain variable regions as *Hind*III to *Bam*H1 fragments.

Figure 3 shows the complete nucleotide (SEQ ID NO: 1) and amino acid sequences of the murine OKT3 heavy chain variable region, including the murine immunoglobulin promoter, a murine signal sequence with intron at the 5' ends, and a splice donor site (Bam HI) at the 3' ends. Restriction enzyme sites are indicated.

Figure 4 shows the complete nucleotide (SEQ ID NO: 3) and amino acid sequences of the murine OKT3 light chain variable region, including the murine immunoglobulin promoter, a murine signal sequence with intron at the 5' ends, and a

splice donor site (Bam HI) at the 3' ends. Restriction enzyme sites are indicated.

Figure 5A shows a graphic map of the vector APEX-1 3F4V_HHuGamma4.

Figure 5B shows the complete nucleotide sequence of the vector (SEQ ID NO: 5) and indicates the amino acid and nucleotide sequences of the hIgG4 insert adjacent to an irrelevant VH region (labeled 3F4VH). The locations of the signal sequence, CH1, hinge, CH2 and CH3 regions are indicated.

Figure 6A shows a graphic map of the vector APEX-1 3F4V_HHuG2/G4.

Figure 6B shows the nucleotide sequence of the vector (SEQ ID NO: 7) and the amino acid and nucleic acid sequence of the G2/G4 insert, and indicates the locations of the signal sequence, irrelevant Vh (herein labeled 3F4Vh), CH1, hinge, CH2 and CH3 regions.

Figure 7 shows a graphic map of the heavy chain expression vector pSVgptHuG2/G4.

Figure 8 shows the complete nucleotide sequence (SEQ ID NO: 9).of the HuG2/G4 fragment excised from the APEX-1 3F4V_HHuG2/G4 vector and modified for insertion into a PUC 19 cloning vector by the addition, at the 5' end, of a Bam HI site and 5' untranslated inron sequences from native human IgG4 and, at the 3' end, of a Bgl II site and 3' untranslated sequence from natural human IgG4.

Figure 9 shows a graphic map of the expression vector pSVgptHuCk and indicates the position of the light chain variable and constant regions.

Figure 10 shows the amino acid sequences of the murine OKT3 variable heavy chain (SEQ ID NO: 10) and the amino acid sequences of several of the deimmunized heavy chain variable regions (SEQ ID NOS: 11-17) constructed in the Example.

Figure 11 shows the amino acid sequences of the murine OKT3 variable light chain (SEQ ID NO: 18) and the amino acid sequences of two deimmunized light chain variable regions (SEQ ID NOS: 19 and 20) constructed in the Example.

Figure 12 shows the mutagenic oligonucleotides primers used to construct the designed de-immunized sequences by mutagenesis using overlapping PCR.

Figure 13 shows the nucleic acid (SEQ ID NO: 21) and amino acid sequences for the de-immunized VH expression cassette OKT3DIVHV1.

Figure 14 shows the nucleic acid (SEQ ID NO: 23) and amino acid sequences for the de-immunized Vκ expression cassette OKT3DIVKV1

Figure 15 shows bidning of murine OKT3 and chimeric OKT3 to Jurkat, JRT3 and HPB-ALL cells.

Figures 16 and 17 are tables showing the binding of de-immunized anti-CD3 antibodies to HPB-ALL and JRT3 CELLS.

Figures 18, 19, 20 and 21 show the results of competition assays measuring the affinity of the de-immunized antibodies relative to that of chimeric OKT3 and murine OKT3.

Figure 22 is a table summarizing the IC50 of the de-immunized antibodies relative to that of murine OKT3.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

De-immunized anti-CD3 antibodies are described. The term "anti-CD3 antibodies" means any antibody or functional antibody fragment that recognizes the CD3 antigen complex or interferes with the cell surface expression of a component of

Samsung Bioepis Exhibit No. 1002
Page 2137

the CD3 antigen complex. The anti-CD3 antibody can be recombinant or naturally occurring. The anti-CD3 antibody can be human, non-human, chimeric or humanized. Anti-CD3 antibodies are known to those skilled in the art and include, for example, the antibodies described in U.S. Patent No. 5,527,713 entitled "Methods for inducing a population of T cells to proliferate using agents which recognize TCR/CD3 and ligands which stimulate an accessory molecule on the surface of the T cells"; U.S. Patent No. 6,352,694 entitled "Methods for inducing a population of T cells to proliferate using agents which recognize TCR/CD3 and ligands which stimulate an accessory molecule on the surface of the T cells"; U.S. Patent No. 6,406,696 entitled "Methods of stimulating the immune system with anti-CD3 antibodies"; U.S. Patent No. 6,143,297 entitled "Methods of promoting immunopotentiation and preparing antibodies with anti-CD3 antibodies"; U.S. Patent No. 6,113,901 entitled "Methods of stimulating or enhancing the immune system with anti-CD3 antibodies"; U.S. Patent No. 6,491,916 entitled "Methods and materials for modulation of the immunosuppresive activity and toxicity of monoclonal antibodies"; U.S. Patent No. 5,929,212 entitled "CD3 specific recombinant antibody"; U.S. Patent No. 5,834,597 entitled "Mutated nonactivating IgG2 domains and anti CD3 antibodies incorporating the same"; U.S. Patent No. 5,527,713 entitled "Anti-CD3 antibody-aminodextran conjugates for induction of T-cell activation and proliferation"; U.S. Patent No. 5,316,763 entitled "Short-term anti-CD3 stimulation of lymphocytes to increase their in vivo activity"; U.S. Patent No. 5,821,337 entitled "Immunoglobulin variants". Each of these patents is incorporated herein in its entirety by this reference.

The anti-CD3 antibody is de-immunized. De-immunization renders the anti-CD3 antibody non-immunogenic, or less immunogenic, to a given species. De-immunization can be achieved through structural alterations to the anti-CD3 antibody. Any de-immunization technique known to those skilled in the art can be employed. One suitable technique for de-immunizing antibodies is described, for example, WO 00/34317 published June 15, 2000, the disclosure of which is incorporated herein in its entirety. In summary, a typical protocol within the general method described therein includes the following steps.

1. Determining the amino acid sequence of the antibody or a part thereof (if modification ofonly of a part is required);

2. Identifying potential T cell epitopes within the amino acid sequence of the antibody by any method including determination of the binding of peptides to MHC molecules, determination of the binding of peptide:HLA complexes to the T cell receptors from the species to receive the therapeutic protein, testing of the antibody or parts thereof using transgenic animals with HLA molecules of the species to receive the therapeutic protein, or testing such transgenic animals reconstituted with immune system cells from the species to receive the therapeutic protein;

3. By genetic engineering or other methods for producing modified antibodies, altering the antibody to remove one or more of the potential T cell epitopes and producing such an altered antibody for testing;

Samsung Bioepis Exhibit No. 1002
Page 2139

4. Optionally within step 3, altering the antibody to remove one or more of the potential B cell epitopes;

5. Testing altered antibodies with one or more potential T cell epitopes (and optionally B cell epitopes) removed in order to identify a modified antibody which has retained all or part of its desired activity but which has lost one or more T cell epitopes. Potential T-cell epitopes herein are defined as specific peptide sequences which either are predicted to or that bind with reasonable efficiency to HLA class II molecules (or their equivalent in a non-human species), or which in the form of peptide:HLA complexes bind strongly to the T cell receptors from the species to receive the therapeutic protein or which, from previous or other studies, show the ability to stimulate T-cells via presentation on HLA class II molecules present on antigen presenting cells from the species to receive the therapeutic antibody.

This de-immunization method recognizes that an effective T cell-dependant immune response to a foreign protein requires activation of the cellular arm of the immune system. Such a response requires the uptake of the therapeutic (foreign) protein (i.e. therapeutic antibody) by antigen presenting cells (APCs). Once inside such cells, the protein is processed and fragments of the protein form a complex with MHC class II molecules and are presented at the cell surface. Should such a complex be recognized by binding of the T cell receptor from T-cells, such cells can be, under certain conditions, activated to produce stimulatory cytokines. The cytokines will elicit differentiation of B-cells to mature antibody producing cells. In addition, such T cell responses may also mediate other deleterious effects on the patient such as

Samsung Bioepis Exhibit No. 1002
Page 2140

inflammation and possible allergic reaction.

The whole anti-CD3 antibody or only a portion thereof (e.g., the variable portions of the anti-CD3 antibody) can be de-immunized. De-immunization of only a portion of the anti-CD3 antibody is particularly useful where the anti-CD3 antibody is a chimeric antibody (e.g. one with human constant regions).

The term "antibody" as used herein includes whole polyclonal and monoclonal antibodies, single chain antibodies, and other functional antibody fragments. Whole, monoclonal antibodies are preferred.

In general, the construction of the antibodies disclosed herein is achieved by using recognized manipulations utilized in genetic engineering technology. For example, techniques for isolating DNA, making and selecting vectors for expressing the DNA, purifying and analyzing nucleic acids, specific methods for making recombinant vector DNA (e.g. PCR), cleaving DNA with restriction enzymes, ligating DNA, introducing DNA, including vector DNA, into host cells by stable or transient means, culturing the host cells in selective or non-selective media, to select and maintain cells that express DNA, are generally known in the field.

The monoclonal antibodies disclosed herein may be derived using the hybridoma method (Kohler et al., Nature, 256:495, 1975), or other recombinant DNA methods well known in the art. In the hybridoma method, a mouse or other appropriate host animal is immunized with a protein which elicits the production of antibodies by the lymphocytes. Alternatively, lymphocytes may be immunized in vitro. The lymphocytes produced in response to the antigen are then are fused with myeloma cells using a suitable fusing

agent, such as polyethylene glycol, to form a hybridoma cell (Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986). The hybridoma cells are then seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. Preferred myeloma cells are those that fuse efficiently, support stable production of antibody by the selected antibody-producing cells, and are not sensitive to a selective medium such as HAT medium (Sigma Chemical Company, St. Louis, Mo., Catalog No. H-0262). Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-20, NS0 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA.

The hybridoma cells are grown in a selective culture medium (e.g., HAT) and surviving cells expanded and assayed for production of monoclonal antibodies directed against the antigen. The binding specificity of monoclonal antibodies produced by hybridoma cells may be determined by assays, such as, immunoprecipitation, radioimmunoassay (RIA), flow cytometry or enzyme-linked immunoabsorbent assay (ELISA).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986). In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal. The monoclonal antibodies secreted by

the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography. DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as E. coli cells, or mammalian cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells.

Antibodies or antibody fragments can also be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990). Other publications have described the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., Bio/Technology, 10:779-783, 1992), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., Nuc. Acids. Res., 21:2265-2266, 1993). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of antigen-specific monoclonal antibodies.

In another aspect, this disclosure provides recombinant expression vectors which include the synthetic, genomic, or cDNA-derived nucleic acid fragments necessary to produce a de-immunized anti-CD3 antibody. The nucleotide sequence coding for any

Samsung Bioepis Exhibit No. 1002
Page 2143

de-immunized anti-CD3 antibody in accordance with this disclosure can be inserted into an appropriate vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. Any suitable host cell vector may be used for expression of the DNA sequences coding for the de-immunized anti-CD3 antibody. Bacterial (e.g. E.coli) and other microbial systems may be used. Eukaryotic (e.g. mammalian) host cell expression systems may also be used to obtain antibodies of the present disclosure. Suitable mammalian host cell include COS cells and CHO cells (Bebbington C R (1991) Methods 2 136-145); and myeloma or hybridoma cell lines (for example NSO cells; Bebbington, et al., Bio Technology, 10, 169-175. 1992).

The de-immunized anti-CD3 antibodies can also be used as separately administered compositions given in conjunction with therapeutic agents. For diagnostic purposes, the antibodies may either be labeled or unlabeled. Unlabeled antibodies can be used in combination with other labeled antibodies (second antibodies) that are reactive with the de-immunized anti-CD3 antibody, such as antibodies specific for human immunoglobulin constant regions. Alternatively, the de-immunized antibodies can be directly labeled. A wide variety of labels may be employed, such as radionuclides, fluors, enzymes, enzyme substrates, enzyme co-factors, enzyme inhibitors, ligands (particularly haptens), etc. Numerous types of immunoassays are available and are well known to those skilled in the art.

The present de-immunized anti-CD3 antibodies can be administered to a patient in a composition comprising a pharmaceutical carrier. A pharmaceutical carrier can be any compatible, non-toxic substance suitable for delivery of the antibodies to the patient. Sterile water, alcohol, fats, waxes, and inert solids may be included in the

Samsung Bioepis Exhibit No. 1002
Page 2144

carrier. Pharmaceutically accepted adjuvants (buffering agents, dispersing agent) may also be incorporated into the pharmaceutical composition.

The antibody compositions may be administered to a patient in a variety of ways. Preferably, the pharmaceutical compositions may be administered parenterally (e.g., subcutaneously, intramuscularly or intravenously). Thus, compositions for parental administration may include a solution of the antibody, antibody fragment or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, etc. The concentration of antibody or antibody fragment in these formulations can vary widely, e.g., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

Actual methods for preparing parenterally administrable compositions and adjustments necessary for administration to subjects will be known or apparent to those skilled in the art and are described in more detail in, for example, *Remington's Pharmaceutical Science*, 17th Ed., Mack Publishing Company, Easton, Pa (1985), which is incorporated herein by reference.

Samsung Bioepis Exhibit No. 1002
Page 2145

The following examples are intended to illustrate but not limit the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may alternatively be used.

EXAMPLE 1

De-immunized, Chimeric Anti-CD3 Antibody

A de-immunized, chimeric anti-CD3 antibody was prepared. The variable regions selected were derived from the known mouse anti-human CD3 antibody OKT3. The variable regions were de-immunized and combined with an engineered human constant region to prepare the chimeric, de-immunized anti-CD3 antibody. The procedures used to prepare and test the chimeric, de-immunized anti-CD3 antibodies are described below.

The murine OKT3 heavy and light chain variable regions were constructed synthetically by gene synthesis using overlapping 40 mer oligonucleotides and a polymerase chain reaction. The sequences of the heavy and light chain variable regions of this antibody have been previously determined and deposited in the GenBank database (Accession numbers A22261 and A22259 respectively; see Figure 1). Sequences, including the murine immunoglobulin promoter and a murine signal sequence with intron, were added at the 5' ends, and sequences including the splice donor site were added at the 3' end by PCR to form expression cassettes (see Figure 2) for the heavy and light chain variable regions as *Hind*III to *Bam*HI fragments. The entire sequences of the expression cassettes were confirmed to be correct. The complete DNA and amino acid sequences of the murine OKT3 heavy and light chain expression cassettes are shown in Figures 3 and 4, respectively. The heavy chain

Samsung Bioepis Exhibit No. 1002
Page 2146

constant region was engineered to include a human IgG2 portion and a human IgG4 portion ("HuG2G4 constant region"). This constant region was prepared as follows: First, the genomic DNA encoding the human IgG4-derived portions (part of CH2 and CH3 regions) was inserted into the bacterial carrier plasmid pBR322, a plasmid derived from an E. coli species (ATCC 37017; Mandel, M. et al. (1970) J. Mol. Biol. 53, 154). The IgG4-derived insert was released from the plasmid by performing a restriction digest with Hind III and Xho I. The insert was gel purified, excised, and subjected to further restriction analysis to confirm the published sequence of the human IgG4 genomic DNA. The individual genomic IgG4 insert (HindIII/SmaI restriction fragment; the SmaI site is in the 3' untranslated region approximately 30 bp 3' of the translation stop site for each insert) was then subcloned by ligation into the expression cassette APEX-1 to yield APEX-1 3F4 VH HuGamma 4 (see Figure 5). DNA sequence analysis was performed to confirm the correct sequence of the human IgG4 desired regions.

The above procedure was also performed with a pBR322 bacterial plasmid which carried genomic DNA encoding the human IgG2 CH1, hinge region and first part of CH2, which were excised with PmlII and Bst EII and subcloned into APEX-1 3F4 VH HuGamma 4 to replace the corresponding IgG4-derived sequences.. The sequence of the resulting chimeric IgG2/IgG4 human constant region is shown in Figure 6 (APEX-1 3F4 VH G2/G4).

Construction of modified G2G4 constant region

The HuG2G4 constant region was modified for insertion into a heavy chain expression vector as follows:

Samsung Bioepis Exhibit No. 1002
Page 2147

The 5' end of the HuIgG4 constant region (native HuIgG4 5' intron sequence with BamH1 site at 5' end) up to the start of the coding region is amplified in reaction 1. The Hu G2G4 coding sequence (including intron) is amplified from APEX-1 3F4 VH Hu G2/G4 vector in reaction 2 (from start of CH1 to end of CH3 region). The 3' end of native HuIgG4 3' sequence from the end of the CH3 coding region is amplified in reaction 3, using a 3' primer designed to introduce a 3' Bam HI site with a Bgl II site just inside the Bam H1 and an Eco R1 site just inside the Bgl II site. The products of these 3 reactions (which overlap) are combined in a 4th PCR reaction using 5' and 3' primers. The combined product is cloned into the BamH1 site of pUC19 and the DNA sequence of the modified HuG2G4 fragment confirmed. The G2G4 gene is cut out with Bgl II and Bam HI to give a fragment with Bam HI at the 5' end and Bgl II at the 3' end. This is cloned into a heavy chain expression vector cut with Bam HI. A clone with the constant region inserted in the correct orientation (Bam HI site reformed at 5' end, hybrid Bam HI / BglII site at 3' end) is selected (Figure 7). The complete sequence of the Bam HI to Bgl II fragment is shown in Figure 8. Antibody Variable regions can be cloned in directly as Hind III to Bam HI fragments.

Primers (SEQ ID NOS:77 - 83):

TTGTGAGCGGATAACAATTTC M13 –50 REVERSE
GTTTTCCCAGTCACGACGTTGTA M13 –40 FORWARD
CTTGCAGCCTCCACCAAGGGCCCATCCGTC G2G4-1
CCCTTGGTGGAGGCTGCAAGAGAGG G2G4-2
GAGCCTCTCCCTGTCTCTGGGTAAATGAGTGCC G2G4-3
TCATTTACCCAGAGACAGGGAGAGGCTCTTCTGTG G2G4-4
TACCCGGGGATCCAGATCTGAATTCCTCATGTCAC G2G4-6

The light chain constant region was the human kappa constant region. This is included in the expression vector pSV hyg HuCκ as shown in Figure 9.

The amino acid sequence of the variable regions of the murine anti-CD3 antibody OKT3 were analyzed for potential T cell epitopes (MHC Class II binding peptides) by using the peptide threading software as detailed in WO 02/069232 published September 6 2002 and other *in silico* techniques. De-immunized sequences were designed to eliminate the potential T cell epitopes, as far as possible by making conservative amino acid changes. In order to test the effect on antibody binding of alternative substitutions designed to remove T cell epitopes, several versions of the de-immunized heavy and light chain variable region were constructed, as shown in Figures 10 and 11.

The murine OKT3 heavy and light chain variable region cassettes were used as templates for construction of the designed de-Immunized sequences by mutagenesis using overlapping PCR with mutagenic oligonucleotides primers (see Figure 12 The vectors VH-PCR1 and VK-PCR1 (Riechmann et al., 1988) were used as templates to introduce 5' flanking sequences including the leader signal peptide sequence, the leader intron and the murine immunoglobulin promoter, and 3' flanking sequence including the splice site and intron sequences. Sets of mutagenic primer pairs were synthesized encompassing the regions to be altered, such that the target DNA sequence is amplified as a set of fragments.

Adjacent oligos were designed so that the sequences overlap by at least 15bp. The number of these depends on the number of sites to be mutated.

PCR amplifications for each primer pair were set up using the following reagents:

Samsung Bioepis Exhibit No. 1002
Page 2149

1µL template DNA
1 µL (25pmol) forward primer
1 µL (25pmol) reverse primer
1 µL 10mM dNTPs
5 µL 10 x Pfu polymerase buffer
0.5 µL (1unit) Pfu DNA polymerase
H_2O to 50µL

All reagents except the enzyme were mixed in a 0.5 ml thin wall PCR tube and heated to 94°C on the PCR block. The Pfu enzyme was added then samples cycled: 94°C/2min, 15-20 cycles of 94°C/30s, 50°C/30s, 75°C/1 min (depending on the length of extension required), finishing with 75° C 5 min. The annealing temperature may be lower or higher than 50°C depending on the Tm of the oligos.

5µL of each reaction were run on an agarose gel to check that the PCRs had given products of the expected size. If not, I the annealing temperature was lowered by 5°C and/or the number of cycles of PCR was increased and/or the $MgCl_2$ concentration was increased to 5mM. If a primary PCR gave multiple bands, the band of the correct size was gel-purified.

The products were joined in a second PCR using the 2nd round 5' and 3' primers only. This sequence was not present in the original template so only mutagenised DNA can be amplified at this stage. The templates for the 2nd joining PCR were the fragments produced in the first round. The quantities of these were adjusted to add approximately equal amounts. The reagents for the 2nd round PCR were:

Products of 1st round PCR
2 µL (50 pmol) 5' 2nd round primer
2 µL (50 pmol) 3' 2nd round primer
1 µL 10 mM dNTPs
5 µL 10xPfu polymerase buffer
0.5 µL (1 unit) Pfu DNA polymerase
H_2O to 50 µL

Samsung Bioepis Exhibit No. 1002
Page 2150

All reagents except the enzyme were mixed in a 0.5 ml thin wall PCR tube and heated to 94°C on the PCR block. The Pfu enzyme was added then samples cycled: 94°C/2min, 15 cycles of 94°C/ 30s, 50°C/30s, 75°C/ 1min (depending on the length of extension required), finishing with 75°C 5 min.

5 µL of each reaction was run on an agarose gel to check that the PCRs had given products of the expected size (approximately 820 bp for VH expression cassettes and 650 bp for VK expression cassettes). If not, the 2nd round PCR was repeated lowering the annealing temperature by 5°C and/or increasing the number of cycles of PCR.

The remainder of the PCR product was phenol/chloroform extracted and ethanol precipitated, digested with the required enzymes (usually *Hind*111 and *Bam*H1 for expression cassettes) and loaded onto a 1.5% low-melting point agarose gel. DNA bands of the correct size were excised and purified.

The de-immunized VH and Vκ expression cassettes produced (Figure 2) were cloned into the vector pUC19 and the entire DNA sequence was confirmed to be correct for each de-immunized VH and Vκ. As an example, the DNA and amino acid sequences for the de-immunized OKT3 VH and Vk 1 expression cassettes OKT3 DIV H V1 (version 1) and OKT3 DIVK V1 (version 1) are shown in Figures 13 and 14, respectively.

The de-immunized heavy and light chain V-region genes were excised from the vector pUC19 as *Hind*III to *Bam*HI expression cassettes. These were transferred to the expression vectors pSVgpt HuG2G4 and pSVhyg HuCκ (Figures 7 and 9, respectively), which include the previously described HuG2G4 or human κ constant regions,

Samsung Bioepis Exhibit No. 1002
Page 2151

respectively, and markers for selection in mammalian cells. The DNA sequence was confirmed to be correct for the de-immunized VH and Vκ in the expression vectors.

The original murine OKT3 heavy and light chain light chain variable region cassettes were also transferred to the expression vectors pSVgpt HuG2G4 and pSVhyg HuCκ as described above, to generate a chimeric antibody with the murine variable region genes linked to the human constant region G2/G4 construct. This chimeric antibody was used as an isotype matched control for binding experiments with the de-immunized antibodies, as it has with the same effector functions and the same secondary detection reagents are used as for the de-immunized antibodies.

The host cell line for antibody expression was NSO, a non-immunoglobulin producing mouse myeloma, obtained from the European Collection of Animal Cell Cultures, Porton UK (ECACC No 85110503). The heavy and light chain expression vectors were co-transfected into NSO cells by electroporation. The transfection was accomplished as follows: DNA to be transfected was linearized to improve efficiency. PvuI digests of about 3 and 6 mg of the plasmids pSVgpt HuG2/G4 and pSV hyg HUCK, respectively, were prepared. The digested DNA was ethanol precipitated and dissolved in 50 ml dH_2O. Recipient NSO cells were resuspended from a semi-confluent 75 cm^2 flask and collected by centrifugation at 1000 rpm for 5 min. The supernatant was discarded. The cells were resuspended in 0.5 ml DMEM and transferred to a Gene Pulser cuvette (Bio-Rad). The DNA was mixed with the cells by gentle pipetting and left on ice for 5 minutes. The cuvette was inserted between the electrodes of the Bio-rad Gene Pulser and a single pulse of 170 V, 960 mF was applied. The cuvette was then returned to ice for 20 minutes. The cell suspension was transferred to a 75 cm^2 flask

Samsung Bioepis Exhibit No. 1002
Page 2152

containing 20 ml DMEM and allowed to recover for 1-2 days. Cells were harvested and resuspended in 80 ml selective DMEM and a 200 μL aliquot was added to each well of 96-well plates. Selective DMEM is Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) foetal calf serum, 250μg/ml xanthine, 0.8μg/ml mycophenolic acid. Approximately 10 days from the start of selection, colonies were visible to the naked eye. 20 μL of medium from each well was assayed for the presence of human antibodies. On the basis of the level of antibody production and the number of cells in the well, wells were chosen for expansion. To resuspend the cells from the designated wells, the tip of a Gilson P200 pipette (with yellow tip) was rubbed across the surface and the medium transferred to a well of a 24-well tissue culture plate containing 1.5 ml of fresh selective DMEM. Cells were expanded to 25 cm^2 and larger tissue culture flasks in order to lay down liquid nitrogen stocks and to provide medium for antibody purification and testing.

Each of the 7 de-immunized heavy chain genes (Fig. 10) was paired with each of 2 de-immunized light chain genes (Fig. 11) to give a total of 14 de-immunized OKT3 antibodies to be produced. The chimeric heavy and light chain vectors were co-transfected to produce the chimeric antibody. Colonies expressing the gpt gene were selected using selective DMEM. Transfected cell clones were screened for production of human antibody by ELISA for human IgG as follows: An ELISA plate (Dynatech Immulon 2) was coated at 100 μL per well with sheep anti-human κ antibody (The Binding Site Cat No: AU015) diluted 1:1000 in carbonate/bicarbonate coating buffer pH9.6 (Sigma Cat: C-3041). The samples were incubated at 4°C overnight. After

Samsung Bioepis Exhibit No. 1002
Page 2153

washing 3 times with a solution of PBS with 0.05% Tween 20 (RTM), Where transfections were plated into 96-well plates, screening was conducted using 25μL samples of culture medium from each well by transfer into an assay plate containing 75μL per well of PBS/Tween (PBST) solution. Where transfected cells were cultured using 24-well plates, 12.5 μL was added to 87.5 μL in the first well and a doubling dilution series set out across the plate. For all assays, blank wells received PBST only. The samples were incubated at room temperature for 1 hour and washed 3 times with the PBS/Tween solution.. The secondary antibody, a peroxidase-conjugated sheep anti-human IgG γ chain specific reagent (The Binding Site Cat No: AP004), was added at a ratio of 1:1000 in the PBS/Tween solution in an amount of 100 μL per well. The samples were again incubated at room temperature for 1 hour and washed 3 times with the PBS/Tween solution. To prepare the colour substrate , one tablet (20mg) of OPD (o-PHENYLENE DIAMINE) (Sigma Cat No: P-7288) was dissolved in 45ml of H_2O plus 5 ml 10x peroxidase buffer (make 10 x peroxidase buffer with Sigma phosphate citrate buffer tablets pH 5.0, Cat No: P-4809), and 10mL 30% (w/w) hydrogen peroxide (Sigma Cat No: H-1109) was added just before use. The substrate was added at 100 μL per well and incubated at room temperature for 5 min. or as required. When colour developed, the process was stopped by adding 25 μL 12.5% H_2SO_4. The result was read at 492 nm. The standard antibody employed was Human IgG1/κ purified myeloma protein (The Binding Site Cat No: BP078).

Cell lines secreting antibody were expanded and the highest produces selected. De-immunized and chimeric antibodies were purified using Prosep ®-A (Bioprocessing

Samsung Bioepis Exhibit No. 1002
Page 2154

Ltd, Durham, UK) as follows: The NSO transfectoma cell line producing antibody was grown in DMEM 5% FCS in Nunc Triple layer flasks, 250 ml per flask (total volume 1L) for 10-14 days until nearing saturation. The conditioned medium was collected and spun at 3000 rpm for 5 minutes in a bench centrifuge to remove cells. One tenth the volume of 1M Tris-HCL pH8 (Sigma Cat: T3038) was added to the cell supernatant to make this 0.1M Tris-HCL pH8. 0.5 to ml of Prosep A (Millipore Cat: 113111824) was added and stirred overnight at room temperature. Prosep A was collected by spinning at 3000 rpm for 5 minutes then packed into a Biorad Poly-Prep column (Cat: 731-1550). The column was washed with 10ml PBS, then eluted in 1ml fractions with 0.1 M Glycine pH3.0. Each fraction was collected into a tube containing 100μL 1M Tris-HCL pH8 (Sigma, as above). The absorbance of each fraction measured at 280 nm. The fractions containing antibody were pooled and dialysed against PBS overnight at room temperature. The preparation was sterilized by filtration through a 0.2 micron syringe filter and the A_{280} was measured. The concentration was determined by ELISA for human IgG1κ.

Evaluation of Binding of De-immunized Antibodies to Cells Expressing CD3

Each de-immunized antibody was evaluated for its ability to bind the CD3 molecule on T cells as it was possible that the mutations introduced during de-immunization could affect antibody specificity or affinity. Cells of the HPB-ALL (Human peripheral blood acute lymphocytic leukemia) line were obtained from the Cell Resource Center for Biomedical Research, Tohoku University, Japan. Jurkat and J.RT3 cell lines were obtained from American Type Tissue Culture (ATCC), Rockville, MD. Cells were

Samsung Bioepis Exhibit No. 1002
Page 2155

cultured at 2 x 10^5 – 2 x 10^6 cells/ml in RPMI 1640 (Cellgro) containing 10% heat-activated fetal bovine serum (Atlas Biologicals), 1% penicillin-streptamycin, 0.01M Hepes (Sigma), 0.2mM 1-glutamine and 5 x 10^{-5}M 2-mercaptoethanol (Sigma) at 37°C in a humidified chamber containing 5% CO_2 in air. HPB-ALL and Jurkat cells bear high levels of the TCR/CD3 complex on their cell surface, while the J.RT3 cells are a variant of the Jurkat line that do not express CD3. Preparations of the murine OKT3, chimeric OKT3 and de-immunized OKT3 G2/G4 antibodies were evaluated for binding to cells of all three lines by immunocytochemistry and flow cytometry. Briefly, 10^6 cells were plated in individual wells of a 96-well plate and reacted with 1μg of each test antibody or with appropriate human or mouse isotype controls for 20 minutes at 4°C. The cells were then washed three times with PBS containing 2% fetal bovine serum (Atlas Biologicals) followed by reactivity for 20 minutes at 4°C with a phycoerthrin-conjugated secondary antibody (R-PE Affinity pure F(ab)2 goat anti-human IgG (H+L) (Jackson ImmunoResearch, Bar Harbor, Maine) for the detection of the chimeric and de-immunized antibodies and G2/G4 isotype control, and R-PE-conjugated goat anti-mouse IgG (Pharmingen), for the detection of the murine OKT3 and murine IgG2a isotype control). The cells were washed three times with PBS-FBS as above, and then resuspended in PBS for analysis on a flow cytometer (FACs Calibur, Becton Dickenson).

Both the chimeric OKT3 HuG2/G4κ antibody and the murine OKT3 antibody bound comparably to Jurkat and HPB-All cells that express CD3, but showed no binding to the CD3 negative cell line J.RT3. The matched murine and human isotype controls showed no binding to any of the cell types (Figure 15)

Samsung Bioepis Exhibit No. 1002
Page 2156

Conditioned media from NSO cell lines expressing de-immunised OKT3 antibodies were also tested in the flow cytometry binding assay using HPB-ALL and J.RT3 cells. The concentration of antibody in the conditioned medium was determined by ELISA for human IgG using the ELISA procedure previously described. The immunocytochemistry and flow cytometry procedures were performed as described above. The results for the 7 versions of de-immunised heavy chain combined with de-immunised OKT3 light chain version 1 are shown in Figure 16. The results for the de-immunised heavy chains combined with de-immunised OKT3 light chain version 2 are shown in Figure 17. A number of the de-immunised OKT3 antibodies demonstrated binding to HPB-ALL cells equivalent to that observed for murine and chimeric antibodies. However, several of the de-immunized antibodies showed a significantly lower level of binding to HPB-ALL cells (e.g. antibodies derived from clones 24C12, 48G3, and 55B2). The ability of a given antibody to bind to HPB-ALL cells did not correlate with the usage of a particular version of kappa light chain; i.e., both mutated kappa chains, in combination with some, but not all of the mutated VH regions, showed comparable binding in this assay.

Competition Assay Comparing Binding Of Murine, Chimeric And De-Immunized OKT3 Antibodies

To obtain more discrimination between the various de-immunized antibodies, and to determine their binding affinity relative to that of OKT3, a competition binding assay was carried out. Because CD3 is part of a cell-surface complex of proteins, affiniities could not be measured by BIACORE analysis, but instead were measured by flow cytometry. Murine OKT3 was biotinylated using EZ-Link Sulfo-NHS-LC Biotin from

Samsung Bioepis Exhibit No. 1002
Page 2157

Perbio Science, catalogue number 21335, following the protocol provided by the manufacturer. The amount of biotinylated OKT3 to use was determined by titrating HPB-ALL cells with decreasing amounts of antibody. A suitable sub-saturating concentration was determined to be 10 ng of biotinylated antibody per 10^6 cells. This concentration was then used in all experiments. The secondary detection reagent was avidin-FITC, Sigma catalogue number A2050. Competition with dilutions of test (de-immunized or chimeric) antibodies from 100 pg to 1 µg was tested. The results are expressed as percent inhibition of maximal fluorescence activity (determined by binding of biotinylated murine OKT3 in the absence of blocking antibody) and are shown in Figures 18, 19, 20 and 21.

The results show that the chimeric OKT3 antibody and the six de-immunized antibodies OKT3 DIVHv5 to DIVGv7/DIVKv1 and OKT3 DIVH5 to DIVH7/DIVK2 can compete with the binding of the biotinylated murine OKT3 antibody either as efficiently as the murine antibody itself or within two to three fold of it.

Throughout this specification, various publications and patent disclosures are referred to. The teachings and disclosures thereof, in their entireties, are hereby incorporated by reference into this application to more fully describe the state of the art to which the present invention pertains.

Although preferred and other embodiments of the invention have been described herein, further embodiments may be perceived by those skilled in the art without departing from the scope of the invention as defined by the following claims.

Samsung Bioepis Exhibit No. 1002
Page 2158

We claim:

1. A de-immunized anti-CD3 antibody.

2. A de-immunized anti-CD3 antibody heavy chain variable region comprising a sequence selected from the group consisting of SEQ. ID NOS: 11, 12, 13, 14, 15, 16 and 17.

3. A de-immunized anti-CD3 antibody light chain variable region comprising a sequence selected from the group consisting of SEQ. ID NOS: 19 and 20.

4. A method comprising:

selecting an anti-CD3 antibody; and

rendering the anti-CD3 antibody less immunogenic to a given species.

5. A method as in claim 4 wherein the step of rendering the anti-CD3 antibody less immunogenic to a given species comprises the steps of:

a) determining at least part of the amino acid sequence of the antibody;

(b) identifying in the amino acid sequence one or more potential epitopes for T cells ("T cell epitopes") which are found in an endogenous protein of the given species; and

(c) modifying the amino acid sequence to eliminate at least one of the T cell epitopes identified in step (b) thereby to reduce the immunogenicity of the antibody or part thereof when exposed to the immune system of the given species.

6. A method comprising the steps of:

(a) producing an expression vector having a DNA sequence which includes a sequence that encodes an anti-CD3 antibody, at least a portion of which has been a de-immunized;

(b) transfecting a host cell with the vector; and

(c) culturing the transfected cell line to produce a de-immunized anti-CD3 antibody molecule.

7. A pharmaceutical composition comprising a de-immunized anti-CD3 antibody and a pharmaceutically acceptable carrier.

8. A composition as in claim 7 wherein the de-immunized anti-CD3 antibody includes a heavy chain variable region comprising a sequence selected from the group consisting of SEQ. ID NOS: 11, 12, 13, 14, 15, 16 and 17.

9. A composition as in claim 7 wherein the de-immunized anti-CD3 antibody includes a light chain variable region comprising a sequence selected from the group consisting of SEQ. ID NOS: 19 and 20.

10. A method comprising administering an anti-CD3 antibody, the anti-CD3 antibody including an engineered heavy chain constant region having a first portion derived from one or more human IgG2 antibodies and a second portion derived from

Samsung Bioepis Exhibit No. 1002
Page 2160

one or more human IgG4 antibodies, at least a portion of the antibody being de-immunized.

11. A method as in claim 10 wherein at least the light chain variable region of the antibody is de-immunized.

12. A method as in claim 10 wherein at least the heavy chain variable region of the antibody is de-immunized.

13. A method as in claim 10 wherein both the light and heavy chain variable regions of the antibody are de-immunized.

14. A method as in claim 10 wherein the anti-CD3 antibody includes a heavy chain variable region comprising a sequence selected from the group consisting of SEQ. ID NOS: 11, 12, 13, 14, 15, 16 and 17.

15. A method as in claim 10 wherein the anti-CD3 antibody includes a light chain variable region comprising a sequence selected from the group consisting of SEQ. ID NOS: 19 and 20.

16. A method as in claim 10 wherein at least a portion of the antibody is de-immunized by a process comprising the steps of:

rendering the antibody, or part thereof, non-immunogenic, or less immunogenic,

Samsung Bioepis Exhibit No. 1002
Page 2161

to a given species by

a) determining at least part of the amino acid sequence of the antibody;

(b) identifying in the amino acid sequence one or more potential epitopes for T cells ("T cell epitopes") which are found in an endogenous protein of the given species; and

(c) modifying the amino acid sequence to eliminate at least one of the T cell epitopes identified in step (b) thereby to reduce the immunogenicity of the antibody or part thereof when exposed to the immune system of the given species.

17. Nucleic acid encoding a de-immunized anti-CD3 antibody.

18. Nucleic acid in accordance with claim 17 which encodes an antibody heavy chain variable region comprising a sequence selected from the group consisting of SEQ. ID NOS: 11, 12, 13, 14, 15, 16 and 17.

19. Nucleic acid in accordance with claim 17 which encodes an antibody light chain variable region comprising a sequence selected from the group consisting of SEQ. ID NOS: 19 and 20.

20. A pharmaceutical composition comprising an anti-CD3 antibody encoded by the nucleic acid in accordance with any of claims 17 through 19 and a pharmaceutically acceptable carrier.

Samsung Bioepis Exhibit No. 1002
Page 2162

Amino Acid and nucleotide sequence of the murine OKT3 heavy chain variable region (Accession #A22621)

MERHWIFLLLLSVTAGVHSQVQLQQSGAELARPGASVKMSCKASYTFTRYTMHWVKQRPGQGLEWIGYINPSRGYTN
YNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSSAKTTAPSVYPLAPVCGD
TTGSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPASSTKVD
KKIEPRGPTIKPCPPCKCPAPNLLGGPSVFIFPPKIKDVLMISLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQ
TQTHREDYNSTLRVVSALPIQHQDWMSGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLPPPEEEMTKKQVTL
TCMVTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFS
RTPGK

ORIGIN

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      1 gaattcccct ctccacagac actgaaaact ctgactcaac atggaaaggc ctggatctt
     61 tctactcctg ttgtcagtaa ctgcaggtgt ccactcccag gtccagctgc agcagtctgg
    121 ggctgaactg gcaagacctg gggcctcagt gaagatgtcc tgcaaggctt ctggctacac
    181 ctttactagg tacacgatgc actgggtaaa acagaggcct ggacagggtc tggaatggat
    241 tggatacatt aatcctagcc gtggttatac taattacaat cagaagttca aggacaaggc
    301 cacattgact acagacaaat cctccagcac agcctacatg caactgagca gcctgacatc
    361 tgaggactct gcagtctatt actgtgcaag atattatgat gatcattact gccttgacta
    421 ctggggccaa ggcaccactc tcacagtctc ctcagccaaa acaacagccc catcggtcta
    481 tccactggcc cctgtgtgtg gagatacaac tggctcctcg gtgactctag gatgcctggt
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FIG. 1A

Samsung Bioepis Exhibit No. 1002
Page 2163

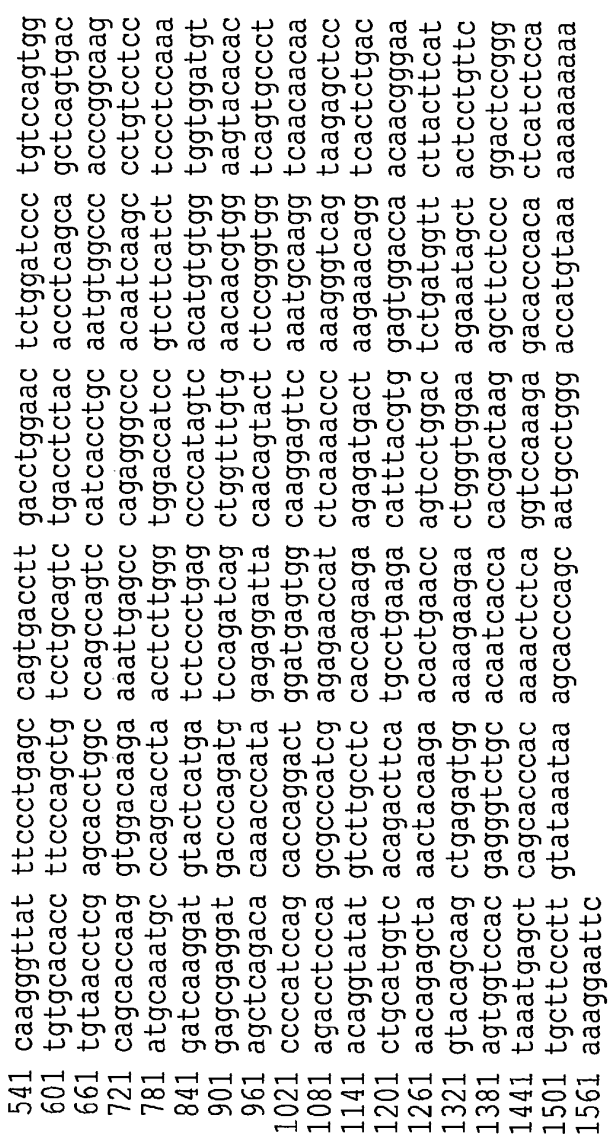

541 caagggttat ttccctgagc cagtgacctt gacctggaac tctggatccc tgtccagtgg
601 tgtgcacacc ttcccagctg tcctgcagtc tgacctctac accctcagca gctcagtgac
661 tgtaacctcg agcacctggc ccagccagtc catcacctgc aatgtggccc acccggcaag
721 cagcaccaag gtggacaaga aaattgagcc cagagggccc acaatcaagc cctgtcctcc
781 atgcaaatgc ccagcaccta acctcttggg tggaccatcc gtcttcatct tccctccaaa
841 gatcaaggat gtactcatga tctccctgag ccccatagtc acatgtgtgg tggtggatgt
901 gagcgaggat gacccagatg tccagatcag ctggtttgtg aacaacgtgg aagtacacac
961 agctcagaca caaacccata gagaggatta caacagtact ctccgggtgg tcagtgccct
1021 ccccatccag caccaggact ggatgagtgg caaggagttc aaatgcaagg tcaacaacaa
1081 agacctccca gcgcccatcg agagaaccat ctcaaaaccc aaagggtcag taagagctcc
1141 acaggtatat gtcttgcctc caccagaaga agagatgact aagaaacagg tcactctgac
1201 ctgcatggtc acagacttca tgcctgaaga catttacgtg gagtggacca acaacgggaa
1261 aacagagcta aactacaaga acactgaacc agtcctggac tctgatggtt cttacttcat
1321 gtacagcaag ctgagagtgg aaaagaagaa ctgggtggaa agaaatagct actcctgttc
1381 agtggtccac gagggtctgc acaatcacca cacgactaag agcttctccc ggactccggg
1441 taaatgagct cagcacccac aaaactctca ggtccaaaga gacacccaca ctcatctcca
1501 tgcttccctt gtataaataa agcacccagc aatgcctggg accatgtaaa aaaaaaaaaa
1561 aaaggaattc

FIG. 1A (Cont.)

Amino Acid and nucleotide sequence of the murine OKT3 light chain variable region (Accession #A22259)

MDFQVQIFSFLLISASVIISRGQIVLTQSPAIMSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYD
TSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEINRADTAPTVSIFPPS
SEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLTKDEYERHNSY
TCEATHKTSTSPIVKSFNRNEC

ORIGIN

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      1 gaattcccaa agacaaaatg gattttcaag tgcagatttt cagcttcctg
        ctaatcagtg
     61 cctcagtcat aatatccaga ggacaaattg ttctcaccca gtctccagca
        atcatgtctg
    121 catctccagg ggagaaggtc accatgacct gcagtgccag ctcaagtgta
        agttacatga
    181 actggtacca gcagaagtca ggcacctccc ccaaaagatg gatttatgac
        acatccaaac
    241 tggcttctgg agtccctgct cacttcaggg gcagtgggtc tgggacctct
        tactctctca
    301 caatcagcgg catggaggct gaagatgctg ccacttatta ctgccagcag
        tggagtagta
    361 acccattcac gttcggctcg gggacaaagt tggaaataaa ccgggctgat
        actgcaccaa
    421 ctgtatccat cttcccacca tccagtgagc agttaacatc tggaggtgcc
        tcagtcgtgt
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FIG. 1B

Samsung Bioepis Exhibit No. 1002
Page 2165

481 gcttcttgaa caacttctac cccaaagaca tcaatgtcaa gtggaagatt
gatggcagtg
541 aacgacaaaa tggcgtcctg aacagttgga ctgatcagga cagcaaagac
agcacctaca
601 gcatgagcag caccctcacg ttgaccaagg acgagtatga acgacataac
agctatacct
661 gtgaggccac tcacaagaca tcaacttcac ccattgtcaa gagcttcaac
aggaatgagt
721 gttagagaca aaggtcctga gacgccacca ccagctccca gctccatcct
atcttccctt
781 ctaaggtctt ggaggcttcc ccacaagcgc ttaccactgt tgcggtgctc
taaacctcct
841 cccacctcct tctcctcctc ctcccttccc ttggctttta tcatgctaat
atttgcagaa
901 aatattcaat aaagtgagtc tttgccttga aaaaaaaaaa aaa

FIG. 1B (Cont.)

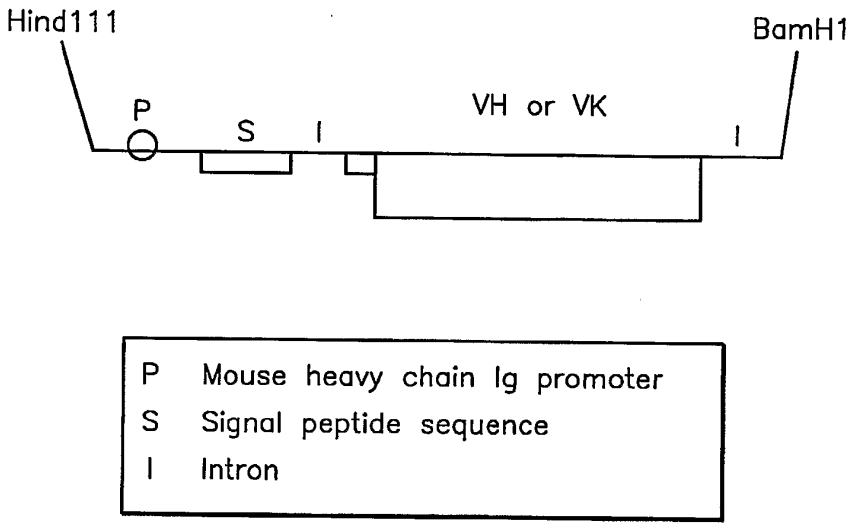

Hind111
BamH1
P
S
I
VH or VK
I
P Mouse heavy chain Ig promoter
S Signal peptide sequence
I Intron

FIG. 2

FIG. 3

Samsung Bioepis Exhibit No. 1002
Page 2168

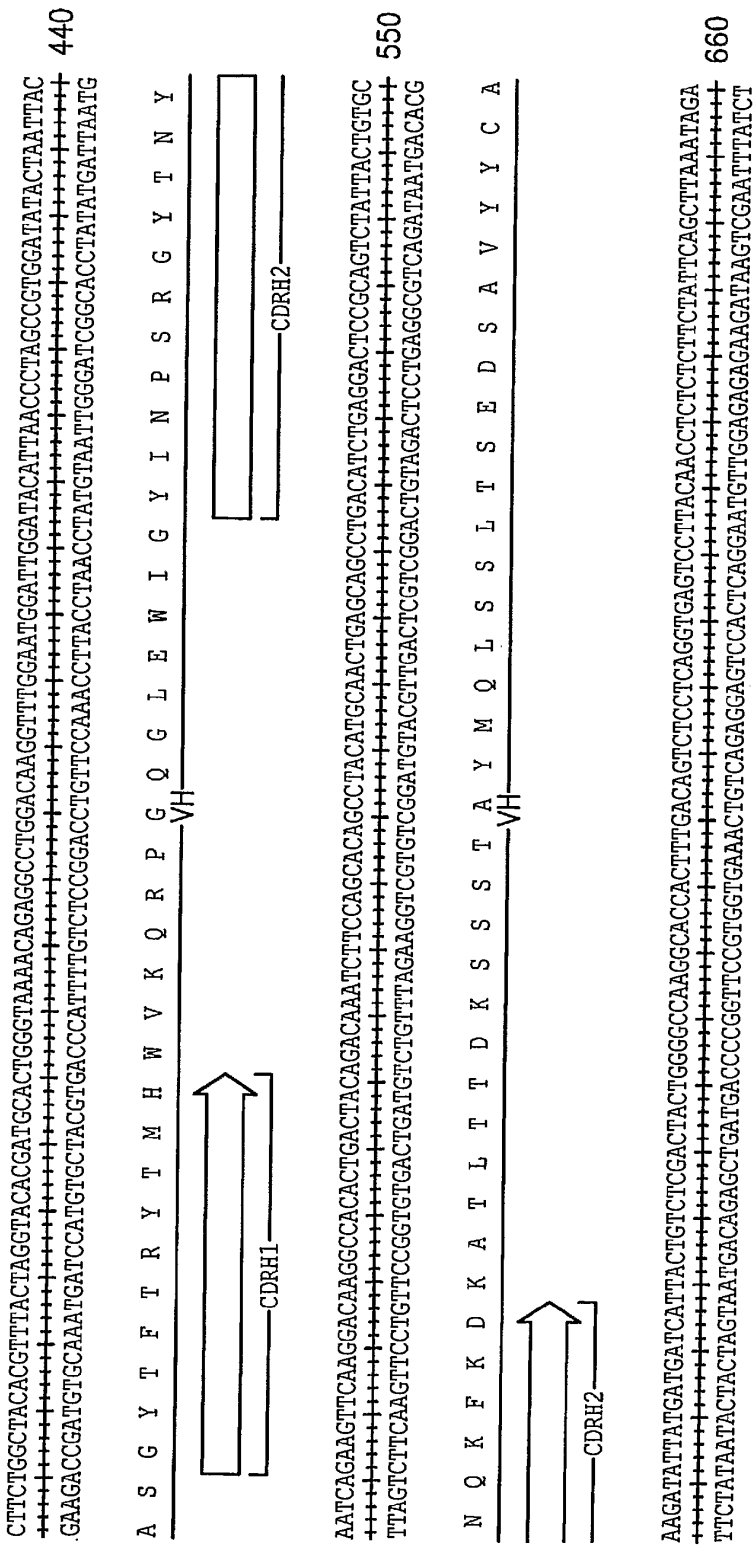


FIG. 3 (Cont.)

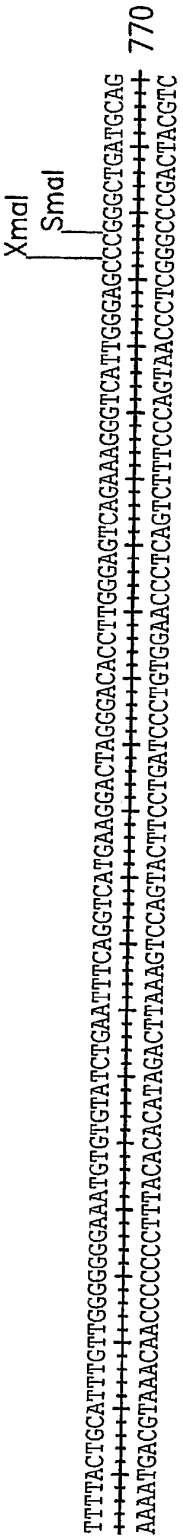


FIG. 3 (Cont.)

Samsung Bioepis Exhibit No. 1002
Page 2170

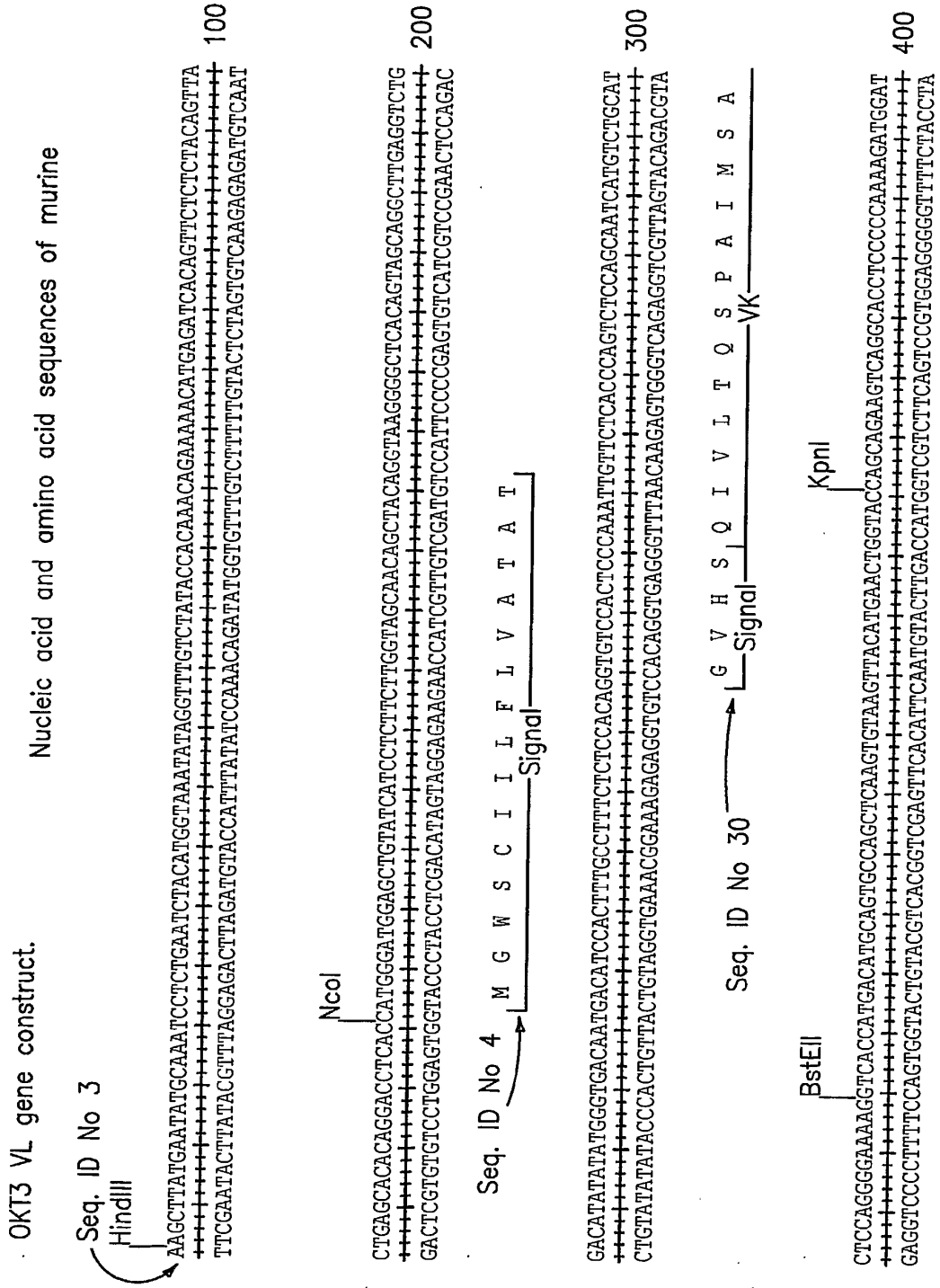

OKT3 VL gene construct.
Nucleic acid and amino acid sequences of murine
Seq. ID No 3
HindIII
AAGCTTATGAATATGCAAATCCTCTGAATCTACATGGTAAATATAGGTTTGTCTATACCACAAACAGAAAAACATGAGATCACAGTTCTCTCTACAGTTA
TTCGAATACTTATACGTTTAGGAGACTTAGATGTACCATTTATATCCAAACAGATATGGTGTTTGTCTTTTTGTACTCTAGTGTCAAGAGAGATGTCAAT
100
NcoI
CTGAGCACACAGGACCTCACCATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTAAGGGGCTCACAGTAGCAGGCTTGAGGTCTG
GACTCGTGTGTCCTGGAGTGGTACCCTACCTCGACATAGTAGGAGAAGAACCATCGTTGTCGATGTCCATTCCCCGAGTGTCATCGTCCGAACTCCAGAC
200
Seq. ID No 4
M G W S C I I L F L V A T A T
Signal
GACATATATATGGGTGACAATGACATCCACTTTGCCTTTCTCTCCACAGGTGTCCACTCCCAAATTGTTCTCACCCAGTCTCCAGCAATCATGTCTGCAT
CTGTATATATACCCACTGTTACTGTAGGTGTTCCACAGGTGAGGGTTTAACAAGAGTGGGTCAGAGGTCGTTAGTACAGACGTA
300
Seq. ID No 30
G V H S Q I V L T Q S P A I M S A
Signal
VK
BstEII
KpnI
CTCCAGGGGAAAAGGTCACCATGACATGCAGTGCCAGCTCAAGTGTAAGTTACATGAACTGGTACCAGCAGAAGTCAGGCACCTCCCCCAAAAGATGGAT
GAGGTCCCCTTTTCCAGTGGTACTGTACGTCACGGTCGAGTTCACATTCAATGTACTTGACCATGGTCGTCTTCAGTCCGTGGAGGGGGTTTTCTACCTA
400

FIG. 4

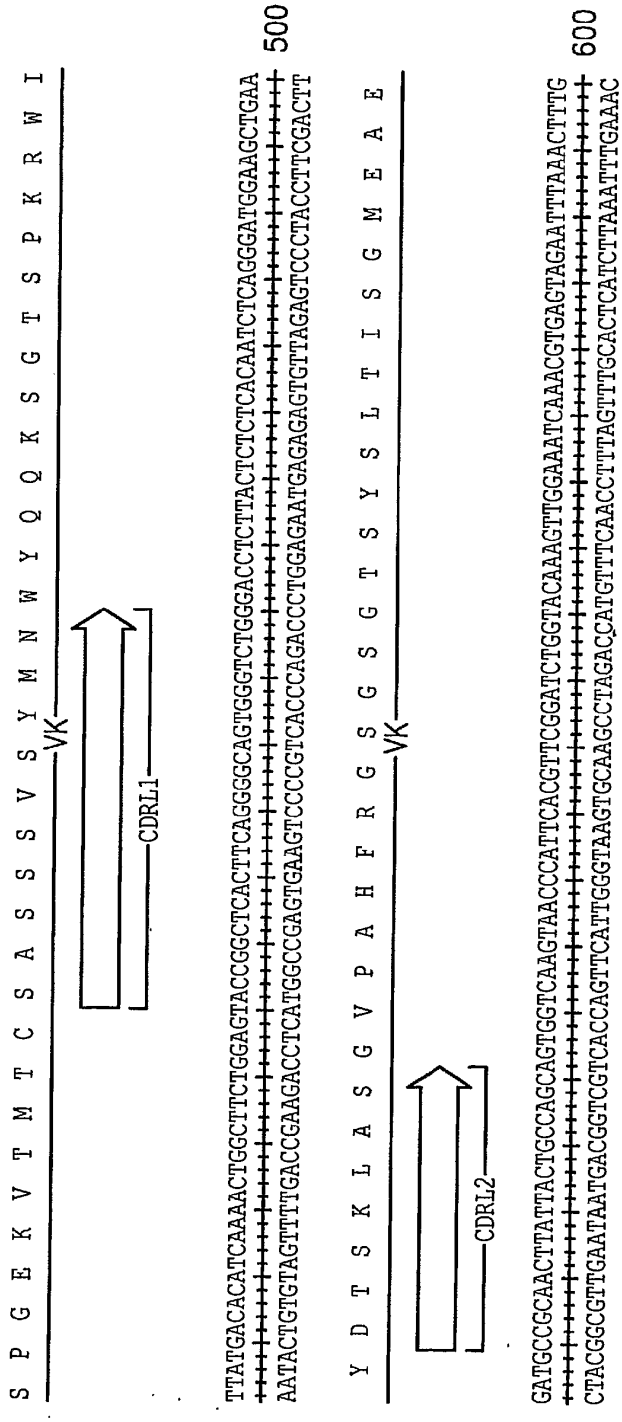


FIG. 4 (Cont.)

Samsung Bioepis Exhibit No. 1002
Page 2172

Schematic map of the vector Apex−1 3F4V_HHuGamma4.

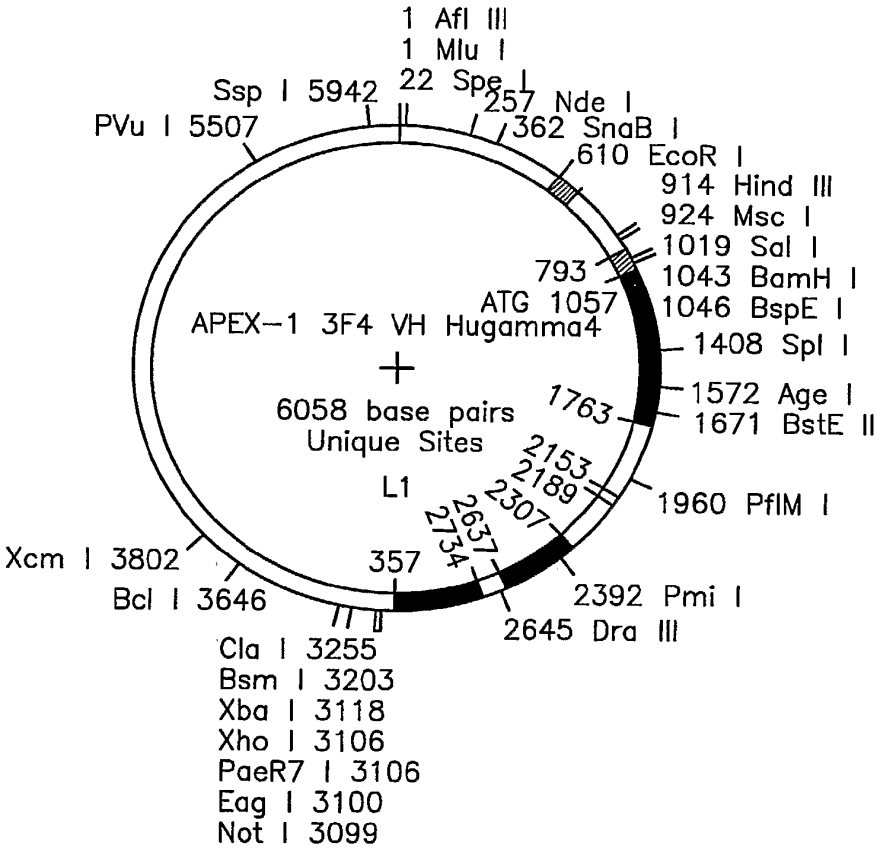


FIG. 5A

Samsung Bioepis Exhibit No. 1002
Page 2173

SUBSTITUTE SHEET (RULE 26)

Vector sequence (APEX−1 3F4V$_H$HuGamma4)

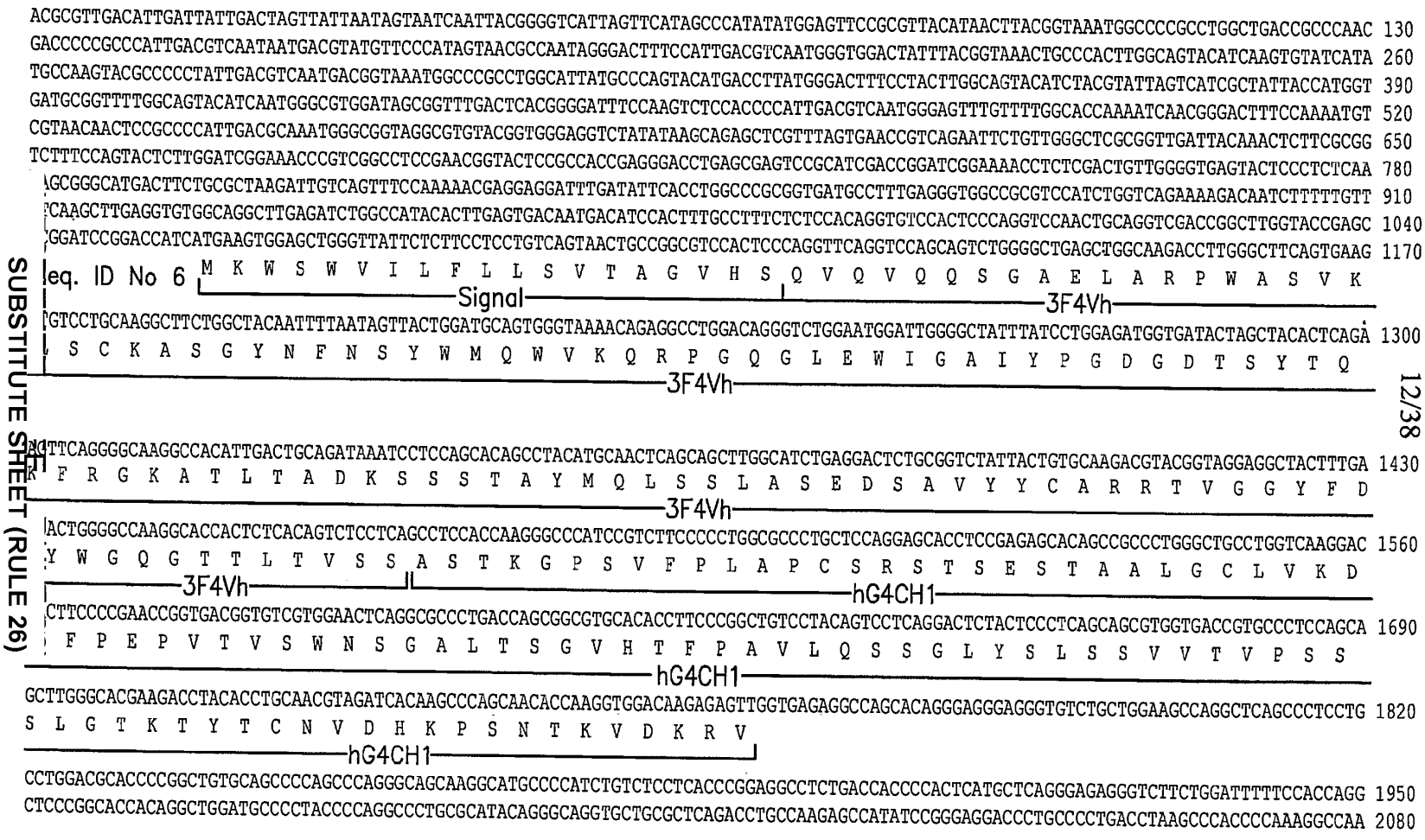


FIG. 5B

Samsung Bioepis Exhibit No. 1002
Page 2174

FIG. 5B (Cont.)

Samsung Bioepis Exhibit No. 1002
Page 2175

GCCCCGCTGTTCGACTTACAAACACAGGCACAGTACTGACAAACCCATACACCTCCTCTGAAATACCCATAGTTGCTAGGGCTGTCTCCCGAACTCATTACACCCTCCAAAGTCAGAGCTGTAATTTCGCC 3510
GTCAAGGGCAGCGAGGGCTTCTCCAGATAAAATAGCTTCTGCCGAGAGTCCCGTAAGGGTAGACACTTCAGCTAATCCCTCGATGAGGTCTACTAGAATAGTCAGTGGCGGCTCCCATTTTGAAAATTCAC 3640
GTTACTTGATCACGCTTCAGAAGATGGCGGAGGGCCTCCAACACAGTAATTTTCCTCCCGACTCTTAAAATAGAAAATGTCAAGTCAGTTAAGCAGGAAGTGGACTAACTGACGCAGCTGGCCGTGCGACAT 3770
TTCTTTTTAATTAGTTGCTAGGCAACGCCCTCCAGAGGGCGTGTGGTTTTGCAAGAGGAAGCAAAAGCCTCTCCACCCAATCATTACTATGACAACACGCTGTTTTTTTTT 3900
TATTAAGCAGAGGCCGGGGACCCCTGGCCCGCTTACTCTGGAGAAAAAGAAGAGAGGCATTGTAGAGGCTTCCAGAGGCAACTTGTCTATTTCTGTCACACTGTCTGGCCC 4030
CTCACAAGGTCCAGCACCTCCATACCCCCTTTAATAAGCAGTTTGGGAACGGGTGCGGGTCTTACTCCGCCCATCCGCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATT 4160
TTTTTATTTATGCAGAGGCCGAGGCCGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGGAGCTCCCAGCAAAAGGCCAGGAACCGTAAAAAG 4290
GCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCC 4420
GTGGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAA 4550
TTGGCCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGC 4680
AGCCAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCT 4810
CTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAA 4940
CGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTAC 5070
TAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGA 5200
ACCGCGGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCGCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGC 5330
GAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACA 5460
TGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCAT 5590
GTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAA 5720
AGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTT 5850
TCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGA 5980
GCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTG 6058

FIG. 5B (Cont.)

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Samsung Bioepis Exhibit No. 1002
Page 2176

Schematic map of the vector Apex−1 3F4V_HHuG2/G4.

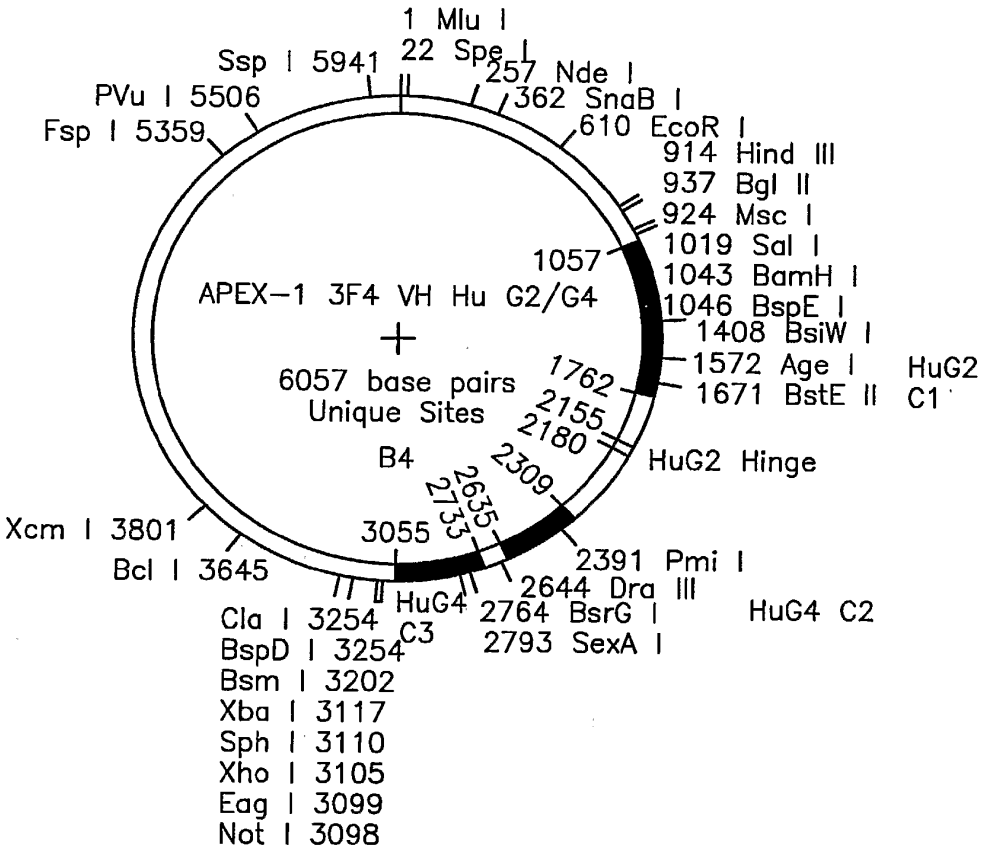


FIG. 6A

Samsung Bioepis Exhibit No. 1002
Page 2177

Vector Sequence (APEX-13F4V$_H$HuG2/G4)

ACGCGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTG 120
ACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGACTATTTACGGTAAACTGCCCACTTGGC 240
AGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACAT 360
CTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAG 480
TTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGT 600
AACCGTCAGAATTCTGTTGGCTCGCGGTTGATTACAAACTCTTCGCGGTCTTTCCAGTACTCTTGGATCGGAAACCCGTCGGCCTCCGCCACCGAGGGACCTGAGC 720
AGTCCGCATCGACCGGATCGGAAAACCTCTCGACTGTTGGGTGAGTACTCCCTCTCAAAAGCGGGCATGACTTCTCGCGCTAAGATTGTCAGTTTCCAAAAACGAGGAGGATTTGATAT 840
ACCTGGCCCGCGGTGATGCCTTTGAGGGTGGCCGCGTCCATCTGGTCAGAAAAGACAATCTTTTTGTTGTCAAGCTTGAGGTGTGGCAGGCTTGAGATCTGGCCATACACTTGAGTGA 960
AATGACATCCACTTTGCCTTTCTCTCCACAGGTGTCCACTCCCAGGTCAACTGCAGGTCGACCGGCTTGGTACCGAGCTCGGATCCGGACCATCATGAAGTGGAGCTGGGTTATTCTC 1080

(Seq. ID No: 8)

M K W S W V I L

Signal

TCCTCCTGTCAGTAACTGCCGGCGTCCACTCCCAGGTTCAGGTCCAGCAGTCTGGGGCTGAGCTGGCAAGACCTTGGGCTTCAGTGAAGTTGTCCTGCAAGGCTTCTGGCTACAATTTT 1200

F L L S V T A G V H S Q V Q V Q Q S G A E L A R P W A S V K L S C K A S G Y N F

Signal 3F4Vh

ATAGTTACTGGATGCAGTGGGTAAAACAGAGGCCTGGACAGGGTCTGGAATGGATTGGGGCTATTTATCCTGGAGATGGTGATACTAGCTACACTCAGAAGTTCAGGGGCAAGGCCACA 1320

N S Y W M Q W V K Q R P G Q G L E W I G A I Y P G D G D T S Y T Q K F R G K A T

3F4Vh

TGACTGCAGATAAATCCTCCAGCACAGCCTACATGCAACTCAGCAGCTTGGCATCTGAGGACTCTGCGGTCTATTACTGTGCAAGACGTACGGTAGGAGGCTACTTTGACTACTGGGGC 1440

L T A D K S S S T A Y M Q L S S L A S E D S A V Y Y C A R R T V G G Y F D Y W G

3F4Vh

CAAGGCACCACTCTCACAGTCTCCTCAGCCTCCACCAAGGGCCCATCCGTCTTCCCCCTGGCGCCCTGCTCCAGGAGCACCTCCGAGAGCACACAGCCGCCCTGGGCTGCCTGGTCAAGGAC 1560

Q G T T L T V S S A S T K G P S V F P L A P C S R S T S E S T A A L G C L V K D

3F4Vh G2G4CH1

FIG. 6B

Samsung Bioepis Exhibit No. 1002
Page 2178

FIG. 6B (Cont.)

Samsung Bioepis Exhibit No. 1002
Page 2179

```
ACCCACGGGGTGCGAGGGCCACACGGACAGAGGCCAGCTCGGCCCACCCTCTGCCCTGGGAGTGACCGCTGTGCCAACCTCTGTCCCTACAGGGCAGCCCCGAGAGCCACAGGTGTACAC 2760
                                                                       (Seq. ID No: 36) ---> G  Q  P  R  E  P  Q  V  Y  T
                                                                                             |___ G2G4CH3 ___
CCTGCCCCCATCCCAGGAGGAGATGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTACCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAA 2880
 L  P  P  S  Q  E  E  M  T  K  N  Q  V  S  L  T  C  L  V  K  G  F  Y  P  S  D  I  A  V  E  W  E  S  N  G  Q  P  E  N  N
____________________________________ G2G4CH3 ____________________________________
CTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAGGCTAACCGTGGACAAGAGCAGGTGGCAGGAGGGGAATGTCTTCTCATGCTCCGTGATGCATGA 3000
 Y  K  T  T  P  P  V  L  D  S  D  G  S  F  F  L  Y  S  R  L  T  V  D  K  S  R  W  Q  E  G  N  V  F  S  C  S  V  M  H  E
____________________________________ G2G4CH3 ____________________________________
GGCTCTGCACAACCACTACACACAGAAGAGCCTCTCCCTGTCTCTGGGTAAATGAGTGCCAGGGCCGGCAAGCCCCCGCTCCCCATCCATCACACTGGCGGCCGCTCGAGCATGCATCTA 3120
 A  L  H  N  H  Y  T  Q  K  S  L  S  L  S  L  G  K  .
__________ G2G4CH3 __________|
GAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATC 3240
TTATCATGTCTGGATCGATCCCGCCATGGTATCAACGCCATATTTCTATTTACAGTAGGGACCTCTTCGTTGTGTAGGTACCGCTGTATTCCTAGGGAAATAGTAGAGGCACCTTGAACT 3360
GTCTGCATCAGCCATATAGCCCCCGCTGTTCGACTTACAAACACAGGCACAGTACTGACAAACCCATACACCTCCTCTGAAATACCCATAGTTGCTAGGGCTGTCTCCGAACTCATTACA 3480
CCCTCCAAAGTCAGAGCTGTAATTTCGCCATCAAGGGCAGCGAGGGCTTCTCCAGATAAAATAGCTTCTGCCGAGAGTCCCGTAAGGGTAGACACTTCAGCTAATCCCTCGATGAGGTCT 3600
ACTAGAATAGTCAGTGCGGCTCCCATTTTGAAAATTCACTTACTTGATCAGCTTCAGAAGATGGCGGAGGGCCTCCAACACAGTAATTTTCCTCCCGACTCTTAAAATAGAAAATGTCAA 3720
GTCAGTTAAGCAGGAAGTGGACTAACTGACGCAGCTGGCCGTGCGACATCCTCTTTTAATTAGTTGCTAGGCAACGCCCTCCAGAGGGCGTGTGGTTTTGCAAGAGGAAGCAAAAGCCTC 3840
CCACCCAGGCCTAGAATGTTTCCACCCAATCATTACTATGACAACAGCTGTTTTTTTTAGTATTAAGCAGAGGCCGGGGACCCCTGGGCCCGCTTACTCTGGAGAAAAAGAAGAGAGGC 3960
ATTGTAGAGGCTTCCAGAGGCAACTTGTCAAAACAGGACTGCTTCTATTTCTGTCACACTGTCTGGCCCTGTCACAAGGTCCAGCACCTCCATACCCCCTTTAATAAGCAGTTTGGGAAC 4080
GGGTGCGGGTCTTACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGA 4200
GCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGGAGCTCCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCC 4320
GCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGA 4440
```

FIG. 6B (Cont.)

CCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTG 4560
GTTCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCA 4680
AGAGCCAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAG 4800
TTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGG 4920
GTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTA 5040
ATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGA 5160
ACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTC 5280
TGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCAC 5400
GCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAA 5520
TAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAG 5640
ATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCT 5760
CAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATG 5880
GCCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTA 6000
TTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTG 6057

FIG. 6B (Cont.)

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Samsung Bioepis Exhibit No. 1002
Page 2181

Map of the heavy chain expression vector pSVgptHuG2/G4 used in

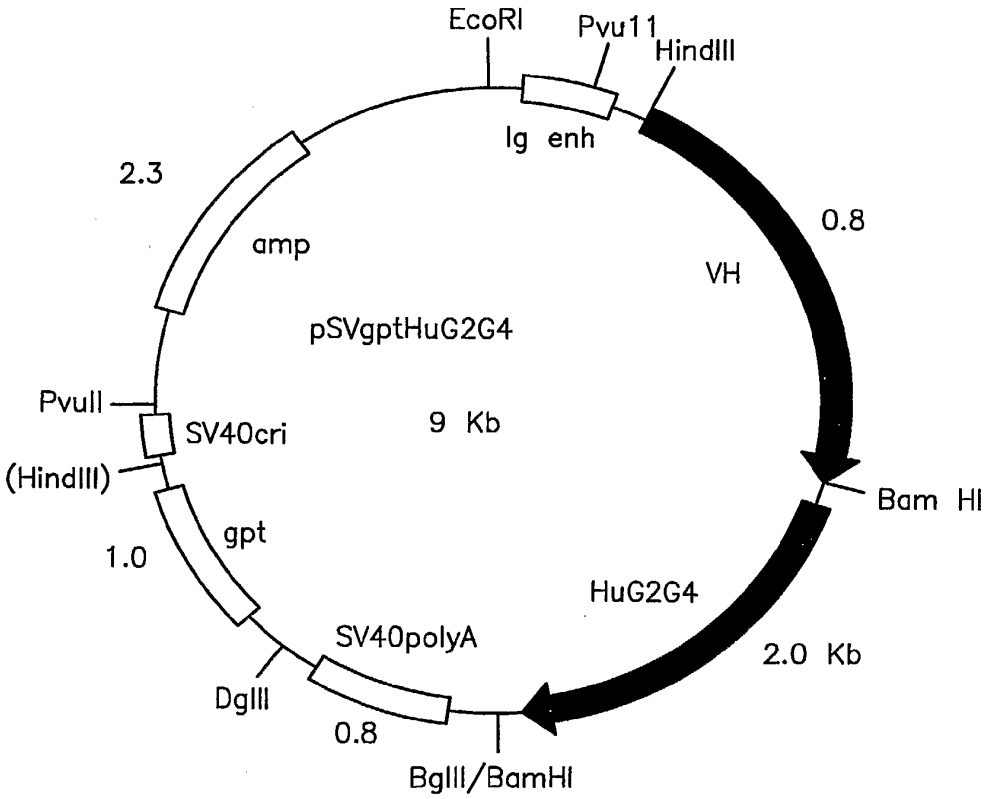


FIG. 7



FIG. 8

SUBSTITUTE SHEET (RULE 26)

Map of the light chain expression vector pSVgptHuCK

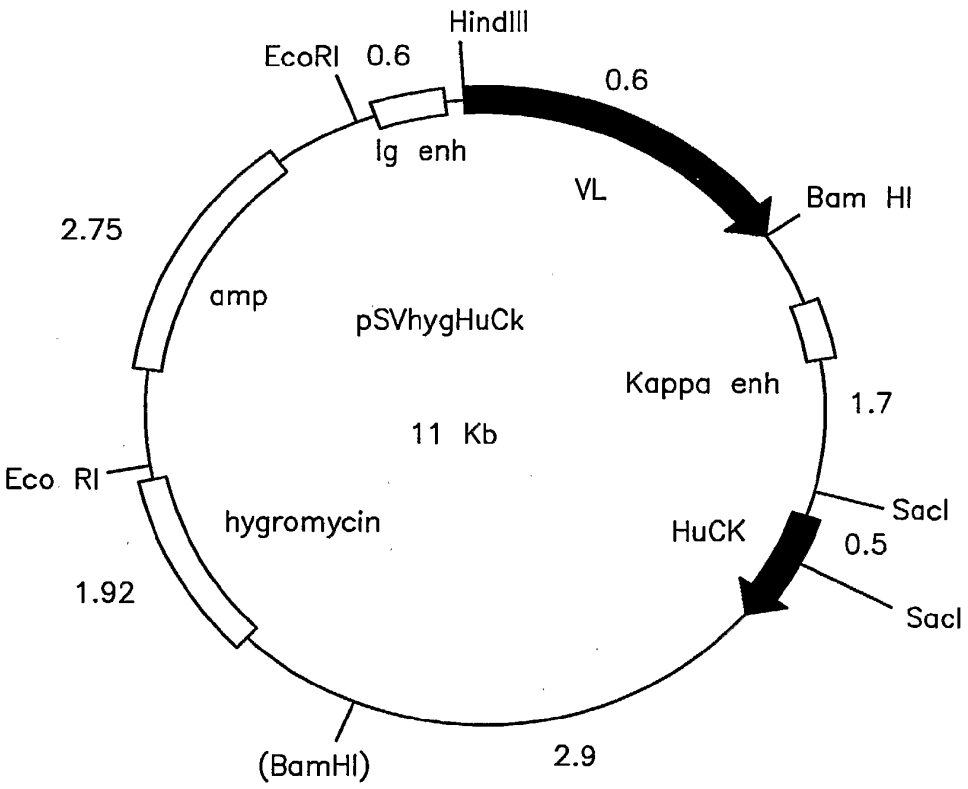


FIG. 9

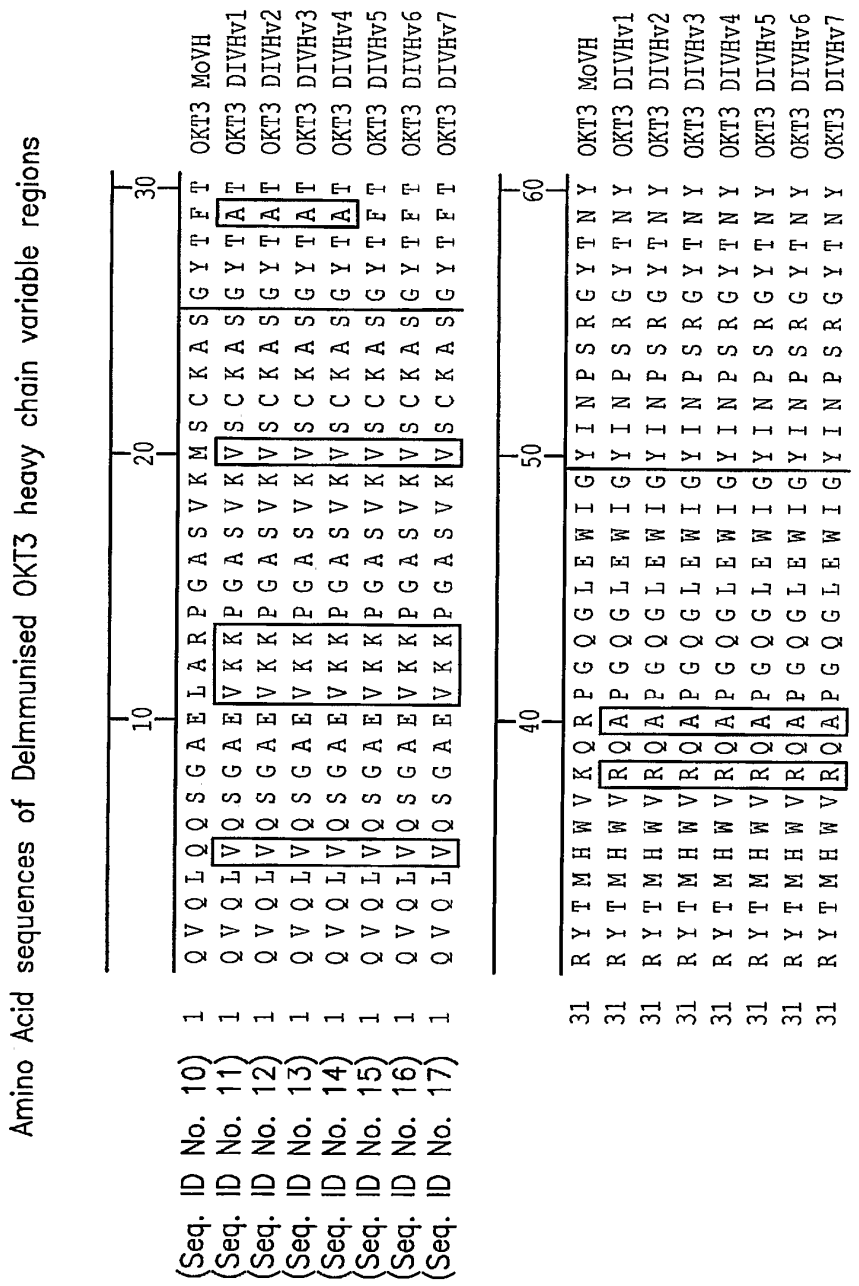

Amino Acid sequences of Delmmunised OKT3 heavy chain variable regions
(Seq. ID No. 10) 1 QVQLQQSGAELARPGASVKMSCKASGYTFT OKT3 MoVH
(Seq. ID No. 11) 1 QVQLVQSGAEVKKPGASVKVSCKASGYTAT OKT3 DIVHv1
(Seq. ID No. 12) 1 QVQLVQSGAEVKKPGASVKVSCKASGYTAT OKT3 DIVHv2
(Seq. ID No. 13) 1 QVQLVQSGAEVKKPGASVKVSCKASGYTAT OKT3 DIVHv3
(Seq. ID No. 14) 1 QVQLVQSGAEVKKPGASVKVSCKASGYTAT OKT3 DIVHv4
(Seq. ID No. 15) 1 QVQLVQSGAEVKKPGASVKVSCKASGYTFT OKT3 DIVHv5
(Seq. ID No. 16) 1 QVQLVQSGAEVKKPGASVKVSCKASGYTFT OKT3 DIVHv6
(Seq. ID No. 17) 1 QVQLVQSGAEVKKPGASVKVSCKASGYTFT OKT3 DIVHv7
10
20
30
40
50
60
31 RYTMHWVKQRPGQGLEWIGYINPSRGYTNY OKT3 MoVH
31 RYTMHWVRQAPGQGLEWIGYINPSRGYTNY OKT3 DIVHv1
31 RYTMHWVRQAPGQGLEWIGYINPSRGYTNY OKT3 DIVHv2
31 RYTMHWVRQAPGQGLEWIGYINPSRGYTNY OKT3 DIVHv3
31 RYTMHWVRQAPGQGLEWIGYINPSRGYTNY OKT3 DIVHv4
31 RYTMHWVRQAPGQGLEWIGYINPSRGYTNY OKT3 DIVHv5
31 RYTMHWVRQAPGQGLEWIGYINPSRGYTNY OKT3 DIVHv6
31 RYTMHWVRQAPGQGLEWIGYINPSRGYTNY OKT3 DIVHv7

FIG. 10

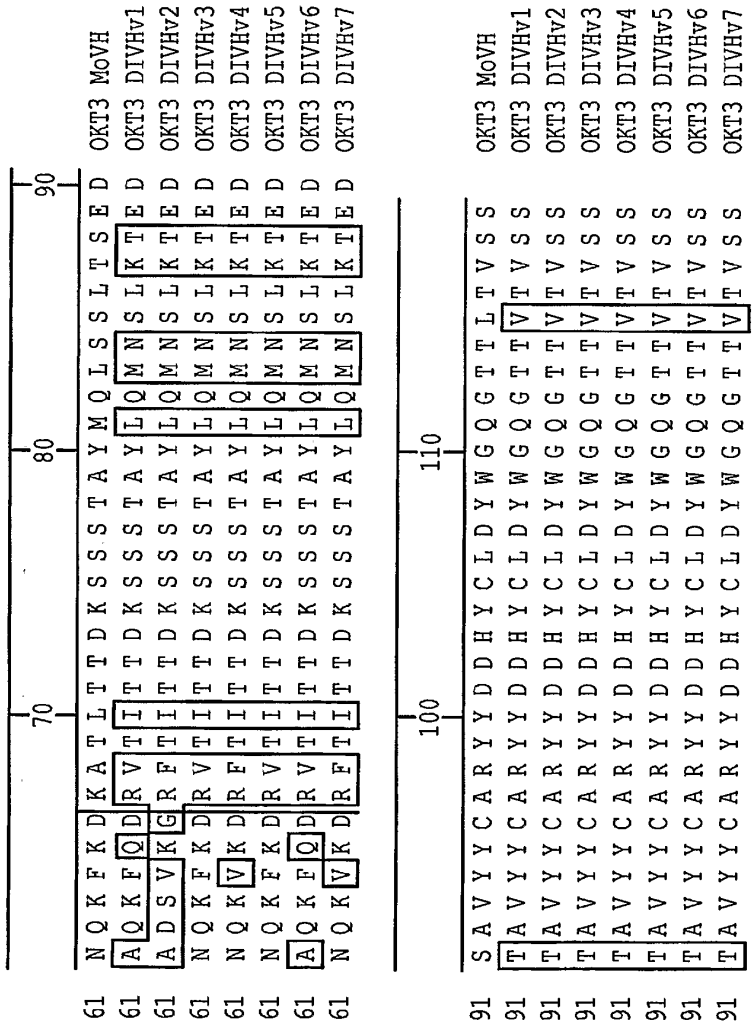

OKT3 MoVH
OKT3 DIVHv1
OKT3 DIVHv2
OKT3 DIVHv3
OKT3 DIVHv4
OKT3 DIVHv5
OKT3 DIVHv6
OKT3 DIVHv7
70
80
90
100
110

FIG. 10 (Cont.)

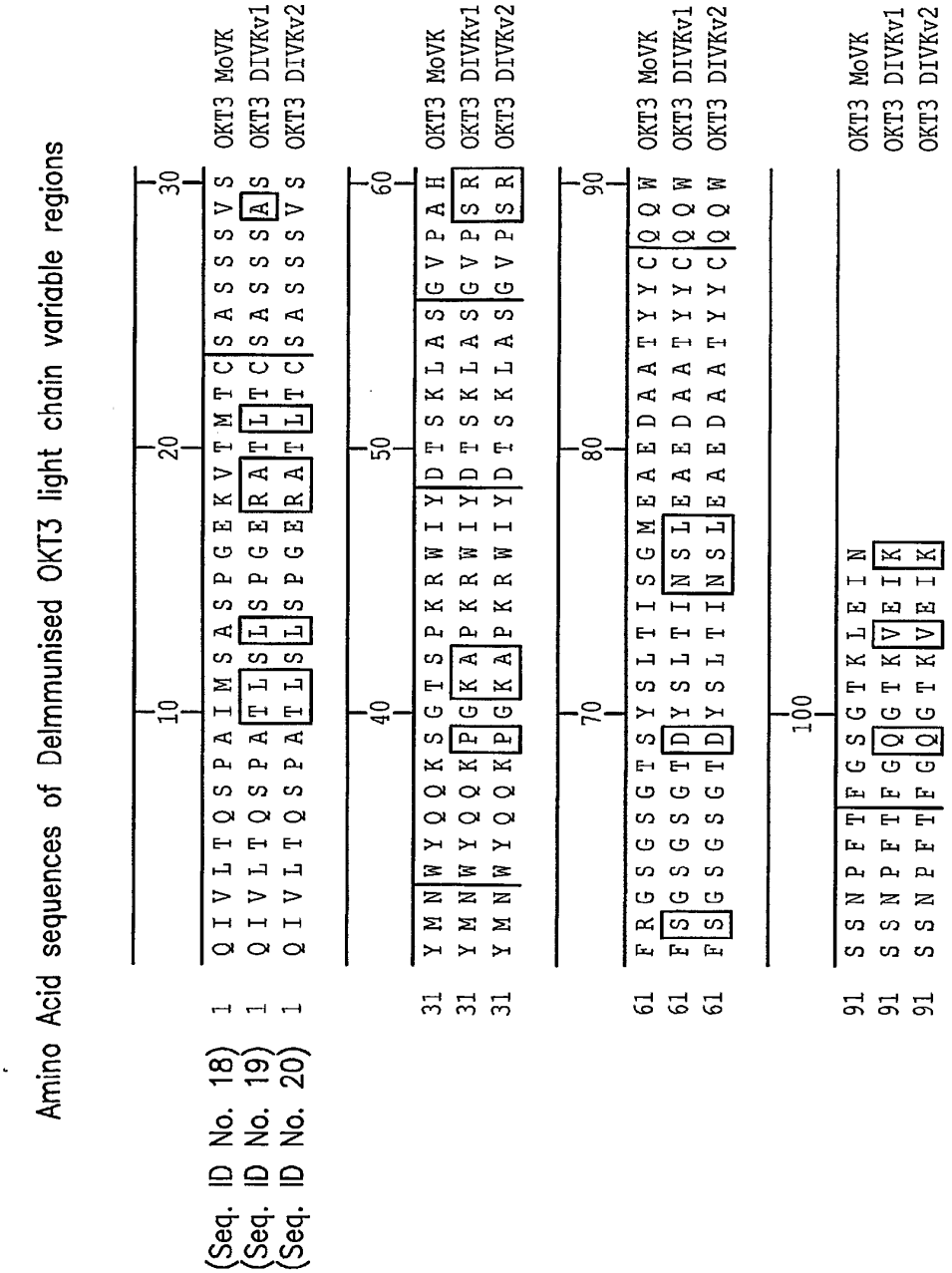

Amino Acid sequences of Delmmunised OKT3 light chain variable regions
(Seq. ID No. 18)
(Seq. ID No. 19)
(Seq. ID No. 20)
OKT3 MoVK
OKT3 DIVKv1
OKT3 DIVKv2

FIG. 11

OLIGOS FOR CONSTRUCTION OF DIVHs (SEQ ID NOS: 37–57)

KTDIVH1 GAAGTCAAGAAACCTGGGGCCTCAGTGAAGGTGTCCTGCAAGG
KTDIVH2
GCCCCAGGTTTCTTGACTTCAGCCCCAGACTGTACCAGCTGGACCTG
KTDIVH3 TGGGTAAGACAGGCGCCTGGACAAGGTTTGG
KTDIVH4 GTCCAGGCGCCTGTCTTACCCAGTGCATC
KTDIVH4A
AGGCGCCTGTCTTACCCAGTGCATCGTGTACCTAGTAGCCGTGTAGCC
KTDIVH5 CAATCAGAAGTTCAAGGACAGGGTCACAATCACTACAGACAAA
KTDIVH5A CGCTCAGAAGTTCCAGGACAGGGTCACAATCACTACAGACAAA
KTDIVH5B CGCTGACAGTGTCAAGGGCAGGTTCACAATCACTACAGACAAA
KTDIVH5C CAATCAGAAGGTCAAGGACAGGTTCACAATCACTACAGACAAA
KTDIVH6 GTCCTTGAACTTCTGATTGTAATTAGTATATCCACGG
KTDIVH6A GTCCTGGAACTTCTGAGCGTAATTAGTATATCCACGG
KTDIVH6B GCCCTTGACACTGTCAGCGTAATTAGTATATCCACGG
KTDIVH6C GTCCTTGACCTTCTGATTGTAATTAGTATATCCACGG
KTDIVH7 AGCCTGAAAACTGAGGACACCGCAGTCTATTACTG
KTDIVH8 GTCCTCAGTTTTCAGGCTGTTCATTTGCAAGTAGGCTGTGCT
KTDIVH9 CCAAGGCACCACTGTGACAGTCTCCTCAGG
KTDIVH10 CCTGAGGAGACTGTCACAGTGGTGCCTTGG
KT3VHY GGTGTCCACTCCCAGGTCCAGCTG
KT3VHZ CAGCTGGACCTGGGAGTGGACACCTGTGG
VHVK1 GCATGTTGACCCTGACGCAAGCTTATGAATATGCAAA
VH12 GCGATAGCTGGACTGAATGGATCCTATAAATCTCTG

OLIGOS FOR CONSTRUCTION OF DIVKs (SEQ ID NOS: 58–74)

KTDIVK1 CCCTCTCTCTTTCTCCAGGGGAACGCGCCACCTTGACATGCAGTG
KTDIVK2 CCTGGAGAAAGAGAGAGGGTTGCTGGAGACTGGGTG
KTDIVK3
CATGAACTGGTACCAGCAGAAGCCCGGCAAAGCTCCCAAAAGATGGAT
KTDIVK4 CGGGCTTCTGCTGGTACCAGTTCATGTAACTTACACTT
KTDIVK4A CTTCTGCTGGTACCAGTTCATGTAACTTGCACTTGAGC
KTDIVK5
GGGTCTGGGACCGATTACTCTCTCACCATCAATAGTCTGGAAGCTGAAG
KTDIVK6
GTAATCGGTCCCAGACCCACTGCCACTGAAGCGAGACGGTACTCCAG
KTDIVK7 TTCACGTTCGGACAAGGTACAAAGGTGGAAATCAAACG
KTDIVK8 CTTTGTACCTTGTCCGAACGTGAATGGGTTACTTGACC
KKT22 GCGGATCCAGTCGACGAAGCA
KT3VKX CTGAATGGATCCAACTGAGGAAGCAAAGTTTAAATTCTACTCACG
KT3VKY CAAATTGTTCTCACCCAGTCTCCAGCAA
KT3VKZ TTGCTGGAGACTGGGTGAGAACAATTTGGGAG
KT3VKZ2 TGGAGACTGGGTGAGAACAATTTGGGAGTGGACACCTGTGG
KT3VKZ3 AGAGAGGGTTGCTGGAGACTGGGTGAGAACAATTTG
VHVK1 GCATGTTGACCCTGACGCAAGCTTATGAATATGCAAA
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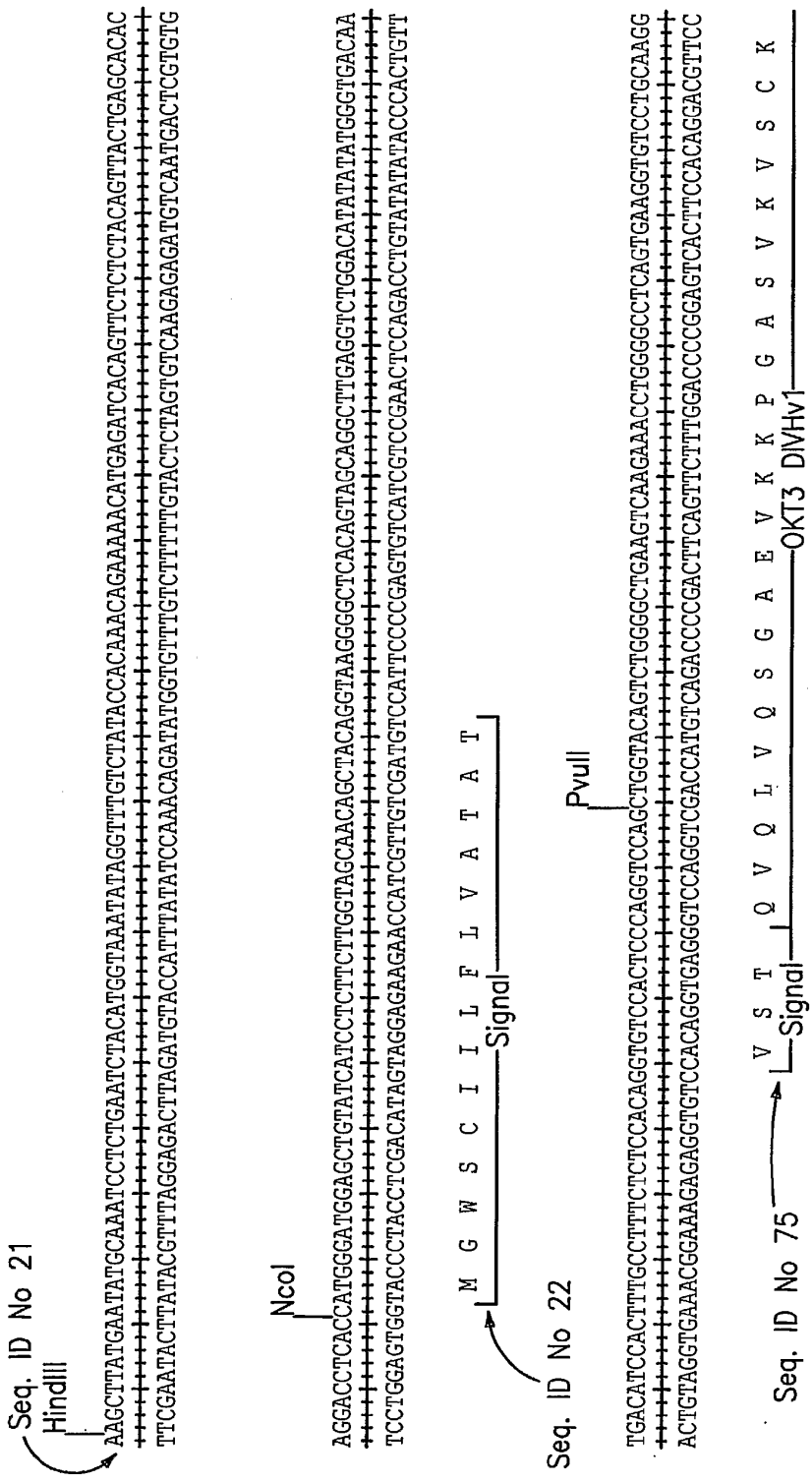


FIG. 13

Samsung Bioepis Exhibit No. 1002
Page 2189

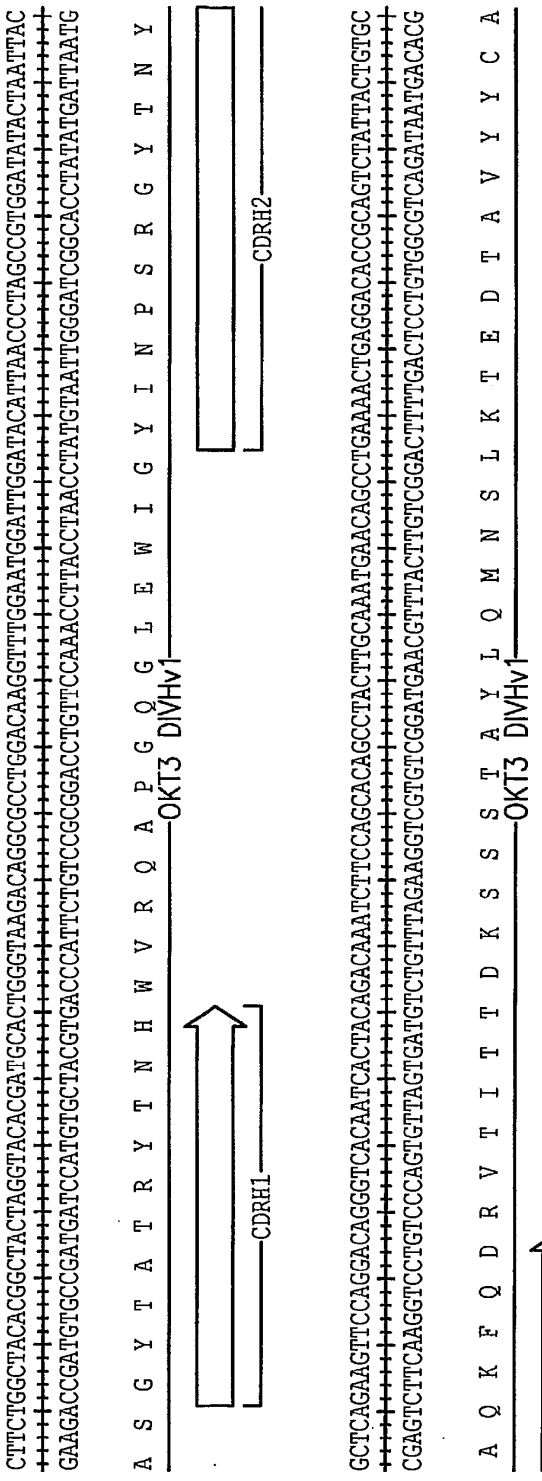


FIG. 13 (Cont.)

Samsung Bioepis Exhibit No. 1002
Page 2190

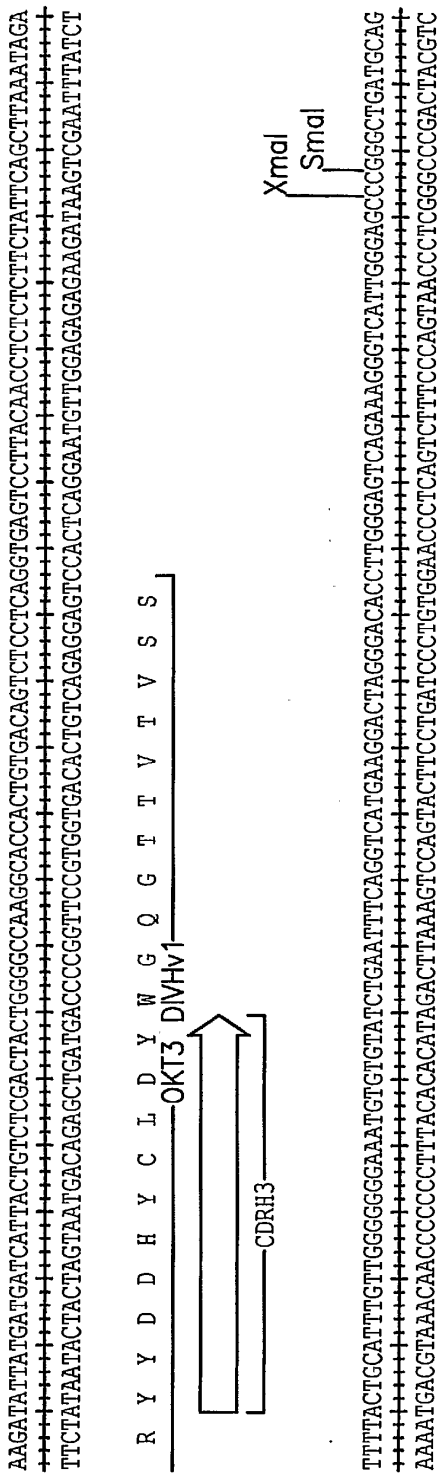


FIG. 13 (Cont.)

Samsung Bioepis Exhibit No. 1002
Page 2191

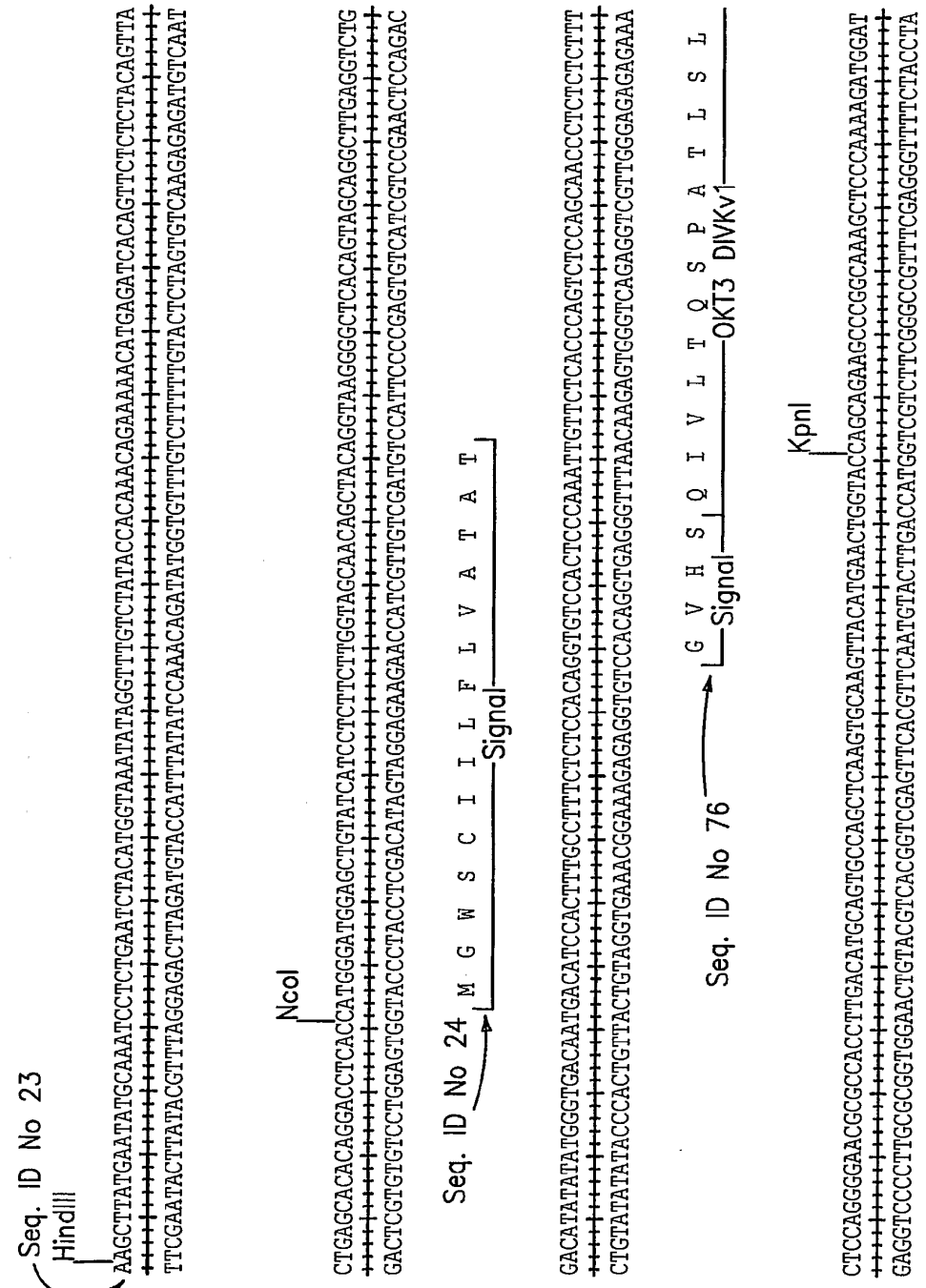


FIG. 14

Samsung Bioepis Exhibit No. 1002
Page 2192

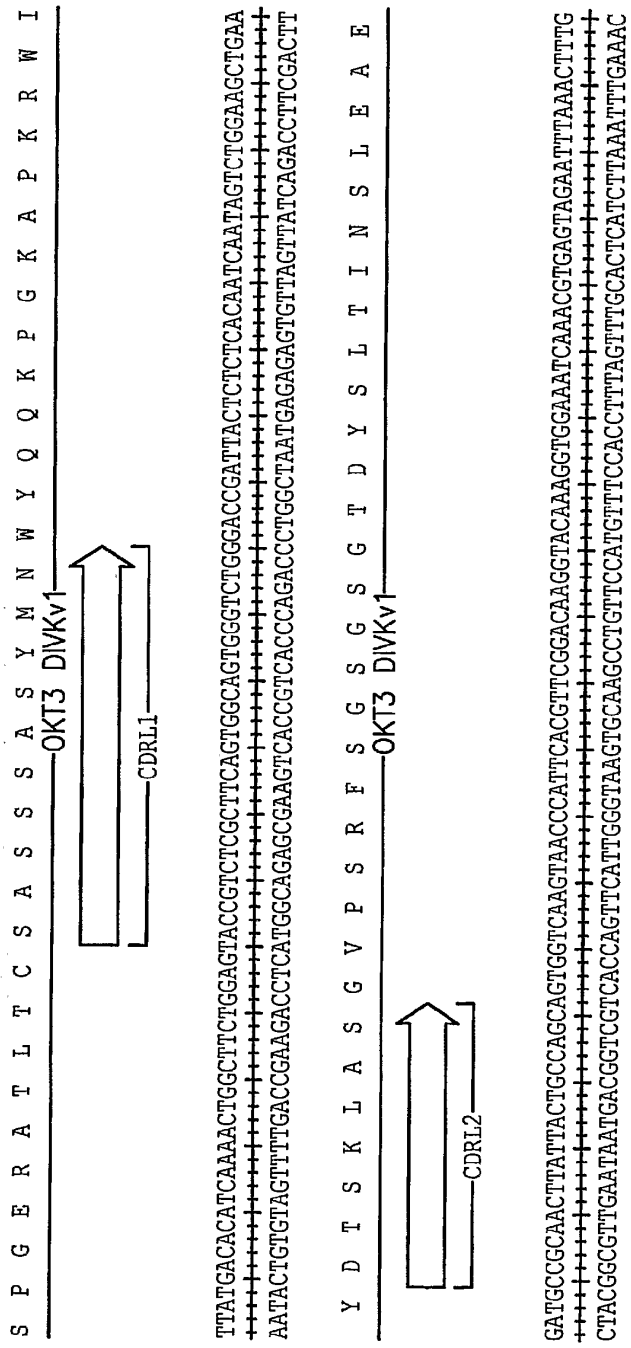

S P G E R A T L T C S A S S S A S Y M N W Y Q Q K P G K A P K R W I
OKT3 DIVKv1
CDRL1
TTATGACACATCAAAACTGGCTTCTGGAGTACCGTCTCGCTTCAGTGGCAGTGGGTCTGGGACCGATTACTCTCTCACAATCAATAGTCTGGAAGCTGAA
AATACTGTGTAGTTTTGACCGAAGACCTCATGGCAGAGCGAAGTCACCGTCACCCAGACCCTGGCTAATGAGAGTGTTAGTTATCAGACCTTCGACTT
Y D T S K L A S G V P S R F S G S G S G T D Y S L T I N S L E A E
OKT3 DIVKv1
CDRL2
GATGCCGCAACTTATTACTGCCAGCAGTGGTCAAGTAACCCATTCACGTTCGGACAAGGTACAAAGGTGGAAATCAAACGTGAGTAGAATTTAAACTTTG
CTACGGCGTTGAATAATGACGGTCGTCACCAGTTCATTGGGTAAGTGCAAGCCTGTTCCATGTTTCCACCTTTAGTTTGCACTCATCTTAAATTTGAAAC
D A A T Y Y C Q Q W S S N P F T F G Q G T K V E I K
OKT3 DIVKv1
CDRL3
BamHI
CTTCCTCAGTTGGATCC
GAAGGAGTCAACCTAGG
617

FIG. 14 (Cont.)

Murine and Chimaeric OKT3 binding to Jurkat, JRT3 and HPB–ALL cells
Values represent the positive % of gated cells in M1

Cell Type	Passage #	Murine OKT3	Mouse Isotype Control	Chimaeric OKT3	Human Isotype Control
Jurkat	12	81.20	0.5	94.68	0.44
JRT3	14	3.45	0.26	4.56	0.43
HPB–ALL	10	99.63	0.62	99.39	0.29

FIG. 15

Antibody	Clone No.	% Cells in M1	
		HPB–ALL	JRT3
Chimaeric OKT3	N/A	99.74	7.74
Control no OKT3 no PE	N/A	2.22	2.3
Control no OKT3 with PE	N/A	2.3	2.21
DMEM Control	N/A	1.91	2.42
DIVH1/DIVK1	19D6	93.87	2.16
DIVH2/DIVK1	24C12	28.47	2.34
DIVH3/DIVK1	27F6	84.75	2.28
DIVH4/DIVK1	30F7	93.06	2.65
DIVH5/DIVK1	35F2	98.15	2.77
DIVH6/DIVK1	37E9	97.85	3.08
DIVH7/DIVK1	42E7	98.62	3.12

FIG. 16

Table 3:

Antibody	Clone No.	% Cells in M1	
		HPB–ALL	JRT3
Chimaeric OKT3	N/A	99.95	0.1
Control no OKT3 no PE	N/A	0.1	0.02
DIVHv1/DIVK2	48G3	20.18	0.1
DIVHv2/DIVK2	52B8	90.04	0.25
DIVHv3/DIVK2	55G5	84.73	0.14
DIVHv4/DIVK2	55B2	69.26	0.13
DIVHv6/DIVK2	66C6	98.16	0.53
DIVHv7/DIVK2	70G10	95.57	0.66

FIG. 17

Competition assay. Inhibition of binding biotinylated mouse OKT3 by chimaeric and DeImmunised OKT3 antibodies, DIVHv1/DIVKv1, DIVHv3/DIVKv1, DIVHv5/DIVKv1, DIVHv6/DIVKv1, OKT3DIVH7/DIVKv1.

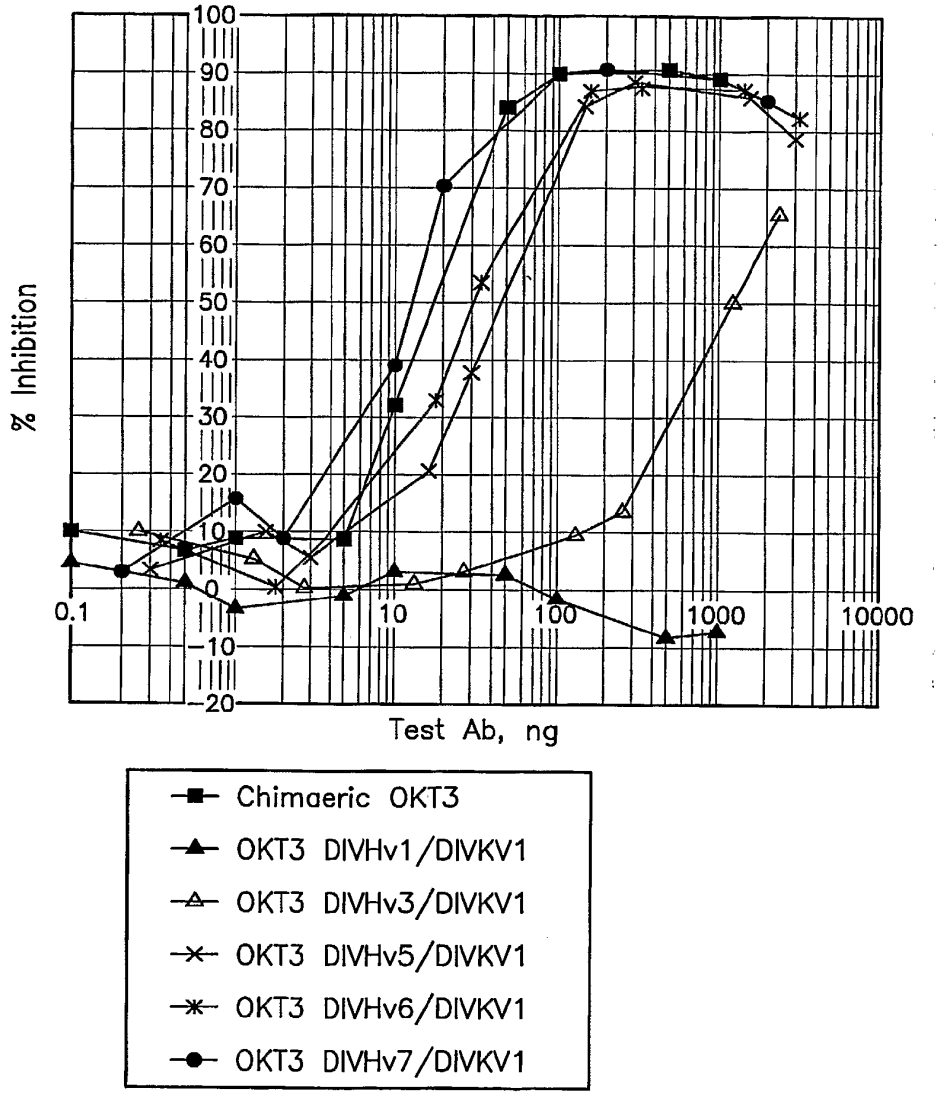


FIG. 18

Competition assay. Inhibition of binding biotinylated mouse OKT3 by mouse, chimaeric and DeImmunised OKT3 antibodies DIVHv2/DIVKv1, DIVHv4/DIVKv1.

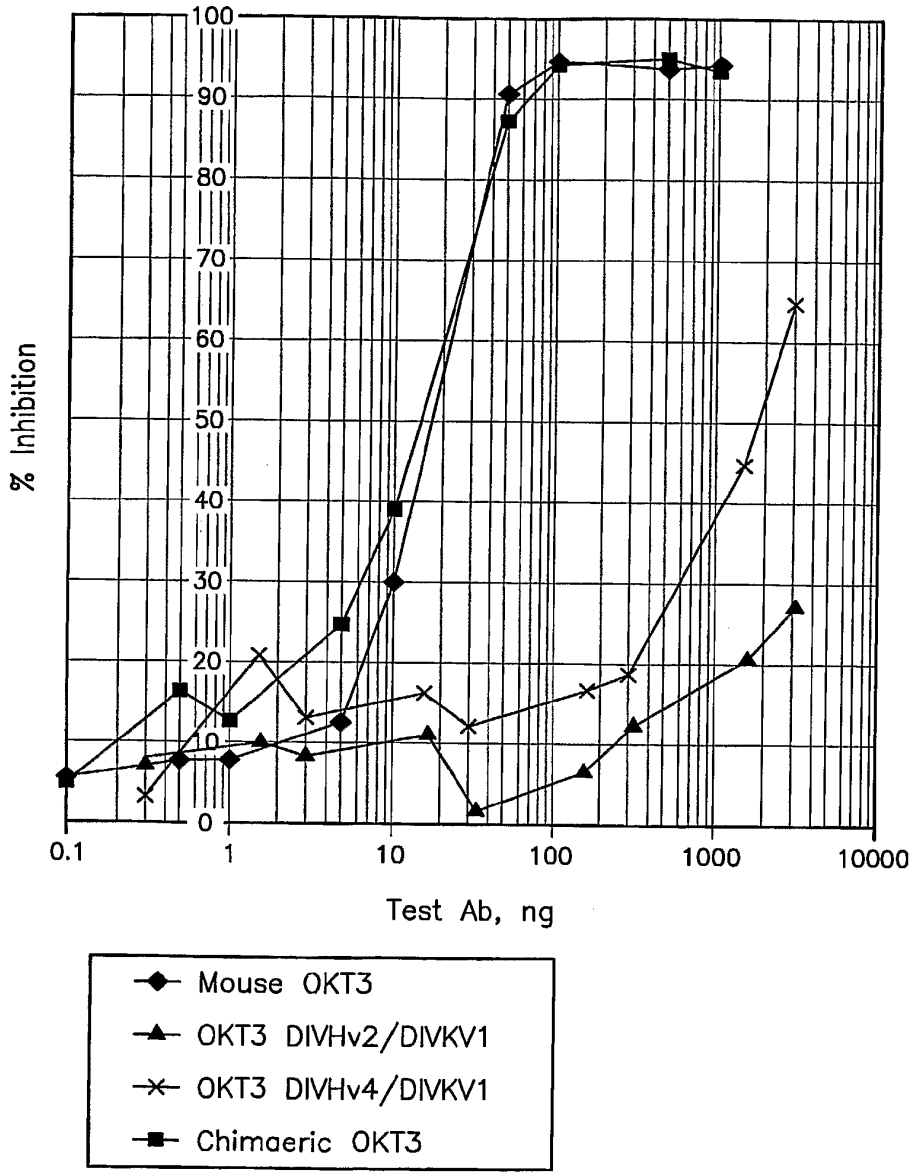


FIG. 19

Competition assay. Inhibition of binding biotinylated mouse OKT3 by mouse, chimaeric and DeImmunised OKT3 antibodies DIVHv3/DIVKv2, DIVHv7/DIVKv2.

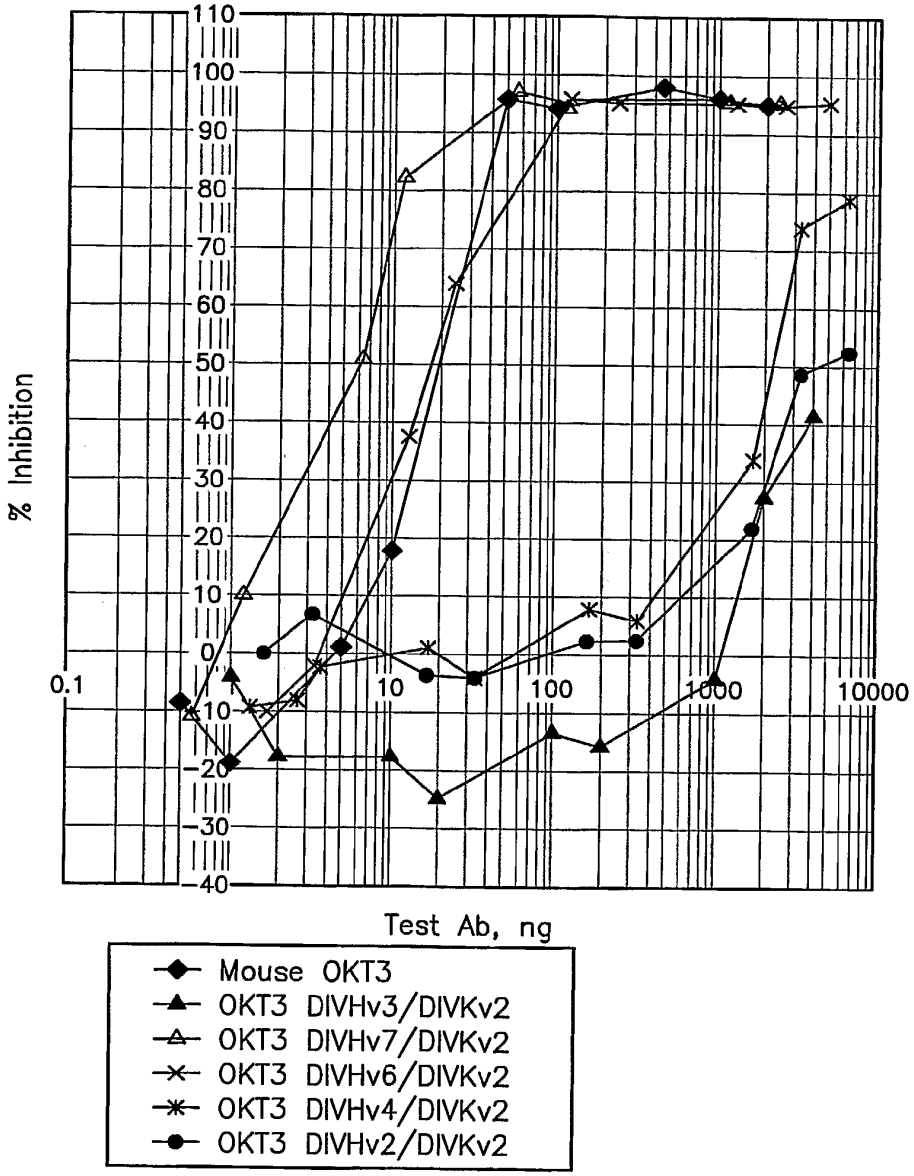


FIG. 20

Samsung Bioepis Exhibit No. 1002
Page 2198

Competition assay. Inhibition of binding biotinylated mouse OKT3 by mouse, chimaeric and DeImmunised OKT3 antibodies DIVHv1/DIVKv2, DIVHv1/DIVKv1, DIVHv5/DIVKv2, DIVHv5/DIVKv1.

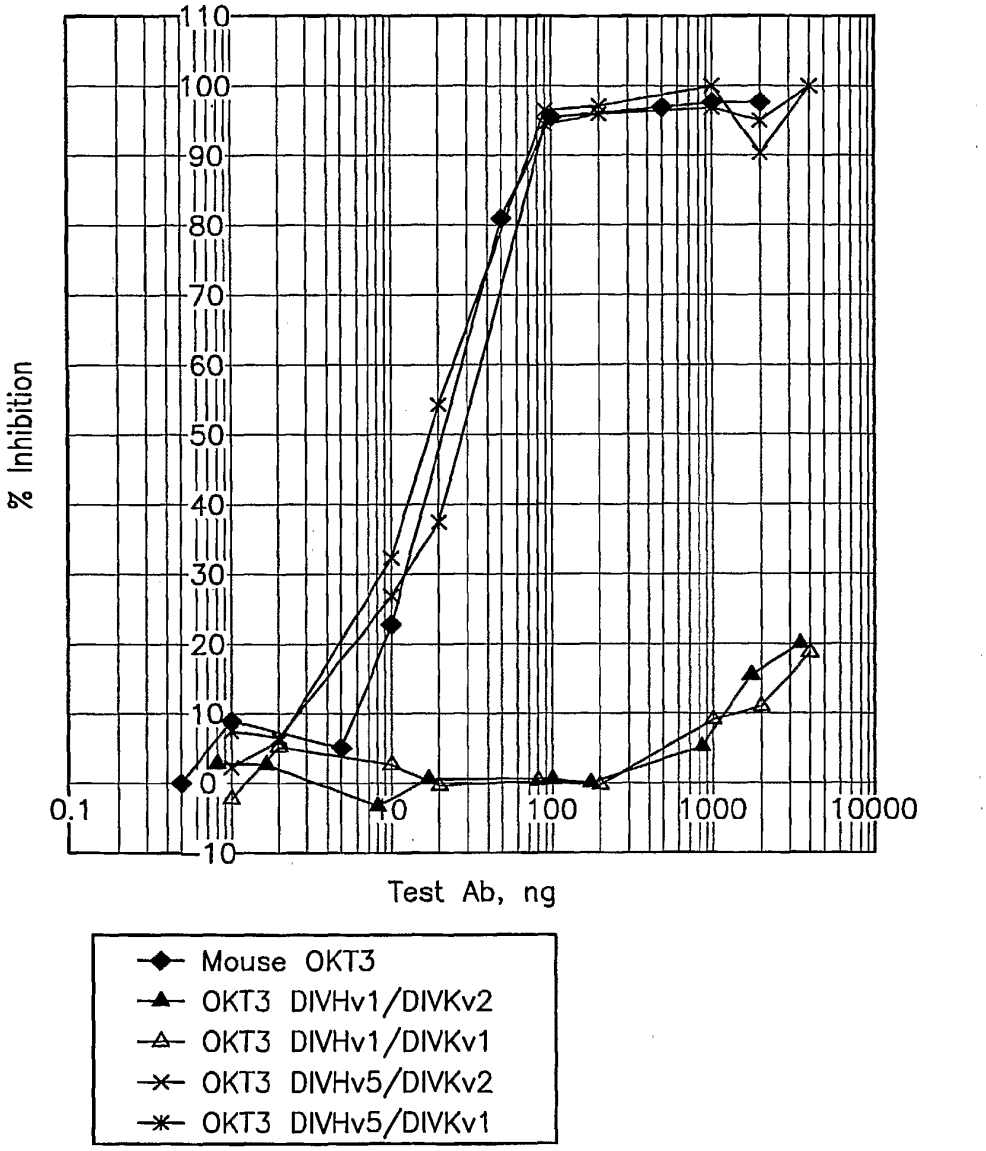


FIG. 21

The IC_{50} determinted from these plots are shown in Table 4.

Table 4:

ANTIBODY	IC50 (ng)
Murine OKT3 1	18
Murine OKT3 2	19
Murine OKT3 3	20
Chimeric OKT3 1	18
Chimeric OKT3 2	15
Di-immunized OKT3 DIVHv1/DIVKv1	N/A
Delm OKT3 DIVHv1/DIVKv1 2nd prep	>2000
De-immunized OKT3 DIVHv2/DIVKv1	>3000
De-immunized OKT3 DIVHv3/DIVKv1	1250
De-immunized OKT3 DIVHv4/DIVKv1	1900
De-immunized OKT3 DIVHv5/DIVKv1	45
Delm OKT3 DIVHv5/DIVKv1 2nd prep	19
De-immunized OKT3 DIVHv6/DIVKv1	30
De-immunized OKT3 DIVHv7/DIVKv1	12
De-immunized OKT3 DIVHv1/DIVKv2	>2000
De-immunized OKT3 DIVHv2/DIVKv2	>3000
De-immunized OKT3 DIVHv3/DIVKv2	>4000
De-immunized OKT3 DIVHv4/DIVKv2	2100
De-immunized OKT3 DIVHv5/DIVKv2	28
De-immunized OKT3 DIVHv6/DIVKv2	18
De-immunized OKT3 DIVHv7/DIVKv2	6

FIG. 22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US04/17219

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 39/395; C07K 16/00; C12N 15/13
US CL : 530/387.3; 424/133.1; 536/23.53; 435/69.6
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 530/387.3; 424/133.1; 536/23.53; 435/69.6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 6,491,916 B1 (BLUESTONE et al.) 10 December 2002 (10.12.2002), see entire document.	1,4,6,7,10-13,17 --- 5,16
Y	US 2003/0100741 A1 (MULLER et al.) 29 May 2003 (29.05.2003), see entire document.	5,16

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
22 September 2004 (22.09.2004)

Date of mailing of the international search report
19 OCT 2004

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
Facsimile No. (703) 305-3230

Authorized officer
Ron Schwadron, Ph.D.
Telephone No. 571-272-1600

Form PCT/ISA/210 (second sheet) (January 2004)

Continuation of B. FIELDS SEARCHED Item 3:
WEST 2.1, MEDICINE/BIOTECH (compendium database on DIALOG) search terns: inventor names, cd3, okt3, de immuniz?, deimmuniz?, reduc?, immunogenic?, anticd3, epitop?, remov?, delet?

Samsung Bioepis Exhibit No. 1002
Page 2202

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US04/17219

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 2,3,8,9,14,15 and 18-20
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
The aforementioned claims were not examined because they recite sequences and no sequence listing has been submitted.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Samsung Bioepis Exhibit No. 1002
Page 2203

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(71) Applicant *(for all designated States except US)*: **PROTEIN DESIGN LABS, INC.** [US/US]; 34801 Campus Drive, Fremont, California 94555 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **RAMAKRISHNAN, Vanitha** [US/US]; 825 Holly Road, Belmont, California 94002 (US). **BHASKAR, Vinay** [US/US]; 534 Clipper Street, San Francisco, California 94114 (US). **HO, Sun** [CN/US]; 208 Black Mountain Circle, Fremont, California 94536 (US). **MURRAY, Richard** [US/US]; 22643 Woodridge Court, Cupertino, California 95014 (US). **LAW, Debbie** [GB/US]; 822 Pacheco Street, San Francisco, California 94080 (US).

(74) Agent: **WHITING, Adam**; Howrey Simon Arnold & White, LLP, 2941 Fairview Park Drive, Box 7, Falls Church, Virginia 22042 (US).

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[Continued on next page]

(54) Title: USE OF ANTI-ALPHA5BETA1 ANTIBODIES TO INHIBIT CANCER CELL PROLIFERATION

(57) Abstract: The present invention provides methods for direct killing of cancer cells using anti-α5β1 antibodies. Generally, the method comprises contacting a cancer cell that expresses α5β1 on its surface with an anti-α5β1 antibody, and thereby inducing the death of the cancer cell. The methods of the invention may be employed at an early stage of cancer development in a patient to prevent tumor establishment. In addition, the methods may be used to treat previously formed tumors especially in cancer that have not proven susceptible to anti-angiogenesis therapy. The methods may be employed as a combination therapy of anti-α5β1 antibodies together with cancer chemotherapeutic agents or other molecular-based cancer therapeutic agents.

A. IIA1 V_H sequences
[NA, SEQ ID NO: 1; AA, SEQ ID NO: 2]

```
1   ATGGCTGTCCTGGGGCTGCTTCTCTGCCTGGTGACTTTCCCAAGCTGTGTCCTGTCCCAG
     M  A  V  L  G  L  L  L  C  L  V  T  F  P  S  C  V  L  S  Q
61  GTGCAGCTGAAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCCTGTCCATCACA
     V  Q  L  K  E  S  G  P  G  L  V  A  P  S  Q  S  L  S  I  T
121 TGCACCATCTCAGGGTTCTCATTAACCGACTATGGTGTTCACTGGGTTCGCCAGCCTCCA
     C  T  I  S  G  F  S  L  T  D  Y  G  V  H  W  V  R  Q  P  P
181 GGAAAGGGTCTGGAGTGGCTGGTAGTGATTTGGAGTGATGGAAGCTCAACCTATAATTCA
     G  K  G  L  E  W  L  V  V  I  W  S  D  G  S  S  T  V  N  S
241 GCTCTCAAATCCAGAATGACCATCAGGAAGGACAACTCCAAGAGCCAAGTTTTCTTAATA
     A  L  K  S  R  M  T  I  R  K  D  N  S  K  S  Q  V  F  L  I
301 ATGAACAGTCTCCAAACTGATGACTCAGCCATGTACTACTGTGCCAGACATGGAACTTAC
     M  N  S  L  Q  T  D  D  S  A  M  Y  Y  C  A  R  H  G  T  Y
361 TACGGTATGACTACGACGGGGGATGCTTTGGACTACTGGGGTCAAGGAACCTCAGTCACC
     Y  G  M  T  T  T  G  D  A  L  D  Y  W  G  Q  G  T  S  V  T
421 GTCTCCTCA
     V  S  S
```

B. IIA1 V_L sequences
[NA, SEQ ID NO: 3; AA, SEQ ID NO: 4]

```
1   ATGGATTTTCAGGTGCAGATTTTCAGCTTCCTGCTAATCAGTGCCTCAGTCATAATGTCC
     M  D  F  Q  V  Q  I  F  S  F  L  L  I  S  A  S  V  I  M  S
61  AGAGGACAAATTGTTCTCACCCAGTCTCCAGCAATCATGTCTGCATCTCTAGGGGAACGG
     R  G  Q  I  V  L  T  Q  S  P  A  I  M  S  A  S  L  G  E  R
121 GTCACCATGACCTGCACTGCCAGTTCAAGTGTAAGTTCCAATTACTTGCACTGGTACCAG
     V  T  M  T  C  T  A  S  S  S  V  S  S  N  Y  L  H  W  Y  Q
181 CAGAAGCCAGGATCCGCCCCCAATCTCTGGATTTATAGCACATCCAACCTGGCTTCTGGA
     Q  K  P  G  S  A  P  N  L  W  I  Y  S  T  S  N  L  A  S  G
241 GTCCCAGCTCGTTTCAGTGGCAGTGGGTCTGGGACCTCTTACTCTCTCACAATCAGCAGC
     V  P  A  R  F  S  G  S  G  S  G  T  S  Y  S  L  T  I  S  S
301 ATGGAGGCTGAAGATGCTGCCACTTATTACTGCCACCAGTATCTTCGTTCCCCACCGACG
     M  E  A  E  D  A  A  T  Y  Y  C  H  Q  Y  L  R  S  P  P  T
361 TTCGGTGGAGGCACCAAGCTGGAAATCAAA
     F  G  G  G  T  K  L  E  I  K
```

WO 2005/092073 A2

PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

USE OF ANTI-α5β1 ANTIBODIES TO INHIBIT CANCER CELL PROLIFERATION

FIELD OF THE INVENTION

The present invention provides antibodies that specifically recognize α5β1 integrin expressed on the surface of cancer cells, and methods for using the antibodies to inhibit proliferation of these cancer cells.

BACKGROUND OF THE INVENTION

The association of α5β1 integrin with tumor angiogenesis is well-established (see, e.g., U.S. Publ. Pat. Appl. US 2002/0172675 A1, filed May 7, 1999, which is hereby incorporated by reference herein). α5β1 is a heterodimeric integrin that specifically binds the ligand fibronectin. α5β1 is expressed on the surface of endothelial cells and mediates adhesion to and migration toward fibronectin. The binding interaction between α5β1 and fibronectin has been shown to be important for tumor angiogenesis. Angiogenesis within a tumor begins when the release of one or more pro-angiogenic growth factors (e.g., FGF, VEGF, PDGF, etc.) locally activates the endothelial cells. These activated endothelial cells then form new blood vessels by binding, via their α5β1 integrin, to the fibronectin in the extracellular matrix. Anti-α5β1 antibodies have been shown to inhibit angiogenesis in *in vivo* tumor models (see, e.g., US 2002/0172675 A1).

Anti-angiogenesis cancer therapy is based on inhibiting tumor vascularization and thereby preventing continued tumor growth and metastasis (for reviews see, e.g., Marx, "A Boost for Tumor Starvation," *Science* **301**, 452 (2003); Sato, "Molecular Diagnosis of Tumor Angiogenesis and Anti-Angiogenic Cancer Therapy," *Int. J. Clin. Oncol.* **8**, 200 (2003); Bissachi *et al.*, "Anti-Angiogenesis and Angioprevention: Mechanisms, Problems and Perspectives," *Cancer Detec. Prev.* **27**, 229 (2003)). More than 60 anti-angiogenesis based therapeutics currently are in clinical development for cancer treatment. While in some cancers, it may be possible to "starve" a tumor by preventing its vascularization, current research shows that there are cancers that do not appear to be vulnerable to anti-angiogenesis treatment (see, Sato, *supra*). For example, the anti-VEGF antibody therapeutic, AVASTIN™ (bevacizumab) succeeded in clinical trials for colon cancer but not breast cancer (see, Marx, *supra*). In addition, anti-angiogenesis based therapeutic

Samsung Bioepis Exhibit No. 1002
Page 2206

methods are not well-suited to early-stage treatment when the tumor vascularization process has not yet begun.

Due to its function in angiogenesis, the α5β1 integrin has been proposed as a therapeutic target for numerous diseases mediated by angiogenic processes including cancerous tumor growth. Chimeric and humanized antibodies to α5β1 have been developed that block specific binding to fibronectin. A chimeric α5β1 antibody, M200 (also known by its generic name, volociximab) has been shown to induce apoptosis of activated endothelial cells *in vitro* regardless of the growth factor stimulus.

Thus, there remains a need for cancer therapies and early-stage treatment methods capable of directly killing cancer cells before the tumor vascularization process even begins, or for when a targeted anti-angiogenesis therapy proves ineffective.

SUMMARY OF THE INVENTION

The present invention provides methods of killing cancer cells using anti-α5β1 antibodies. In a general embodiment, the method comprises contacting a cancer cell that expresses α5β1 on its surface with an anti-α5β1 antibody.

In one preferred embodiment, the invention provides a method of inhibiting proliferation of a cancer (or tumor) cell that expresses α5β1 integrin on its surface comprising contacting the tumor cell with an antibody that binds to the α5β1 integrin expressed on the tumor cell surface. In a preferred embodiment, the tumor cell is in a patient with a refractory solid tumor. In another preferred embodiment, the tumor cell is from a cancer selected from the group consisting of: bladder cancer, breast cancer, colon cancer, fibrosarcoma, lung cancer, metastatic melanoma, pancreatic cancer, prostate cancer, ovarian cancer, renal cell carcinoma and spleen cancer.

In another embodiment, the invention provides a method of inducing death of a tumor cell that expresses α5β1 integrin on its surface comprising contacting the tumor cell with an antibody that binds to the α5β1 integrin expressed on the tumor cell surface. In a preferred embodiment, the tumor cell is in a patient with a refractory solid tumor. In another preferred embodiment, the tumor cell is from a cancer selected from the group consisting of bladder cancer, breast cancer, colon cancer, fibrosarcoma, lung cancer, metastatic melanoma, pancreatic cancer, prostate cancer, ovarian cancer, renal cell carcinoma and spleen cancer.

Samsung Bioepis Exhibit No. 1002
Page 2207

In a further embodiment, the invention provides a method of inhibiting the proliferation of a cancer cell in a patient wherein the cancer cell expresses α5β1 integrin on its surface. In this embodiment, the method comprises administering to the patient a therapeutically effective amount of an antibody, wherein the antibody competitively inhibits binding of M200 to α5β1 integrin on the cancer cell surface. In another embodiment, the antibody comprises a variable region with an amino acid sequence substantially identical to SEQ ID NOs: 2, 4, 6 and 8. In a preferred embodiment of this method, the antibody administered to the patient neutralizes at least one biological activity of α5β1 integrin. In another embodiment, the antibody administered to the patient comprises a therapeutic effector moiety (e.g., an antibody-drug conjugate). In still a further embodiment of this method, the antibody is administered to the patient, serially, or together with, another chemotherapeutic agent. In another preferred embodiment of this method, the antibody is administered to a patient with a refractory solid tumor, serially, or together with, another chemotherapeutic agent.

In another embodiment, the invention provides a method for treating a subject suspected of developing a cancer that expresses α5β1 on its cell surface wherein the subject has not yet developed a tumor comprising, administering to the patient a therapeutically effective amount of a pharmaceutical composition comprising an antibody binds to the α5β1 integrin. In a preferred embodiment, the cancer is selected from the group consisting of bladder cancer, breast cancer, colon cancer, fibrosarcoma, lung cancer, metastatic melanoma, pancreatic cancer, prostate cancer, ovarian cancer, renal cell carcinoma and spleen cancer.

In another embodiment, the invention provides a method of treating a subject with a genetic predisposition for a cancer that expresses α5β1 wherein the subject has not yet developed a tumor comprising administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising an antibody binds to the α5β1 integrin. In a preferred embodiment, the cancer is selected from the group consisting of bladder cancer, breast cancer, colon cancer, fibrosarcoma, lung cancer, metastatic melanoma, pancreatic cancer, prostate cancer, ovarian cancer, renal cell carcinoma and spleen cancer.

The preferred anti-α5β1 antibodies useful with the methods of the invention include IIA1, M200, F200, and antibodies that specifically bind to the same epitope on α5β1 as IIA1, M200, and F200.

Samsung Bioepis Exhibit No. 1002
Page 2208

In another embodiment, the anti-α5β1 antibodies useful with the methods of the invention include antibodies that competitively inhibit binding of IIA1 and/or M200 to α5β1 integrin expressed on the tumor cell surface.

Other antibodies useful with the method of the invention include antibodies comprising a variable region amino acid sequence substantially identical to SEQ ID NOs: 2,4,6 and 8. Also, included are antibodies comprising variable region amino acid sequences with at least about 90%, 95%, 98% or preferably, 99% or greater identity to SEQ ID NOs: 2,4,6 and 8.

In another embodiment, the present invention provides anti-α5β1 antibodies formulated as pharmaceutical compositions. These pharmaceutical compositions are useful in the various methods of the invention disclosed herein. In various embodiments, the anti-α5β1 antibody pharmaceutical compositions may be administered in a therapeutically effective amount to a subject by various routes including, but not limited to, orally, subcutaneously, topically, intravenously, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, intraocularly, intraventricularly, or intrathecally. In a preferred embodiment, the pharmaceutical composition is a liquid formulation comprising between about 1.0 mg/mL and 15 mg/mL of an anti-α5β1 antibody, about 22-27 mM citrate, about 145-165 mM sodium chloride, about 0.04% to 0.06% polysorbate (TWEEN®) 80, at a pH of about 5.5 to 7.5. In another preferred embodiment, the pharmaceutical composition is a liquid formulation comprising about 10 mg/mL anti-α5β1 antibody, about 25 mM citrate, about 150 mM sodium chloride, about 0.05% polysorbate (TWEEN®) 80, at a pH of about 6.5. In a particularly preferred embodiment, the pharmaceutical composition is a liquid formulation comprising about 10 mg/mL M200, about 25 mM citrate, about 150 mM sodium chloride, about 0.05% polysorbate (TWEEN®) 80, with a pH of about 6.5. In other preferred embodiments, each of the pharmaceutical compositions described herein may further comprise a chemotherapeutic agent. In another embodiment, the pharmaceutical composition comprising an anti-α5β1 antibody may be administered to a patient together with a pharmaceutically effective amount of another chemotherapeutic agent.

The pharmaceutical compositions described above may be used in a method of treating a patient diagnosed with or suspected of having a cancer selected from the group consisting of: bladder cancer, breast cancer, colon cancer, fibrosarcoma, lung cancer, metastatic melanoma, pancreatic cancer, prostate cancer, ovarian cancer, renal cell

Samsung Bioepis Exhibit No. 1002
Page 2209

carcinoma and spleen cancer, with the method comprising: administering intravenously to the patient a therapeutically effective dose of the liquid formulation comprising between about 1.0 mg/mL and 15 mg/mL of an anti-α5β1 antibody, about 22-27 mM citrate, about 145-165 mM sodium chloride, about 0.04% to 0.06% polysorbate (TWEEN®) 80, at a pH of about 5.5 to 7.5. In one embodiment of the treatment methods, the therapeutically effective dose administered is about 10 mg/kg. In a preferred embodiment, the patient treated with the pharmaceutical composition has been diagnosed with or is suspected of having renal cell carcinoma or metastatic melanoma, and the therapeutically effective dose is about 10 mg/kg.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts: (A) IIA1 V_H nucleic acid sequence (SEQ ID NO: 1) and amino acid sequence (SEQ ID NO: 2); (B) IIA1 V_L nucleic acid sequence (SEQ ID NO: 3) and amino acid sequence (SEQ ID NO: 4).

Figure 2 depicts: (A) M200 V_H nucleic acid sequence (SEQ ID NO: 5) and amino acid sequence (SEQ ID NO: 6); (B) M200 V_L nucleic acid sequence (SEQ ID NO: 7) and amino acid sequence (SEQ ID NO: 8).

DETAILED DESCRIPTION OF THE INVENTION

For clarity and understanding, the following disclosure describes the invention in detail by way of illustrative embodiments and exemplifications. The embodiments and examples included in this disclosure are not intended to limit the scope of the invention. It will be readily apparent to one of ordinary skill in the art that equivalent materials and/or methods may be employed, and/or obvious changes, variations or modifications may be made in any of the disclosed embodiments and examples without departing from the scope of the appended claims.

All publications and patent applications cited in this disclosure are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Unless defined otherwise, all terms used herein have the common ordinary meaning ascribed to them by a person of ordinary skill in the art to which this invention belongs.

Samsung Bioepis Exhibit No. 1002
Page 2210

Overview

The present invention is based on the surprising discovery that α5β1 integrin is expressed on the surface of tumor epithelial cells for many types of cancer. Further, it has been found that targeted antibody binding to this surface α5β1 results in direct killing of these cancer cells. Because this method of attacking and killing cancer cells is direct, it is amenable to very early-stage treatment (i.e., prior to substantial tumor formation). Furthermore, the direct cancer cell killing method of the present invention may be particularly useful for treating cancers that express α5β1 on the cell surface but have not proven susceptible to anti-angiogenic approaches. Cancers in this category include, but are not limited to, bladder cancer, breast cancer, renal cancer, pancreatic cancer, lung cancer, prostate cancer, ovarian cancer, and metastatic melanoma.

The present invention provides methods of killing or otherwise preventing the proliferation of cancer cells using anti-α5β1 antibodies. In its most general embodiment, the method comprises contacting a cancer cell that expresses α5β1 on its surface with an anti-α5β1 antibody, and thereby inducing the death (e.g., via apoptosis) of the cancer cell.

The methods of the invention may be employed to kill cancer cells *in vivo* (e.g., in a patient) and thereby prevent or attenuate tumor formation and growth. In addition, the method may be used to treat previously formed tumors, and may be employed together with other cancer therapies (e.g., chemotherapeutic agents or other molecular-based cancer therapeutic agents). For example, a patient suffering from the growth of a cancerous tumor may be treated with a formulation of the antibody M200 in tandem with a chemotherapeutic compound such as doxorubicin. Because M200 is a chimeric antibody with relatively low toxicity in humans, this combination treatment may provide comparable cancer cell killing power without the toxic side effects associated with a higher dosage of the chemotherapeutic alone.

The present invention provides a method in which anti-α5β1 antibodies kill and/or inhibit the proliferation of cancer cells directly, even in the absence of any tumor vasculature that may be susceptible to the anti-angiogenic effect of these antibodies. Therefore, the method is particularly well-suited for the prophylaxis or therapeutic treatment of cancers that express α5β1 but do not form heavily vascularized tumors, and/or are not otherwise susceptible to anti-angiogenesis therapeutics e.g., pancreatic cancer, renal cancer, metastatic melanoma, lung cancer and breast cancer.

Samsung Bioepis Exhibit No. 1002
Page 2211

Furthermore, because of the direct cancer cell killing ability of M200 (and other anti-α5β1 antibodies disclosed herein) it is possible to use these antibodies in early stage cancer therapy prior to the formation of vascularized tumors. An early-stage treatment method is particularly significant in view of the advent of new, more sensitive diagnostic tests for cancers that have been developed with the genetic marker information mined from the human genome sequence. It is likely that many common cancers will be detected and diagnosed at a very early stage, i.e., a pretumor stage wherein cancer cells may be present in tissue and/or circulating but have not established a tumor structure detectable by less sensitive non-genetic diagnostics. In such an early stage diagnosis scenario, anti-angiogenesis therapies may have little or no effect when a tumor vasculature has not yet been established and common chemotherapeutics may create too many toxic side-effects to warrant their use. Because the method of the present invention results in antibody-targeted direct killing of cancer cells that express α5β1 integrin on their surface it is particularly well-suited for early-stage preventative treatment.

Those subjects most likely to benefit from the early-stage method of treatment would include, but are not limited to: 1) a subject who has had pretumor tests indicating a high probability of the development and/or presence of tumors (or microtumors); 2) a subject exposed to a very potent carcinogenic environment whose probability of tumor progression is high; and, 3) a subject whose has a high genetic predisposition to develop a cancer wherein the cancer cells express α5β1 on their surface.

Anti-α5β1 Antibodies

The methods of the present invention employ anti-α5β1 antibodies as direct cancer cell killing agents. As used herein, the term "antibody" refers to an immunoglobulin molecule that specifically binds to, or is immunologically reactive with a particular antigen, and includes polyclonal, monoclonal, genetically engineered and otherwise modified forms of antibodies, including but not limited to chimeric antibodies, humanized antibodies, heteroconjugate antibodies (*e.g.*, bispecific antibodies, diabodies, triabodies, and tetrabodies), and antigen binding fragments of antibodies, including e.g., Fab', $F(ab')_2$, Fab, Fv, rIgG, and scFv fragments. The term "scFv" refers to a single chain Fv antibody in which the variable domains of the heavy chain and the light chain from a traditional antibody have been joined to form one chain. In addition, the term "antibody," as used in the context of the invention disclosed herein encompasses mixtures of more

Samsung Bioepis Exhibit No. 1002
Page 2212

than one antibody reactive with a specific antigen (e.g., a cocktail of different types of monoclonal antibodies reactive with α5β1 integrin).

Preferably, the anti-α5β1 antibodies used in the methods of the present invention are monoclonal antibodies. Monoclonal antibodies useful with the methods present invention may be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow and Lane, "Antibodies: A Laboratory Manual," Cold Spring Harbor Laboratory Press, New York (1988); Hammerling et al., in: "Monoclonal Antibodies and T-Cell Hybridomas," Elsevier, New York (1981), pp. 563-681 (both of which are incorporated herein by reference in their entireties). Production of antibodies by selection of libraries of recombinant antibodies in phage or similar vectors, *see, e.g.,* Huse *et al., Science* 246:1275-1281 (1989); Ward *et al., Nature* 341:544-546 (1989); and Vaughan *et al., Nature Biotech.* 14:309-314 (1996), or by immunizing an animal with the antigen or with DNA encoding the antigen.

In preferred embodiments, the direct cancer cell killing methods of the present invention may be carried out using the previously characterized anti-α5β1 antibodies, IIA1, M200, or F200. IIA1 is the parent mouse IgG1 class antibody that has been shown to inhibit α5β1 integrin binding to fibronectin (see, e.g., U.S. Publ. Pat. Appl. US 2002/0172675 A1, filed May 7, 1999, which is hereby incorporated by reference herein). M200 is a chimeric IgG4 antibody derived from IIA1. F200 is a Fab fragment derived from M200. These antibodies have been generated, functionally characterized and their specific amino acid sequences disclosed in U.S. Pat. Appl. Ser. Nos. 10/724,274, filed Nov. 26, 2003, and 10/830,956, filed April 23, 2004, each of which is hereby incorporated by reference herein. Both M200 and F200 have been shown to exhibit *in vivo* anti-angiogenic efficacy in monkey eye and rabbit eye models (see, U.S. Pat. Appl. Ser. Nos. 10/724,274, filed Nov. 26, 2003, and 10/830,956, filed April 23, 2004).

Antibodies useful with the method of the present invention also include those that specifically bind to the same epitope on α5β1 as IIA1, M200, and F200. An "epitope" (or "antigenic determinant") refers to a site on an antigen to which an antibody binds. Epitopes may be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. For example, the epitope on α5β1 integrin

Samsung Bioepis Exhibit No. 1002
Page 2213

may comprise amino acids on each of the α and β polypeptide chains that make up the heterodimer structure. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5 or 6-10 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., Epitope Mapping Protocols in Methods in Molecular Biology, Vol. 66, Glenn E. Morris, Ed (1996).

Two antibodies are said to bind to the same epitope of a protein if amino acid mutations in the protein that reduce or eliminate binding of one antibody also reduce or eliminate binding of the other antibody. Also, it may be concluded that two antibodies bind the same epitope if the two antibodies compete for binding to the protein, i.e., binding of one antibody to the protein competitively inhibits, reduces or eliminates binding of the other antibody. Consequently, the methods of the present invention may be carried out with an antibody that has been determined to competitively inhibit the binding of IIA1, M200 (volociximab), or F200 to α5β1 integrin expressed on the cancer cell surface.

The anti-α5β1 antibodies useful with the methods of the present invention are not limited to IIA1, M200 and F200, but may include antibodies comprising a variable region, framework region, or CDR amino acid sequence substantially identical to those of IIA1, M200 and F200. The extent of the variable regions, framework regions and CDRs are well-known to those of ordinary skill in the art (see e.g., Kabat, et al., "Sequences of Proteins of Immunological Interest", 5th ed., National Institutes of Health, Bethesda, MD (1991)). As used herein, the variable region of the heavy chain ("V_H" or "VH") or light chain ("V_L" or a "VL") of an antibody, includes the heavy or light chains of an antigen binding fragment, e.g., Fv, scFv, dsFv or Fab. Antibody light and heavy chain variable regions also contain four "framework" regions interrupted by three hypervariable regions, also called "complementarity-determining regions" or "CDRs." The CDRs are primarily responsible for binding to an epitope of an antigen.

A "substantially identical" variable, constant, framework region, or CDR refers to an antibody region wherein at least about 85-90%, and preferably at least 95% of the amino acid sequence is identical to a natural or unaltered antibody variable or constant

Samsung Bioepis Exhibit No. 1002
Page 2214

region. The terms "identical" or percent "identity," in the context of two or more amino acid or nucleotide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (i.e., about 60% identity, preferably 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity over a specified region, when compared and aligned for maximum correspondence over a comparison window or designated region) as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual alignment and visual inspection (see, e.g., description of BLAST at the National Center for Biotechnology Information (NCBI) web site located at www.ncbi.nlm.nih.gov).

Identical or substantially identical sequences include sequences having deletions and/or additions, as well as those that have substitutions, as well as naturally occurring, e.g., polymorphic or allelic variants, and man-made variants such as conservatively modified variants. The well-known algorithms for measuring sequence identity can account for gaps and the like. Preferably, sequence identity exists over a region that is at least about 25 amino acids or nucleotides in length, or more preferably over a region that is 50-100 amino acids or nucleotides in length.

The amino acid sequences of the anti-$\alpha 5\beta 1$ antibodies useful with the methods of the present invention are not confined to the sequences found in natural antibodies; antibodies can be redesigned to obtain desired characteristics using well-known recombinant DNA techniques. Such "genetically altered antibodies" include those where the amino acid sequence has been varied from that of a parent (i.e., unaltered) antibody. The possible variations range from the changing of just one or a few amino acids to the complete redesign of, for example, the variable or constant region. Changes, by site-directed mutation, in the constant region may be made in order to improve or alter the functional characteristics of a therapeutic antibody such as immunogenicity, pharmacokinetic characteristics (e.g., serum half-life), complement fixation, interaction with membranes and other effector functions. Generally, changes to the antibody variable region may be made in order to improve the antigen binding characteristics.

In one preferred embodiment, the chimeric antibody, M200, may be employed in the direct cancer cell killing methods of the present invention. The term "chimeric antibody" refers to an immunoglobulin molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable

Samsung Bioepis Exhibit No. 1002
Page 2215

region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, e.g., an enzyme, toxin, hormone, growth factor, drug, etc.; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity. Methods for producing chimeric antibodies are well-known to those of ordinary skill in the art. See e.g., Morrison *et al.*, Science 229:1202-1207 (1985); Oi *et al.*, BioTechniques 4:214-221 (1986); Gillies et al., J. Immunol. Methods 125:191-202 (1989); and U.S. Patent Nos. 5,807,715; 4,816,567; and 4,816,397, each of which is hereby incorporated herein by reference in its entirety.

In another preferred embodiment, humanized anti-$\alpha 5\beta 1$ antibodies may be employed in the direct cancer cell killing methods of the present invention. The additional human sequences in humanized antibodies further decrease the possible immunogenicity of the antibody when it is used as a human therapeutic. Humanized versions of IIA1 are disclosed in U.S. Pat. Appl. Ser. Nos. 10/724,274, filed Nov. 26, 2003, and 10/830,956, filed April 23, 2004, each of which is hereby incorporated by reference herein. The term "humanized antibody" refers to an immunoglobulin comprising a human framework, at least one and preferably all CDRs from a non-human antibody, and in which any constant region present is substantially identical to a human immunoglobulin constant region, i.e., at least about 85-90%, and preferably at least 95% identical. Hence, all parts of a humanized immunoglobulin, except the CDRs, are substantially identical to corresponding parts of one or more native human immunoglobulin sequences. Accordingly, such humanized antibodies are chimeric antibodies, wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. Framework residues in the human framework regions may be substituted with the corresponding residue from the CDR donor antibody to alter, preferably improve, antigen binding. These framework substitutions may be identified by methods well known in the art, e.g., by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. See, e.g., Queen et al., U.S. Patent Nos: 5,530,101; 5,585,089; 5,693,761; 5,693,762; 6,180,370 (each of which is incorporated by reference in its entirety). Antibodies may be humanized using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT

Samsung Bioepis Exhibit No. 1002
Page 2216

publication WO 91/09967; U.S. Patent Nos. 5,225,539; 5,530,101 and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596; Padlan, Mol. Immunol., 28:489-498 (1991); Studnicka et al., Prot. Eng. 7:805-814 (1994); Roguska et al., Proc. Natl. Acad. Sci. 91:969-973 (1994), and chain shuffling (U.S. Patent No. 5,565,332), all of which are hereby incorporated by reference in their entireties.

In another embodiment, human antibodies (i.e., antibodies comprising both a human variable and constant region) to $\alpha 5\beta 1$ may be employed for therapeutic treatment of human patients according to the methods of the present invention. Human antibodies can be made or obtained by a variety of methods known in the art including phage display methods described above using antibody libraries derived from human immunoglobulin sequences. See U.S. Patent Nos. 4,444,887 and 4,716,111; and PCT publications WO 98/46645; WO 98/50433; WO 98/24893; WO 98/16654; WO 96/34096; WO 96/33735; and WO 91/10741, each of which is incorporated herein by reference in its entirety. Human antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For an overview of this technology for producing human antibodies, see Lonberg and Huszar, Int. Rev. Immunol. 13:65-93 (1995). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e.g., PCT publications WO 98/24893; WO 92/01047; WO 96/34096; WO 96/33735; European Patent No. 0 598 877; U.S. Patent Nos. 5,413,923; 5,625,126; 5,633,425; 5,569,825; 5,661,016; 5,545,806; 5,814,318; 5,885,793; 5,916,771; and 5,939,598, which are incorporated by reference herein in their entireties. Completely human antibodies that recognize a selected epitope also can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, e.g., a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope (Jespers et al., Biotechnology 12:899-903 (1988).

In an alternative embodiment, primatized antibodies (i.e., an antibody comprising monkey variable regions and human constant regions) may be employed for therapeutic treatment according to the methods of the present invention. Methods for producing primatized antibodies are known in the art. See e.g., U.S. Patent Nos. 5,658,570; 5,681,722; and 5,693,780, which are incorporated herein by reference in their entireties.

Antibody Function

Samsung Bioepis Exhibit No. 1002
Page 2217

The methods of the present invention employ functional antibodies that exhibit specific binding to α5β1 integrin. Avidity testing of antibodies for specific binding to antigen allows one skilled in the art to identify antibodies specifically recognizing one or more epitopes of α5β1 integrin. Antibodies are defined to be specifically binding if: 1) they exhibit a threshold level of binding activity; and/or 2) they do not significantly cross-react with related polypeptide molecules.

First, anti-α5β1 antibodies useful for the methods disclosed herein specifically bind (or "specifically react" or "specifically immunoreact") if they bind to a α5β1 integrin polypeptide, peptide or epitope with a binding affinity (K_a) of 10^6 mol^{-1} or greater, preferably 10^7 mol^{-1} or greater, more preferably 10^8 mol^{-1} or greater, and most preferably 10^9 mol^{-1} or greater. The binding affinity of an antibody can be readily determined by one of ordinary skill in the art, for example, by Scatchard analysis (Scatchard, Ann. NY Acad. Sci. 51: 660-72, 1949), or by surface plasmon resonance using BIAcore. A variety of antibody binding assays useful for characterizing anti-α5β1 antibodies are disclosed in U.S. Pat. Appl. Ser. Nos. 10/724,274, filed Nov. 26, 2003, and 10/830,956, filed April 23, 2004, each of which is hereby incorporated by reference herein.

Second, antibodies specifically bind if they do not significantly cross-react with related polypeptides. Antibodies do not significantly cross-react with related polypeptide molecules, for example, if they detect α5β1 integrin polypeptide but not known related polypeptides using a standard Western blot analysis (see, e.g., "Current Protocols in Molecular Biology," eds. Ausubel et al., 1995). Examples of known related polypeptides include orthologs, proteins from the same species that are members of the integrin family of proteins, or mutant α5β1 integrin polypeptides where the mutation alters the anti-α5β1 epitope. Moreover, antibodies may be "screened against" known related polypeptides to isolate a population that specifically binds to the α5β1 integrin. For example, antibodies raised to human α5β1 integrin polypeptides are adsorbed to related polypeptides adhered to insoluble matrix; antibodies specific to human α5β1 integrin polypeptides will flow through the matrix under the proper buffer conditions. Such screening allows isolation of polyclonal and monoclonal antibodies non-crossreactive to closely related polypeptides (Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988; Current Protocols in Immunology, Cooligan, et al. (eds.), National Institutes of Health, John Wiley and Sons, Inc., 1995). Screening and isolation

Samsung Bioepis Exhibit No. 1002
Page 2218

of specific antibodies is well known in the art (see, Fundamental Immunology, Paul (eds.), Raven Press, 1993; Getzoff et al., Adv. in Immunol. 43: 1-98, 1988; Monoclonal Antibodies: Principles and Practice, Goding, J. W. (eds.), Academic Press Ltd., 1996; Benjamin et al., Ann. Rev. Immunol. 2: 67-101, 1984). Representative examples of such assays include: concurrent immunoelectrophoresis, radioimmuno-assay, radioimmuno-precipitation, enzyme-linked immuno-sorbent assay (ELISA), dot blot or Western blot assay, inhibition or competition assay, and sandwich assay. For a review of immunological and immunoassay procedures, see *Basic and Clinical Immunology* (Stites & Terr eds., 7th ed. 1991).

Specific binding anti-α5β1 antibodies useful with the methods of the present invention may be prepared through a process of selection for α5β1 binding. Generally, polyclonal antibodies raised to specifically bind to a particular protein, or its polymorphic variants, alleles, orthologs, conservatively modified variants, splice variants, may be selected to obtain only those antibodies that are specifically immunoreactive with the selected protein (e.g., α5β1 integrin) and not with other proteins. Specific binding selection is achieved by subtracting out antibodies that cross-react with other molecules. A variety of immunoassay formats may be used to select antibodies specifically reactive with the α5β1 integrin polypeptide. For example, solid-phase ELISA immunoassays may be used to select antibodies specifically reactive with protein (see, e.g., Harlow & Lane, Antibodies, A Laboratory Manual (1988) for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity).

Antibody Drug Conjugates

In some embodiments, the method of killing cancer cells may employ an anti-α5β1 antibody conjugated to an effector moiety. The effector moiety may be any number of molecules, including labeling moieties such as radioactive labels or fluorescent labels, or preferably may be a therapeutic moiety. The effector moiety (or "effector component") may be bound (or linked, or conjugated), to the anti-5β1 antibody either covalently, through a linker or a chemical bond, or noncovalently, through ionic, van der Waals, electrostatic, or hydrogen bonds.

In one aspect the therapeutic moiety is a small molecule that modulates the activity of the α5β1 integrin. In another aspect, the therapeutic moiety affects the activity of molecules or cells associated with or in close proximity to the α5β1 integrin. For

Samsung Bioepis Exhibit No. 1002
Page 2219

example, the therapeutic moiety may be a cytotoxic agent. The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g., I^{131}, I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof. Suitable toxins and their corresponding fragments include diphtheria A chain, exotoxin A chain, ricin A chain, abrin A chain, curcin, crotin, phenomycin, enomycin, auristatins (e.g., auristatin E, or auristatin F), and the like. Targeting the therapeutic moiety to the α5β1 integrin expressed on the surface of a cancer cell not only serves to increase the local concentration of therapeutic moiety in the cancer afflicted area, but also serves to reduce deleterious side effects that may be associated with the therapeutic moiety.

Examples of chemotherapeutic agents that may used as therapeutic moieties with the anti-α5β1 antibodies of the present invention include, but are not limited to, adriamycin, doxorubicin, doxil, epirubicin, 5-fluorouracil, cytosine arabinoside ("Ara-C"), cyclophosphamide, thiotepa, busulfan, cytoxin, taxoids, e.g., paclitaxel (TAXOL™, Bristol-Myers Squibb Oncology, Princeton, N.J.), and doxetaxel (TAXOTERE™, Rhone-Poulenc Rorer), toxotere, methotrexate, cisplatin, melphalan, vinblastine, bleomycin, etoposide, ifosfamide, mitomycin C, mitoxantrone, vincristine, vinorelbine, carboplatin, teniposide, daunomycin, carminomycin, aminopterin, dactinomycin, mitomycins, esperamicins (see U.S. Pat. No. 4,675,187), 5-FU, 6-thioguanine, 6-mercaptopurine, actinomycin D, VP-16, chlorambucil, melphalan, and other related nitrogen mustards. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors such as tamoxifen and onapristone.

Alternatively, the method of the invention may be carried out wherein the chemotherapeutic agents disclosed above are administered in a formulation together with the anti-α5β1 antibody although not as a conjugate.

Cancer Indications

The present invention is directed to methods of killing cancer cells, inhibiting cancer cell proliferation and/or metastasis, by targeting α5β1 integrin on the cancer cell's surface with an antibody. Cancer is a physiological condition typically characterized by cells undergoing unregulated growth. Cancer includes all malignant neoplasms including but not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More

Samsung Bioepis Exhibit No. 1002
Page 2220

particular examples of cancers that express α5β1 on the cell surface that may be targeted using the methods disclosed herein include, but are not limited to, bladder cancer, breast cancer, colon cancer, fibrosarcoma, lung cancer, metastatic melanoma, pancreatic cancer, prostate cancer, ovarian cancer, renal cell carcinoma and spleen cancer.

A tumor is a proliferating mass of cells lacking normal growth controls. A tumor may benign or malignant, and may include pre-cancerous, or cancerous, cells and tissues. Cancer cells typically form tumors as a cancer progresses. Tumor growth is accompanied by an increase in vascular density. This tumor neovasculature provides required nourishment that allows tumors to grow. The anti-angiogenic antibody therapeutics anti-α5β1 and anti-VEGF (VEGF = vascular endothelial growth factor) have demonstrated efficacy in impeding tumor growth in a variety of cancer models (see, e.g., Kim *et al.*, *J Clin Invest.*, 110(7):933-41 (2002); Ferrara, *Nat Rev Cancer*, 2(10):795-803 (2002)). The anti-α5β1 antibody, M200, has been shown to inhibit angiogenesis *in* vitro and *in vivo* in rabbit and monkey models of angiogenic ocular diseases (e.g., advance macular degeneration; see, U.S. Pat. Appl. Ser. Nos. 10/724,274, filed Nov. 26, 2003, and 10/830,956, filed April 23, 2004, each of which is hereby incorporated by reference herein).

The present invention is based on the surprising discovery that for many types of cancer α5β1 integrin is expressed on the surface of tumor epithelial cells (in addition to the endothelial cells of the tumor vasculature), and that targeted binding to this surface α5β1 with an antibody results in direct killing of these cancer cells. Consequently, cancers characterized by proliferating epithelial cells expressing α5β1 may be treated and/or targeted with anti-α5β1 antibodies even absent any tumor vasculature formation. Because this method of attacking and killing cancer cells is direct, it is amenable to very early stage treatment (i.e., prior to substantial tumor formation).

Patients most likely to benefit from the early-stage method of treatment would include, but are not limited to: 1) a patient who has had a pretumor diagnostic tests indicating a high probability of the development and/or presence of tumors (or microtumors); 2) a patient exposed to a very potent carcinogenic environment whose probability of tumor progression is high; and, 3) a patient whose genetic predisposition (e.g., as evidenced by a genetic marker for a cancer) makes the likelihood of cancer development high wherein the cancer cells express α5β1 on their surface. For example, a patient with a genetic predisposition for breast cancer (e.g., BRCA gene positive), or in

Samsung Bioepis Exhibit No. 1002
Page 2221

whom some pretumor cancer marker has been detected (e.g., micro-metastases detected by PCR), will be particularly well-suited for the early-stage preventative direct cancer-cell killing method wherein a pharmaceutical composition of an anti-α5β1 antibody is administered.

Genes indicating an increased cancer predisposition, and tumor markers indicating a likelihood of developing a tumor may be found in the cancer biomedical literature and databases. In addition, lists of carcinogens and their exposure levels that greatly increase cancer risk are available in the biomedical literature well-known to those of ordinary skill in the art. One of ordinary skill may use these literature resources together with the well-known methods for detecting α5β1 integrin expression on cancer cells, disclosed below, to determine whether a subject may be amenable to the early-stage cancer treatment methods of the present invention. As described in Example 2 below, flow cytometry analysis reveals α5β1 integrin is expressed on the surface of cell lines originating from at least the following cancers: bladder cancer, breast cancer, colon cancer, fibrosarcoma, lung cancer, metastatic melanoma, pancreatic cancer, prostate cancer, ovarian cancer, and renal cell carcinoma.

Furthermore, the direct cancer cell killing method of the present invention may be particularly useful for treating cancers that express α5β1 but have not proven susceptible to anti-angiogenic approaches. Cancers in this category include, but are not limited to, bladder cancer, breast cancer, fibrosarcoma, renal cancer, pancreatic cancer, prostate cancer, ovarian cancer, lung cancer, and metastatic melanoma. In a particularly preferred embodiment, the methods of the present invention may be used to treat any of the above listed cancers wherein the patient exhibits refractory solid tumors. The anti-α5β1 antibody, M200 (volociximab) has demonstrated some efficacy for treating human patients with a variety of cancers exhibiting refractory solid tumors, including: colorectal cancer (CRC), melanoma (MEL), renal cell carcinoma (RCC), hepatocellular cancer (HCC), lung cancer (NSCLC), pancreatic cancer (PC), parotid cancer (PARO) and breast cancer (BC).

One of ordinary skill may determine those cancers that express α5β1 on the surface of tumor epithelial cells by using an anti-α5β1 antibody (e.g., IIA1 or M200) to probe tumor biopsy samples according to standard methods of immunohistochemistry. As described in Example 1 below, immunohistochemical (IHC) analysis of tumor biopsy samples taken from melanoma, lung, renal, pancreatic and breast cancer patients all

Samsung Bioepis Exhibit No. 1002
Page 2222

showed α5β1 integrin expression on tumor epithelial cells. It is reasonable to expect that there other cancers will be found to express α5β1 on the surface of tumor epithelial cells using IHC (or other methods), and it is recognized that these cancers will be susceptible to the cancer cell kill method of the present invention.

In addition to IHC of tumor samples, cancer cell lines may be screened for α5β1 cell surface expression using an anti-α5β1 antibody and standard flow cytometry techniques well-known to those of ordinary skill in the art. As detailed in Example 2, below, flow cytometry screening revealed α5β1 expression on the surface of 21 well-known cancer cell lines. Based on this result, these cell lines are susceptible to direct cell killing using anti-α5β1 antibodies according to the methods of the invention. Furthermore, to the extent a cancer cell line is determined to express α5β1 on its surface, and the cell-line corresponds to, originated from, or is derived from, cancer cells present in a cancer patient, the methods of the present invention may be used to treat such a patient. For example, the NW231 cell line, which was found in Example 2 to express α5β1 integrin on its surface, originated from breast cancer tumors. Consequently, one of ordinary skill would immediately recognize that an anti-α5β1 antibody may be used to treat a breast cancer patient in accordance with the methods taught herein.

Compositions, Formulations and Administration of Antibodies

The anti-α5β1 antibodies useful in the methods of the present invention may be used in an isolated and purified form and directly contacted with cancers cells or tumors. Methods of purifying anti-α5β1 antibodies (e.g., IIA1, M200, and F200) are disclosed in U.S. Pat. Appl. Ser. Nos. 10/724,274, filed Nov. 26, 2003, and 10/830,956, filed April 23, 2004, each of which is hereby incorporated by reference herein. F200 may also be prepared as a Fab'-NAC fragment according to the methods disclosed in U.S. Pat. Appl. 60/583,127, filed June 25, 2004, which is hereby incorporated by reference herein. The F200-Fab'-NAC exhibits increased stability in liquid and lyophilized formulations of the antibody. Purity and homogeneity may be determined using standard analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. An antibody that is the predominant species present in a preparation is considered to be substantially purified. For example, antibody solution that exhibits essentially one band in an electrophoretic gel is substantially purified.

Samsung Bioepis Exhibit No. 1002
Page 2223

Preferably, the antibody used in the pharmaceutical compositions of the invention is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure.

In preferred embodiments, the direct cancer cell killing method is carried out wherein the purified anti-α5β1 antibodies are formulated into a pharmaceutical composition that is administered to a subject in a therapeutically effective amount. As used herein, "therapeutically effective amount" refers to the amount of a pharmaceutical formulation or composition that is sufficient to cure, alleviate, attenuate or at least partially arrest the cancer and/or its symptoms, and/or complications. Clinical methods for determining the therapeutically effective amount of an anti-α5β1 antibody for treatment of cancer are well-known to those of ordinary skill in the art and may be determined empirically using routine experimentation. For example, in the context of cancer treatment, a "therapeutically effective amount" is an amount capable of invoking one or more of the following effects: (1) inhibition, to some extent, of tumor growth, including, slowing down and complete growth arrest; (2) reduction in the number of cancer cells; (3) reduction in tumor size; (4) inhibition (i.e., reduction, slowing down or complete stopping) of cancer cell infiltration into peripheral organs; (5) inhibition (i.e., reduction, slowing down or complete stopping) of cancer cell metastasis; (6) enhancement of anti-cancer immune response, which may, but does not have to, result in the regression or rejection of a tumor; and/or (7) relief, to some extent, of one or more symptoms associated with the disorder.

The pharmaceutical compositions for administration will commonly comprise an anti-α5β1 antibody dissolved in a pharmaceutically acceptable carrier or excipient, preferably an aqueous carrier. Acceptable carriers, excipients, or stabilizers, for the pharmaceutical composition are those which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids, antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming, counter-ions such as sodium; and/or nonionic surfactants such as TWEEN®, polyethylene glycol (PEG), and

Samsung Bioepis Exhibit No. 1002
Page 2224

PLURONICS™. A variety of aqueous carriers can be used, e.g., buffered saline and the like. These solutions should sterile and generally free of undesirable matter. The pharmaceutical compositions may be sterilized by conventional, well known sterilization techniques.

The pharmaceutical compositions also may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, e.g., sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and other pharmaceutically acceptable salts, which is meant to include both acid and base addition salts. The pharmaceutical compositions may also include one or more of the following: carrier proteins such as serum albumin; buffers; fillers such as microcrystalline cellulose, lactose, corn and other starches; binding agents; sweeteners and other flavoring agents; coloring agents; and polyethylene glycol. The concentration of antibody in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs (e.g., "Remington's Pharmaceutical Science" (15th ed., 1980) and Goodman & Gillman, "The Pharmacologial Basis of Therapeutics" (Hardman et al., eds., 1996)).

In a preferred embodiment of the methods of the present invention, the anti-α5β1 antibody is formulated as a pharmaceutical composition comprising a solution of between about 1.0 mg/mL and 15.0 mg/mL antibody, about 22 mM to 28 mM citrate, about 135 mM to 165 mM Sodium Chloride, 0.04%-0.06% Polysorbate (TWEEN®) 80, at a pH of 5.5 to 7.5. Preferably, the pH range of the liquid formulation is between about pH 6.0 and pH 7.0, and most preferably between about pH 6.3 and pH 6.7. In a particularly preferred embodiment the pharmaceutical composition comprises a solution of about 10.0 mg/mL antibody, about 25 mM citrate, about 150 mM sodium chloride, about 0.05% polysorbate (TWEEN®) 80, at a pH of about 6.5. In other embodiments, the above anti-α5β1 antibody pharmaceutical composition may further comprise a chemotherapeutic agent, or alternatively, may be administered to a patient together with a pharmaceutically effective amount of another chemotherapeutic agent.

Preferably the liquid formulation of the pharmaceutical composition is a stable, colorless, or clear to slightly opalescent solution exhibiting no more than 10%, and preferably 5% or less of degaded antibody monomer as measured by SEC-HPLC.

Samsung Bioepis Exhibit No. 1002
Page 2225

Preferably, no more than 10%, and preferably 5% or less of hydrolysis clipping is observed, and no more than 10%, and preferably 5% or less of antibody aggregation is formed. Preferably, the concentration, pH and osmolality of the formulation have no more than ± 10% change. Potency is within 70-130%, preferably 80-120% of the control.

The administration of the pharmaceutical compositions comprising anti-α5β1 antibodies to a subject may be carried in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intranasally, topically, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, intraocularly, intraventricularly, or intrathecally. It is well recognized that antibodies when administered orally, should be protected from digestion. This is typically accomplished either by complexing the molecules with a composition to render them resistant to acidic and enzymatic hydrolysis, or by packaging the molecules in an appropriately resistant carrier, such as a liposome or a protection barrier. Means of protecting agents from digestion are well known in the art.

The pharmaceutical compositions may be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable for oral administration include, but are not limited to, powder, tablets, pills, capsules and lozenges.

The exact dosage to be used in a particular embodiment of the method of the invention will depend on the purpose of the treatment, and may be ascertained by one skilled in the art using well-known techniques (e.g., Ansel et al., "Pharmaceutical Dosage Forms and Drug Delivery;" Lieberman, "Pharmaceutical Dosage Forms" (vols. 1-3, 1992), Dekker, ISBN 0824770846, 082476918X, 0824712692, 0824716981; Lloyd, "The Art, Science and Technology of Pharmaceutical Compounding" (1999); and Pickar, "Dosage Calculations" (1999)). As is known in the art, adjustments for cancer degradation, systemic versus localized delivery, and rate of new protease synthesis, as well as the age, body weight, general health, sex, diet, time of administration, drug interaction and the severity of the condition may be necessary, and will be ascertainable with routine experimentation by those skilled in the art.

In one embodiment of the methods of the present invention, a pharmaceutical compositions comprising an anti-α5β1 antibody is administered to a patient based on the weight of antibody (in mg) per patient body weight (in kg). Thus, preferred dose levels include at least about 0.5 mg/kg, 1.0 mg/kg, 2.5 mg/kg, 5.0 mg/kg, 10.0 mg/kg, and 15

Samsung Bioepis Exhibit No. 1002
Page 2226

mg/kg. Preferably, the dose is administered to the patient as an intravenous infusion over 1 hour. Additional doses may be administered over an extended time period such that a steady state serum concentration is established in the patient. For example, an infusion of 10 mg/kg may be administered once a week over the course of a year.

In one preferred embodiment, the anti-α5β1 antibody dosing level and schedule are selected to ensure that the dose produces a maximum serum concentration below the safe mean peak serum concentrations seen in pharmacokinetic studies carried out in monkeys (e.g., cynomolgus). For example, in cynomolgus monkeys, the mean peak level of M200 at a dose of 50 mg/kg after 4 weekly doses was 1862 μg/mL (range: 1000-2606 μg/mL). The monkeys showed no toxicity at these serum concentrations. In addition, or alternatively, a dose may be selected so that the trough serum level is > 1 μg/mL, the concentration that produces 80% inhibition of binding of α5β1 to fibronectin in an *in vitro* activity assay.

In accordance with one embodiment of the methods of therapeutic cancer cell killing of the present invention, a pharmaceutical composition comprising an anti-α5β1 antibody is administered to a patient intravenously at a fixed dosage, typically about 0.1 to 10 mg per patient per day. In embodiments where the pharmaceutical composition is administered to a secluded site, such as into a body cavity or into a lumen of an organ, and not into the blood stream, fixed dosages from 0.1 mg up to about 100 mg per patient per day may be used. Substantially higher dosages are possible for embodiments where topical administration is desired. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art, e.g., "Remington's Pharmaceutical Science," and Goodman and Gillman, "The Pharmacologial Basis of Therapeutics," *supra*.

The pharmaceutical compositions employed in the cancer cell killing method of the invention may be administered as part of a therapeutic or prophylactic treatment. In a therapeutic method, the pharmaceutical composition is administered to a patient already suffering from a cancer in an amount sufficient to cure, or at least partially arrest the progress of the disease and its complications. Generally, in a therapeutic treatment context, the progress of the therapy may be measured as decrease in tumor size or a decrease in the rate of tumor growth. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the cancer and the general state of the patient's health. Single or multiple

Samsung Bioepis Exhibit No. 1002
Page 2227

administrations of the pharmaceutical compositions may be employed depending on the dosage and frequency tolerated by the patient.

A early-stage treatment method is directed to preventing or slowing the development of cancer in a subject that is suspected of developing the disease, or in the very early stage of the disease. The particular dose required for an early-stage treatment will depend upon the medical condition and history of the patient, the particular cancer being prevented, as well as other factors such as age, weight, gender, administration route, efficiency, etc. An early-stage treatment method also may be used prophylactically, e.g., in a patient who has previously had cancer to prevent a recurrence of the cancer, or in a patient who is suspected of having a significant likelihood of developing cancer. For example, a patient with a genetic predisposition for breast cancer, in whom some pre-tumorous cancer marker has been detected (e.g., micro-metastases detected by PCR), will be particularly well-suited for the early-stage treatment method.

In an alternative embodiment of the present invention, the direct cancer cell killing method may be carried out wherein a chemotherapeutic agent is administered in addition to the anti-$\alpha5\beta1$ antibody. Typical chemotherapeutic agents useful in this embodiment are disclosed *supra*. This combination therapy method may be particularly preferred in an early-stage, or prophylactic treatment context where the patient lacks fully developed disease symptoms. At this early stage, or in a preventative context, many patients may not agree to undergo the toxic side effects that accompany the use of a standard chemotherapy agent alone. By administering a lower dosage of the standard chemotherapeutic together with a relatively non-toxic anti-$\alpha5\beta1$ antibody, the cancer may be treated prophylactically, or at a very early-stage, with a regimen that is effective yet much more tolerable for the patient.

The following examples are intended to illustrate but not limit the invention disclosed herein.

Examples

Example 1: $\alpha5\beta1$ expression on tumor samples detected by IHC

Tumor biopsy samples taken from melanoma, lung, renal, pancreatic and breast cancer patients were surveyed for $\alpha5\beta1$ integrin expression by immunohistochemistry (IHC).

Samsung Bioepis Exhibit No. 1002
Page 2228

Materials and Methods

Frozen tissue samples (obtained from the Mayo Clinic or Cleveland Clinic), were frozen in optimal cutting temperature (OCT) compound and stored at –70°C. Cryostat tissue sections (7 μm) were fixed in 75% acetone/25% ethanol for 1 minute. Samples were incubated with either the anti-α5β1 mouse antibody IIA1 (5 μg/ml) or control mouse IgG1 (anti-trinitrophenyl, ATCC hybridoma clone 1B7.11) for 30 minutes. Antibody binding was detected using the biotinylated secondary antibody, goat-anti-mouse IgG (3 μg/ml, 30 minutes; Jackson ImmunoResearch), and developed using the Vectastain Elite ABC Kit (Vector Laboratories) and stable DAB (diaminobenzidine and H_2O_2; Research Genetics). Staining was performed using the DAKO Autostainer at room temperature.

Results

As shown in Table 1, nearly all of the tumor sections analyzed stained positive for α5β1 on the tumor vasculature. Surprisingly, a significant proportion of tumor samples also exhibited positive staining for α5β1 integrin expression on the tumor epithelium itself. These results indicate that an anti-α5β1 antibody will directly target cancer cells in addition to the invading neovasculature.

Table 1: IHC results for tumor samples stained with anti-α5β1 antibody IIA1

Tumor type (# samples)	**Tumor Vasculature**					**Tumor Epithelium**				
	Neg	**1+**	**2+**	**3+**	**4+**	**Neg**	**1+**	**2+**	**3+**	**4+**
Renal (n=39)	2	2	13	18	4	27	8	4	0	0
Pancreatic (n=32)	0	1	17	12	2	15	2	7	7	1
Lung (n=39)	0	1	0	28	1	19	14	3	2	1
Colon (n=21)	2	2	3	8	6	19	0	1	1	0
Melanoma (n=19)	3	0	2	13	1	13	5	1	0	0
Ovarian omental mets (n=4)	0	0	1	3	0	2	2	0	0	0
Bladder (n=8)	0	0	4	3	1	5	2	1	0	0

Samsung Bioepis Exhibit No. 1002
Page 2229

Example 2: α5β1 expression on cancer cell lines detected by flow cytometry

A panel of 24 cancer cell lines was surveyed by flow cytometry for α5β1 integrin expression on the cell surface. As shown in Table 2, these 24 cell lines originated in a variety of different cancers including: bladder, breast, colon, fibrosarcoma, lung, melanoma, pancreas, prostate, ovarian, renal, and spleen.

Materials and Methods

Cells were removed with 5 mM EDTA in Tris-HCl (pH 8.0) and blocked by centrifugation in Hank's balanced salt solution containing 3% heat inactivated FBS, 1% normal goat serum (Sigma) and 1% BSA at 4°C for 5 minutes. Cells were incubated for 30 – 60 minutes at 4°C with the mouse anti-α5β1 antibody, IIA1 (10 μg/ml) in FACS buffer (PBS containing 0.1% BSA). Excess monoclonal antibody was removed by centrifugation and cells were washed two times with FACS buffer prior to resuspending in PE-anti-mouse IgG (H+L) antibody (Southern Biotech, 1:400 dilution) for 30 – 60 minutes at 4°C. After washing cells were re-suspended in FACS buffer containing propidium iodide (1 μg/ml). Mean fluorescence intensity (MFI) was measured on a FACSCalibur (Becton Dickinson). Background MFI was ~5.

Results

As shown in Table 2, significant detectable surface expression of α5β1 integrin was observed for 21 of the 24 cell lines assessed. Three cancer cell lines CHL-1, COLO 357 and C32 exhibited MFI values that were very close to background indicating little or no surface expression of α5β1 integrin. The 21 cell lines expressing α5β1 integrin on their surface should be vulnerable to direct cell killing by an anti-α5β1 antibody. In addition, the cancer from which these cell lines were originated may be susceptible to treatment with an anti-α5β1 antibody therapeutic.

Table 2: Results for FACS analysis of various cancer cell lines with IIA1, and *in vitro* proliferation assay with M200.

Cell Line	Cancer of Origin	α5β1 Surface Expression (MFI)	% Growth Inhibition	
			- Serum	+ Serum
A549	lung	71.41	40	0
H460	lung	87.5	40	0
SW839	renal	65.8	30	0
MIA PACA-2	pancreas	61.9	20	0

Samsung Bioepis Exhibit No. 1002
Page 2230

HCT-116	colon	42.5	15	0
786-0	renal	144.4	10	0
EKVX	lung	36.7	10	0
SW1990	spleen	nd[1]	10	0
SN12C	renal	129.1	5	0
A498	renal	67.1	5	0
A375	melanoma	156.1	5	0
A2058	melanoma	36.2	0	0
CHL-1	melanoma	6.8	0	0
TK-10	renal	36.6	0	0
COLO 357	pancreas	7.8	0	0
HT144	melanoma	102.1	0	0
C8161	melanoma	122	0	0
HT1376	bladder	111.9	0	0
DU145	prostate	87.5	0	0
UACC-62	melanoma	88.5	0	0
HT1080	fibrosarcoma	421.7	0	0
ES-2	ovarian	132.8	0	0
CAPAN-1	pancreas	nd[1]	0	0
CAPAN-2	pancreas	nd[1]	0	0
ASPC-1	pancreas	nd[1]	0	0
C32	melanoma	5.7	0	0
LOX	melanoma	261.6	40[2]	35
NW231	breast	132.2	10[2]	35

[1] nd = not determined
[2] Values for % growth inhibition were determined in a 2-day rather than 4-day assay.

Example 3: M200 inhibition of *in vitro* cancer cell proliferation

A panel of 28 cancer cell lines was assessed for sensitivity to the chimeric anti-α5β1 antibody M200 in a cell proliferation assay in the presence or absence of serum.

Materials and Methods

Cancer cell lines were plated at a density of 2500 cells/well in 96-well plates in IMDM with supplements in the presence or absence of 10% FBS. At the time of plating, cells were challenged with various concentrations M200 or a non-function blocking anti-α5 antibody, VC5. Four days later, cell viability was assessed by the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instructions. All growth studies were done at least 3 times in triplicate.

Samsung Bioepis Exhibit No. 1002
Page 2231

Results

As shown in Table 2, M200 inhibited growth of thirteen cancer cell lines in the absence of serum and two cell lines in the presence of serum. Two of the cell lines, LOX and NW231 were found to be sensitive to M200, under both conditions. Based on these results, the cancers from which these cell lines originate (melanoma and breast) are likely to respond to treatment with M200.

Example 4: *In vivo* inhibition of tumor cell proliferation in NW231 and LOX xenograft models

NW231 and LOX cells were grown as orthotopic xenografts in SCID mice and challenged with the anti-α5β1 antibodies M200 and IIA1 by intraperitoneal injection.

Materials and Methods

Immune compromised mice CB-17 SCID (strain C.B-Igh1/IcrTac-Prkdc) were obtained from Taconic Farms (Germantown, NY). Studies were initiated using female mice between the ages of 6-10 weeks (~20 grams in weight). Animals were pre-dosed with M200, IIA1 or control IgG intraperitoneally at a dose of 10 mg/kg 1 hour prior to NW231 inoculation ($1x10^7$ cells in IMDM) into the mammary fat pad. Dosing continued for 3 weeks at a frequency of 3 times/week at 10 mg/kg. Tumor volume was measured twice weekly by caliper and calculated by $\pi/6$ x length x width x height. Clinical and mortality observations were performed daily according to IACUC regulations.

Results

IIA1, was found to reproducibly inhibit both NW231 and LOX xenograft tumor growth in this model. M200 was not found to reproducibly inhibit tumor growth in these models. Since IIA1 does not recognize mouse α5β1 in the xenograft tumor vasculature, the entire inhibitory effect of IIA1 on tumor growth may be attributed to a direct anti-proliferative effect on the cancer cells of the tumor.

Example 5: IIA1 in combination with DOXIL® prevents tumor establishment in an *in vivo* NW231 xenograft model

The following experiment was conducted in order to determine the efficacy of the anti-α5β1 integrin antibody, IIA1 in combination with the chemotherapeutic agent, DOXIL®, for preventing establishment of human NW231 tumors *in vivo*. DOXIL® is a liposome encapsulated formulation of doxorubicin HCl, a cytotoxic anthracycline

Samsung Bioepis Exhibit No. 1002
Page 2232

antibiotic isolated from *Streptomyces peucetius*. The NW231 cell line originated from breast cancer and is considered a good model for studying treatments for breast cancer.

Materials and Methods

Four to six week old female SCID mice obtained from Taconic Farms and maintained in micro-isolator cages were inoculated in the mammary fat pads with $1x10^7$ NW231 cells. Animals received either TIB control (n=20) or M200 (n=20) at 5 mg/kg for the first injection at the time of tumor cell inoculation. Subsequent treatments were at 0.7 mg/kg per injection. DOXIL® treatment was commenced 5 days post tumor cell inoculation. Chemotherapeutic doses were at 4 mg/kg for the first injection and at 2 mg/kg for subsequent injections. Reagents were delivered by intraperitoneal injection for four doses. Tumor volume was measured twice weekly and clinical and mortality observations were performed daily according to IACUC regulations.

Results

IIA1 treatment exhibited a significant effect in slowing the establishment of the NW231 tumors in mice. At 24 days post-implant of NW-231 tumors, the mean tumor volume of control TIB treated xenografts had increased in an exponential fashion to ~425 mm^3 whereas the IIA1 treated xenografts had increased to a mean volume of ~175 mm^3.

DOXIL® treatment by itself also exhibited a significant effect in preventing tumor establishment. For the DOXIL® alone treated xenografts, mean tumor volume initially increased to only ~25 mm^3 during the first 45 days post-implant, and then increased gradually up to ~275 mm^3 by day 74.

Treatment of mice with the combination therapy of IIA1 and doxil had an even greater inhibitory effect on the rate of tumor establishment compared to either treatment alone. For the IIA1 plus DOXIL® treated xenografts, mean tumor volume remained near zero out to day 54, then increased gradually but only up to ~125 mm^3 by day 74. These results indicate increased efficacy (i.e., a combined tumor inhibitory effect) *in vivo* for the combined treatment of an anti-$\alpha5\beta1$ antibody, IIA1 and a chemotherapeutic agent, DOXIL®.

Example 6: M200 efficacy measured in an VX2 rabbit tumor model

Although M200 does not cross react with mouse or rat $\alpha5\beta1$ integrin, it does recognize $\alpha5\beta1$ integrin found in rabbit. Thus, the rabbit VX2 carcinoma may represent a good model for determining the *in vivo* direct cancer cell killing efficacy of M200. VX2

Samsung Bioepis Exhibit No. 1002
Page 2233

is a widely accepted rabbit model for studies on treatment of primary tumors of various locations (see e.g., Chen JG, et al, Lab Anim. 2004 Jan;38(1):79-84; Purdie, TG et al, Phys Med Biol. 2001 Dec;46(12):3161-75); Geschwind, JH, et al, Cancer Res. 2002 Jul 62(1): 3909-3913).

A. M200 VX2 Pilot Study

An initial Pilot Study was carried out to determine the general parameters for conducting the M200 efficacy study in the rabbit VX2 tumor model.

Tumor inoculation

Rabbits were inoculated on Day 0 with a cell suspension (100 µl) subcutaneously (left hind limb), and intramuscularly at a depth of about 3 mm (right hind limb). Intramuscular inoculation was carried out as follows. With the rabbit under ketamine/isoflurane anesthesia, a ~2 cm incision was made parallel to the right femur with a scalpel on the anterior, lateral aspect of the femoral axis, ~1/3 of the total femur length distally from the femoral-pelvic (hip) joint. Muscle groups were separated to create a ~0.5 cm deep cavity. One VX2 tumor fragment from a donor animal was placed into the cavity. Skin was securely closed with sterile surgical staples or sutures and a topical antibiotic was applied to the wound site.

Tumor measurements

Starting on Day 5, tumor dimensions (Length, Width and Height) were measured in millimeters via electronic vernier calipers connected to a laptop computer at a minimum frequency of twice weekly. For consistency, the same trained technician performed the tumor measurements throughout the course of the study. Tumor volume was calculated using the formula: Length x Width x Height x 0.52. Upon animal termination, tumors were carefully excised, trimmed and weighed. In addition, animals were weighed a minimum of once weekly. Representative samples of each tumor were preserved in sample cassette in formalin or OCT and flash frozen in liquid nitrogen.

In vivo passaging of VX2 tumors

The VX2 tumors used in both the Pilot and M200 efficacy study were maintained by *in vivo* passaging on a monthly basis. Either a cell suspension (100 µl) or pieces of tumor (~5-10 mm^3) were used for intramuscular inoculation in each hind limb. The animals and tumors were visually monitored and tumors removed before reaching 2 cm in diameter for *in vivo* passaging. *In vivo* passage was carried out by sacrificing the animal,

Samsung Bioepis Exhibit No. 1002
Page 2234

removing the tumor, and processing the tumor into 5-10 mm^3 pieces. The pieces were then re-implanted into the next group of 2 rabbits.

IHC analysis of VX2 tumors from M200 treated rabbits

M200 was administered intravenously at 10 mg/kg dosage to a tumor-bearing rabbit and the tumor was excised 1 hour later. Tumor sections were stained with either: (i) an anti-human secondary antibody specific for tumor bound M200; (ii) IIA1 followed by an anti-mouse secondary to detect total $\alpha5\beta1$; or (iii) a control IgG and anti-mouse secondary.

Results

Both the subcutaneous tumors and the intramuscular tumors were found to be accessible to M200 injected intravenously into the animals, as assessed by IHC. IHC analysis of the stained sections revealed high levels of $\alpha5\beta1$ expression both in VX2 tumor cells and rabbit tumor vasculature.

B. M200 VX2 Efficacy Study

Based on the pilot study IHC results, the VX2 rabbit model was used to assess M200 efficacy *in vivo*.

Methods

Rabbits (30 total) were inoculated, either subcutaneously or intramuscularly with either a VX2 cell suspension (100 µl) or pieces of tumor (~5-10 mm^3). The test group of 20 was treated with M200 at 10 mg/kg intravenously, twice weekly for 3 weeks. The control group of 10 animals was treated with a control IgG delivered by the same method as the M200. Tumor measurements, termination, weighing, tumor preservation and study duration were carried out according to the methods described above for the Pilot Study. In addition, one milliliter of blood was taken from an ear vessel on a weekly basis for serum analysis.

Results

A strong correlation was observed between inhibition of tumor growth and circulating levels of M200 in the animals. In general, when the levels of M200 were maintained at or above 50 µg/mL, then tumor growth was inhibited. Since M200 is immunogenic in rabbits, some of the test group rabbits generated an immune response to M200 as early as two weeks following M200 administration, and eventually all of the animals were found to seroconvert. In the test group animals that generated an anti-

Samsung Bioepis Exhibit No. 1002
Page 2235

idiotype response resulting in the clearance of M200 (i.e. M200 < 50 μg/mL at day 14), larger tumor growth was observed. Thus, the results observed with the VX2 carcinoma model indicate that M200 is able to inhibit tumor growth in a robust *in vivo* oncology model.

Example 7: M200 and IIA1 efficacy in a SCID mouse VX2 xenograft model

As described in Example 6, M200 exhibited efficacy in a rabbit VX2 carcinoma model. A further measure of the direct cancer cell killing ability of M200 and IIA1 was carried out in a SCID mouse VX2 xenograft model. Because M200 and IIA1 do not recognize mouse α5β1 integrin present on the VX2 xenograft tumor vasculature, any inhibitory effect on VX2 xenograft tumor growth may be attributed to a direct anti-proliferative effect that M200 and IIA1 has on VX2 cancer cells.

Materials & Methods

Immune compromised mice CB-17 SCID (strain C.B-Igh1/IcrTac-Prkdc) were obtained from Taconic Farms (Germantown, NY). Studies were initiated using female mice between the ages of 6-10 weeks (~20 grams in weight). Animals were pre-dosed with M200, IIA1, or control IgG intraperitoneally at a dose of 10 mg/kg one hour prior to VX2 inoculation ($1x10^7$ cells in Iscove's Modified Dulbecco's Medium) into the mammary fat pad. Dosing was continued for 3 weeks at a frequency of 3 times/week at 10 mg/kg. Tumor volume was measured twice weekly by caliper and calculated by $\pi/6$ x length x width x height. Clinical and mortality observations were performed daily according to IACUC regulations.

Results

Dosing with M200 or IIA1 did not correlate with a decreased rate of VX2 tumor growth or an overall decreased tumor size versus the mice dosed with control IgG. These results suggest that neither M200 and IIA1 are able to directly kill VX2 cells in the mouse xenograft model.

Example 8: Phase I Dose-Escalation Study of M200 in Human Patients with Refractory Solid Tumors

A. Overview

A two-part Phase I open-label study was carried to determine the effects of treatment with up to 6 dose levels, from 0.5 mg/kg to 15.0 mg/kg of M200 (volociximab)

Samsung Bioepis Exhibit No. 1002
Page 2236

in 21 human patients with a variety of refractory solid tumor types. The total duration of treatment for the first part was 6 weeks with physical assessments through 45 days after the last dose. The second part was an extension study wherein 6 of the 11 patients who exhibited a stable disease response in the first part received continued M200 dosing for up to 52 weeks.

B. Study Parameters and Protocols

1. Patient selection

Patients selected for the study had to meet the following inclusion/exclusion criteria:

a. At least one measurable lesion on routine computed tomography (CT) imaging or magnetic resonance imaging (MRI).
b. An estimated survival of $\geq$ 3 months; Eastern Collaborative Oncology Group (ECOG) performance status $\leq$ 2;
c. No central nervous system (CNS) tumors or metastasis (documented at screening by head imaging);
d. No major surgery within 4 weeks prior to entry;
e. No minor surgery within one week prior to entry; no chemotherapy, immunotherapy, or radiotherapy within 4 weeks prior to entry;
f. No active bleeding disorder or thromboembolic events;
g. No other clinically significant or unstable medical condition.
h. Patients who had previously received murine or chimeric mAb therapy were required to test negative in screening for anti-M200 antibodies (i.e., cross-reactive human-anti-murine-antibody [HAMA] or human-anti-chimeric antibody [HACA]).

A total of 22 patients were enrolled in the study. Tumor types of the 22 patients included: colorectal (4 patients; CRC), melanoma (4 patients; MEL), renal cell carcinoma (3 patients; RCC), esophageal (2 patients; EC), hepatocellular (2 patients; HCC), lung (1 patient; NSCLC), prostate (1 patient; PRO), thyroid (1 patient; THY), pancreatic (2 patients; PC), parotid (1 patient; PARO) and breast (1 patient; BC). Twenty-one of the 22 patients were treated, and 20 of the 21 treated patients completed all 5 doses plus follow-up through day 45. The 21 treated patients (12 male, 9 female) had a median age of 59 years (range 29-81), and a mean Eastern Collaborative Oncology Group (ECOG) score of 1 (range 0-2).

Samsung Bioepis Exhibit No. 1002
Page 2237

2. Selection of M200 Dosing Levels and Schedule

Dosing levels and schedule were chosen to ensure that the highest dose (15.0 mg/kg) would produce maximum serum concentrations well below the safe mean peak serum concentrations seen in cynolmolgus monkeys, and so that the trough serum levels for doses of ≥ 1.0 mg/kg would produce serum concentrations > 1 μg/mL, the concentration that produces 80% inhibition of binding of α5β1 to fibronectin in an *in vitro* activity assay.

The dosing levels and schedule used in the study were as follows. M200 was administered to the 21 patients on days 1, 15, 22, 29, and 36. The dosing levels and numbers of patients per level were: 0.5 mg/kg (1 patient), 1.0 mg/kg (2 patient), 2.5 mg/kg (3 patients), 5.0 mg/kg (3 patients), 10.0 mg/kg (6 patients) and 15 mg/kg (6 patients). The dose was administered as an intravenous infusion over 1 hour. The first and second doses were separated by a two week period to allow sampling for single-dose pharmacokinetic data. The real-time PK measurements performed following the first dose were used to predict each patient's peak serum concentration following the fifth dose. If the predicted peak serum concentration following the fifth dose was <750 μg/mL, the patient received all 5 doses of M200. The remaining 3 doses were given weekly and followed by a 45-day evaluation period.

Human serum concentrations of M200 were predicted based on the above dosing scheme and applying the same range of variabilty that was observed in the cynomolgus monkeys (i.e., 54%-140% of the mean). At the highest dose in humans (15.0 mg/kg), the mean peak after 5 doses was predicted to be 592 μg/mL with the range of variability in humans predicted as 320 to 829 μg/mL. Table 3, was compiled using PK data from the monkey studies to predict the peak and tough serum concentrations (Cmax and Cmin, respectively) for each dose at each dose level in humans.

TABLE 3: Estimated Peak and Trough Serum Levels of M200 in Humans

Level	Dose (mg/kg)	PK Value[a]	Dose Number				
			1	2	3	4	5
1	0.5	Cmax	11	11	12	13	13
		Cmin[b]	1.5	1.6	2	2.1	2.2
2	1.0	Cmax	22	22	25	26	26
		Cmin	3	3	4	5	5
3	2.5	Cmax	54	57	64	67	68
		Cmin	9	10	13	14	15
4	5.0	Cmax	108	114	132	141	147

Samsung Bioepis Exhibit No. 1002
Page 2238

		Cmin	20	24	33	39	43
5	10.0	Cmax	216	236	282	317	346
		Cmin	51	66	101	131	156
6	15.0	Cmax	324	366	450	524	592
		Cmin	91	126	200	268	332
		Cmax range (54-140%)	175-453	197-512	243-629	283-733	320-829

[a]Values expressed in μg/mL.
[b]Calculated for values expected 1 week after first dose.

Based on the above table, the terminal half-life in humans at 15.0 mg/kg was predicted to be about 13 days, and less for lower doses. M200 accumulation in serum was predicted to be substantive at dose levels ≥ 5.0 mg/kg, with a steady state concentration reached within 4 weeks for all doses < 10.0 mg/kg and within 5 weeks for doses ≥10.0 mg/kg.

3. M200 Formulation and Administration

M200 bulk biologic was manufactured in accordance with current Good Manufacturing Practices (cGMP). The composition of the M200 formulation used in the present study was 10 mg/mL M200, 25 mM Citrate, 150 mM Sodium Chloride, 0.05% Polysorbate (TWEEN®) 80, with a pH of 6.5. This formulation is a sterile, colorless, clear-to-slightly opalescent, preservative-free liquid for I.V. use. Each 20-mL single-use vial was filled to deliver 15 mL of M200 at 10.0 mg/mL. Each 10-mL single-use vial was filled to deliver 10 mL of M200 at 10.0 mg/mL. Intact vials were stored in a refrigerator at 2°C to 8°C (36°F-46°F) and maintained without freezing or shaking.

Once prepared, the M200 was administered within 6 hours if stored at room temperature (25°C) or 48 hours if refrigerated (between 2-8°C). After that time, the prepared solution was discarded.

The appropriate dose of M200 to be administered to the patient was calculated by multiplying the patient's weight (kg) by the appropriate dose level (mg/kg) for the patient. The patient's pre-dose weight on Study Day 1 was used to calculate the dose throughout the study. For patients enrolled in the 0.5 mg/kg through the 10.0 mg/kg dose cohorts and for patients weighing ≤80 kg enrolled in the 15.0 mg/kg dose cohort, the dose was administered in a fixed total volume of 120 mL over one hour.

Although 120 mL of the diluted study drug was administered to the patient, the infusion bag was prepared to contain a total of 150 mL. The additional 30 mL was used to prime the infusion line and was not be administered to the patient. Thus, the total dose of

Samsung Bioepis Exhibit No. 1002
Page 2239

study drug placed into the infusion bag was the patient's dose (i.e., the patient's weight (kg) × dose level [mg/kg] multiplied by 1.25). The total volume in the infusion bag was brought up to 150 mL by adding Sodium Chloride for Injection, USP (0.9%).

Patients weighing >80 kg enrolled in the 15.0 mg/kg dose cohort had their dose administered in a fixed total volume of 180 mL over one hour. Although 180 mL of the diluted study drug was administered to the patient, the infusion bag was prepared to contain a total of 210 mL. The additional 30 mL was used to prime the infusion line and was not administered to the patient. Thus, the total dose of study drug placed into the infusion bag was the patient's dose (i.e., the patient's weight (kg) x dose level [mg/kg] multiplied by 1.167). The total volume in the infusion bag was brought up to 210 mL by adding Sodium Chloride for Injection, USP (0.9%).

The pre-filled infusion line was attached directly to the patient's IV access (e.g., heparin lock). The infusate was administered at a rate of 2 mL/min (or 3 mL/min for 15.0 mg/kg cohort patients weighing >80 kg) over one hour using an infusion pump.

4. Primary Endpoints and Adverse Events

The study endpoints included maximum tolerated dose, dose-limiting toxicity, safety profile, immunogenicity, pharmacokinetics (PK) , monocyte saturation (monocytes express the $\alpha 5\beta 1$ receptor), and tumor response based on Response Evaluation Criteria in Solid Tumors (RECIST) criteria (see, Therasse P, *et al.,* "New guidelines to evaluate the response to treatment in solid tumors," *Journal of the National Cancer Institute,* 92(3):205-216 (2000), which is hereby incorporated by reference herein in its entirety).

Adverse events were graded using the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE), version 3.0. Dose-limiting toxicity was defined as any Grade-3 or Grade-4 adverse event, excluding scheduled hospitalizations or elective surgeries. Grade-3 or Grade-4 adverse events thought to be unrelated to M200 were excluded where appropriate following a review with the medical monitor and regulatory agencies.

Patient evaluations during the study included the following:

a. Screening (within 14 days of study entry) of: medical history and physical examination, ECOG performance status, routine chemistry panel, complete blood count (CBC) with differential and platelets, sensitive C-reactive protein (CRP), urinalysis with microscopic analysis (UA/micro), electrocardiogram (ECG), chest radiograph (CXR), disease-directed body CT or MRI, urine

Samsung Bioepis Exhibit No. 1002
Page 2240

pregnancy test within 48 hours prior to dosing, and coagulation studies (prothrombin [PT] and partial thromboplastin time [PTT]), anti-M200 antibodies (i.e., to detect cross-reactive HAMA or HACA) for patients who previously received murine or chimeric antibodies, head CT for patients with tumors that commonly metastasize to brain or CNS, and, in some patients, a biopsy to evaluate tumor vessel vascularity.

b. On-study laboratory evaluations: routine chemistry panel, urate, serum amylase, CBC with differential and platelets, UA/micro.

c. Routine disease-directed radiographic imaging (e.g., CT scanning) before and after treatment with M200 was used to evaluate tumor response using uni-dimensional, RECIST criteria. Target lesions were selected on the basis of their size (lesions with the longest diameter (LD)) and their suitability for accurate repetitive measurements (either by imaging techniques or clinically). A sum of the LD for all target lesions was calculated and reported as the baseline sum LD. The baseline sum LD was used as reference by which to characterize the objective tumor response. The following response criteria for the target lesions was used to assess the best overall response.

- Complete Response (CR): Disappearance of all target lesions confirmed by repeat imaging 25 to 28 days after CR first documented.
- Partial Response (PR): At least a 30% decrease in the sum of LD of target lesions, taking as reference the baseline sum LD, confirmed by repeat imaging 25 to 28 days after PR first documented.
- Stable Disease (SD): Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as references the smallest sum LD.
- Progressive Disease (PD): At least a 20% increase in the sum of LD of target lesions, taking as reference the smallest sum LD recorded since treatment started, or the appearance of one or more new lesions.

d. Blood drawn for serum PK measurements, immunogenicity assays (anti-M200 antibodies), vascular growth factors, and measurement of bound and unbound $\alpha 5\beta 1$ on peripheral blood monocytes.

Samsung Bioepis Exhibit No. 1002
Page 2241

e. A 3-mm punch biopsy of superficial tumor metastasis was obtained and frozen after the last treatment with M200 from patients who consented (not required for enrollment) to the procedure. This biopsy specimen could be used evaluate tumor vessel vascularity following treatment with M200.

5. Pharmacokinetic Measurements

The first human PK data at low doses was used to predict the human serum levels at higher doses (described above). The serum for measuring M200 concentrations was obtained immediately before and after the first dose, and at 4, 24, 48, and 168 hours after completion of the first dose. Serum samples for measuring M200 concentrations were also taken immediately before, at the end, and 4 hours after the completion of each subsequent infusion. These samples were split and aliquots assayed before the next dose and again at the end of the study: end of 1st dose, 168 hours after 1st dose, immediately pre-dose (trough) and immediately post-dose (peak) for the 2nd, 3rd, 4th and 5th doses. The serum levels of M200 were measured by ELISA.

The plasma concentration-time data of M200 from each patient was subjected to a PK analysis. The following parameters were included in the calculations: peak (Cmax) and trough (Cmin) levels, terminal phase half-life ($T_{1/2\,\beta}$), area under the plasma-concentration time curve (AUC), elimination clearance (CL), and volume of distribution (V). Summary parameters were computed across patients from each treatment group. Changes in the PK parameters as a function of dose and time were also evaluated.

6. Immunogenicity

Immunogenicity of M200 was determined by a double-antigen bridging ELISA assay. Serum samples for anti-M200 antibodies were obtained from patients before treatment, prior to the second dose, and at study exit, 45 days after the last dose of M200. In addition, serum for anti-M200 antibodies was drawn if the interval between doses was $\geq$ 2 weeks. Samples were stored and evaluated at the end of the study. Samples that tested positive for antibodies to M200 were re-assayed for neutralizing antibodies to M200. Patients with known exposure to murine or chimeric monoclonal antibodies were screened for the presence of anti-M200 antibodies prior to receiving the first dose of M200.

7. Flow Cytometry Analysis

Samsung Bioepis Exhibit No. 1002
Page 2242

The integrin α5β1 is also expressed on monocytes in the peripheral blood. To determine the dose at which the α5β1 sites on these circulating cells is saturated by M200, samples of peripheral blood were taken before the first dose on Study Day 1, Study Days 2 and 8, just before each subsequent dose, and on Study Day 43. The saturation of α5β1 sites on monocytes was determined by flow cytometry. The monocytes were identified by using antibodies to CD14. Bound M200 on the monocyte surface was detected by adding labeled anti-human IgG4. Unoccupied (free) α5β1 on the cells was detected by adding labeled IIA1. The percentages of monocytes and lymphocytes expressing α5β1 was also assessed.

C. Results – Dose Escalation Study

M200 was well tolerated by patients at doses up to 15 mg/kg and no dose-limiting M200 toxicities were observed. The overall response outcome for the study was: stable disease (SD) in 11 patients and progressive disease (PD) in 10 patients. Table 4 shows a breakdown of the response outcome by dosage and patient tumor type.

TABLE 4: Response Outcomes by Dosage and Tumor Type

0.5 mg/kg	1.0 mg/kg	2.5 mg/kg	5.0 mg/kg	10.0 mg/kg	15.0 mg/kg
CRC (PD)	HCC (SD)	CRC (PD)	HCC (SD)	CRC (SD)	EC (PD)
	PC (SD)	NSCLC (SD)	EC (PD)	CRC (SD)	RCC (SD)
		MEL Ocular (PD)	THY (PD)	PARO (SD)	PRO (PD)
				BC (SD)	MEL (PD)
				RCC (SD)	MEL (SD)
				MEL (PD)	RCC (PD)

Abbreviations: CRC = colorectal; MEL = melanoma; RCC = renal; EC = esophogeal; HCC = hepatocellular; NSCLC = lung; PRO = prostate; THY = thyroid; PC = pancreatic; PARO = parotid; BC = breast; SD = Stable Disease; PD = Progressive Disease

Adverse events were generally mild to moderate in intensity and included fatigue, nausea, constipation, headache and anorexia. There were no severe or serious adverse events that were dose limiting or considered by investigators to be related to M200. Three patients at the 0.5 mg/kg and 1.0 mg/kg dose levels tested positive for anti-M200 antibodies, but there were no apparent associated adverse events. No patients in the

Samsung Bioepis Exhibit No. 1002
Page 2243

higher dose cohorts tested positive for anti-M200. The one patient who received 0.5 mg/kg had fever after the first dose, which was recorded as an infusion reaction. The patient, however, did complete all 5 doses of M200 without subsequent episodes of fever or other signs of infusion reaction.

The study data indicated that M200 exhibits non-linear pharmacokinetics. Slower-clearance was observed at the higher concentrations with the $T_{1/2}$ = 15.7 days at the 10 mg/kg dosage level. In addition, The 10 mg/kg dosage resulted in monocyte saturation, and had a mean trough level of 82 μg/mL two weeks after the 1st dose, which is above the minimum effective *in vitro* concentration of 2-3 μg/mL.

D. Results – Extension Study

Six of the 11 patients exhibiting a stable disease response or better entered an extension study and continued dosing. As shown in Table 5, below, 5 of the 6 patients exhibited stable disease (SD) or better response. One of these patients with renal cell cancer (RCC) in the 15.0 mg/kg cohort achieved a partial response.

Table 5: Extension Study Outcomes

Dose Cohort (# patients; tumor type)	Best Response Outcome	Time to Progression* (Days)
2.5 mg/kg (1; NSCLC)	SD	129
5 mg/kg (1; HCC)	SD	122
10 mg/kg (1; CRC)	PD	70
10 mg/kg (1; PARO)	SD	143
15 mg/kg (1; RCC)	PR	214
15 mg/kg (1; MEL)	SD	172+ (on Study)

* Includes 43 days on dose escalation study plus days on extension study.
SD defined as stabilization for ≥16 weeks (112 days)

Example 9: Phase II Open-Label Study of M200 in Human Patients with Metastatic Renal Cell Carcinoma

A. Overview

Based on the efficacy of M200 demonstrated by the Phase I study of Example 8, a Phase II, open-label, multi-center, single-arm, 2-stage study of the efficacy of M200 for

Samsung Bioepis Exhibit No. 1002
Page 2244

treating human patients has been undertaken. The primary objective of the study is to evaluate the efficacy (tumor response) of M200 in patients with metastatic renal cell carcinoma (RCC), as defined using Response Criteria for Solid Tumors (see RECIST, Example 8 above). The study also has as secondary objectives evaluation of other efficacy measures (ie, time to disease progression and duration of response), and further evaluation of the safety, immunogenicity and PK parameters of M200 that were initially evaluated in the Phase I study of Example 8. An additional exploratory objective is to measure detectable biomarkers in serum and plasma. The study will enroll up to 40 patients at up to eight investigational sites. Twenty patients will be enrolled into stage one of the study. If one confirmed complete response (CR) or partial response (PR) is observed by 4 months (16 weeks) or if one stable disease (SD) is observed at 4 months, an additional 20 patients will be enrolled (stage 2). All patients will receive M200 (10 mg/kg) as an intravenous infusion over 30 minutes once every other week for up to 52 weeks or until disease progression, whichever occurs first. The study exit visit will occur 45 days after the last dose or at the time of early termination if the patient is unable to return. A follow-up visit will occur at 3 months after the last dose. If a visit is not feasible, follow-up will be conducted by telephone. Long-term follow-up will be conducted by telephone at 6 months after the last dose.

B. Study Parameters and Protocols

The Phase II study is being conducted according to the parameters and protocols described in Table 6 below.

Table 6: Parameters of Phase II Study in Patients with Metastatic Renal Cell Carcinoma

Study Population:	Males and females of at least 18 years of age with metastatic RCC of clear cell histology.
Key Patient Inclusion/Exclusion Criteria:	**Inclusion:** Males and females of at least 18 years of age with metastatic RCC of predominantly clear cell histology who have received 0 to 2 prior regimens for metastatic disease; measurable disease according to Response Criteria for Solid Tumors (RECIST); Eastern Cooperative Oncology Group (ECOG) performance status ≤1; negative pregnancy test (women of childbearing potential only); pretreatment laboratory levels that meet specific criteria; and signed informed consent, including permission to use protected health information (PHI). **Exclusion:** Any of the following histologies of RCC: papillary, chromophobe, collecting duct, or unclassified; known sensitivity

Samsung Bioepis Exhibit No. 1002
Page 2245

	to murine proteins or chimeric antibodies or other components of the product; use of any investigational drug within 4 weeks prior to screening or 5 half-lives of the prior investigational drug (whichever is longer); systemic chemotherapy, immunotherapy, radiation therapy, or monoclonal antibody therapy within 4 weeks of M200 administration; documented CNS tumor or CNS metastasis; history of thromboembolic events and bleeding disorders within the past year; and medical conditions that may be exacerbated by bleeding.
Sample Size:	Up to 40 patients may be enrolled. Twenty patients will be enrolled into stage one. If one confirmed complete response (CR) or partial response (PR) is observed by 4 months (16 weeks) or if one stable disease (SD) is observed at 4 months, an additional 20 patients will be enrolled (stage 2).
Dosage Form and Strength/Formulation:	M200 is a sterile, colorless, clear-to-slightly opalescent, preservative-free liquid for IV use. Each 10-mL single-use vial is filled to deliver 10 mL of M200 at 10 mg/mL. The composition of each vial is 10 mg/mL M200, 25 mM citrate, 150 mM sodium chloride, and 0.05% Polysorbate (Tween®) 80, with a pH of 6.5.
Storage and Filtration:	Intact vials will be stored in a refrigerator at 2°C to 8°C (36°F to 46°F). Do not freeze or shake. M200 must be administered within 6 hours if stored at room temperature (25°C) or 48 hours if refrigerated (between 2°C to 8°C). After that time, the prepared solution must be discarded.
Dosing Regimen/Route of Administration:	All eligible patients will receive 10 mg/kg M200 by intravenous (IV) infusion over 30 minutes every other week for up to 52 weeks or until disease progression, whichever occurs first. The dose of M200 to be administered to the patient will be calculated by multiplying the patient's weight (kg) by the dose level (10 mg/kg). The patient's predose weight on Study Day 0 will be used to calculate the dose throughout the study, provided the weight does not vary by more than 10%. The appropriate volume of the 10 mg/mL M200 formulation will be removed and diluted with 0.9% sodium chloride to a final volume of 120 mL for IV infusion into the patient over the 30 minute period.
Duration of Treatment and Follow-up:	All patients will receive M200 (10 mg/kg) intravenously once every other week for up to 52 weeks or until disease progression, whichever occurs first. The study exit visit will occur 45 days after the last dose or at the time of early termination if the patient is unable to return. A follow-up visit will occur at 3 months after the last dose. If a visit is not feasible, follow-up will be conducted by telephone. Long-term follow-up will be conducted by telephone at 6 months after the last dose.

Samsung Bioepis Exhibit No. 1002
Page 2246

Endpoints:	The primary endpoint of this study is the proportion of patients with a confirmed tumor response at any time during the study. The secondary endpoints are as follows: (1) time to disease progression; (2) duration of tumor response; (3) PK of M200; and (4) immunogenicity. The exploratory endpoint is the measurement of detectable biomarkers in serum and plasma.
Efficacy Measurements:	Disease-directed radiographic imaging every 8 weeks (2 months) to evaluate tumor response using uni-dimensional RECIST. At screen, CT or MRI of the head, chest, abdomen, and pelvis will be obtained. Every 8 weeks, a complete physical examination and imaging scans of all lesions present at screen plus all disease-directed anatomical lesions will be performed. For any PR or CR, confirmatory radiographic imaging will be repeated one month (between 28 to 35 days) post-PR or -CR. All measurable lesions up to a maximum of 5 lesions per organ and 10 lesions in total, representative of all involved organs, should be identified as target lesions and will be recorded and measured at baseline. Measurable lesions are defined as follows: 2.0 cm in one dimension using conventional CT/MRI and 1.0 cm in one dimension using spiral CT. A sum of the longest diameter (LD) for all target lesions will be calculated and reported as the baseline sum LD. The baseline sum LD will be used as the reference by which objective tumor response will be characterized.
Safety Measurements:	The following safety measurements will be monitored: adverse events (AE); serious adverse events (SAE); physical examination findings; vital signs; and abnormal laboratory values.
Pharmacokinetic Measurements:	Pharmacokinetic (PK) measurements will be taken in all patients at predose (within 15 minutes prior to dosing) and postdose (one hour post-end of infusion): Day 0, Weeks 2, 4, and 6, once every other month (Weeks 8, 16, 24, 32, 40, 48), Week 52, study exit visit, and 3-month follow-up (if possible). Samples obtained for PK may be used for anti-Ab analyses, if appropriate.
Immunogenicity:	Anti-Ab measurements will be taken in all patients within 15 minutes prior to dosing on Day 0, Week 8, study exit visit, and 3-month follow-up (if possible). Samples obtained for PK may be used for anti-Ab analyses, if appropriate.
Other Measurements:	An exploratory assay will evaluate the presence of biomarkers in serum and plasma. The following cancer markers are included in the assay: CEA, CA 19-9, Syndecan, IGFBP-2, and LFL2. The following cytokines/growth factors are included: MIA, IL-6, TNF-alpha, PGF, VEGF, EGF, and bFGF.
Statistical Methods:	This study design will yield a power of at least 79.5% for detecting an overall response rate of ≥20% with up to 20% of the responders displaying stable disease (SD). Summary statistics and 95% confidence intervals will be provided for dichotomous

Samsung Bioepis Exhibit No. 1002
Page 2247

	endpoints. Kaplan-Meier methods will be used to summarize temporal variables.

It is understood that the examples described above in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All publications, sequences of accession numbers, and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Samsung Bioepis Exhibit No. 1002
Page 2248

CLAIMS

We claim:

1. A method of inhibiting the proliferation of a cancer cell in a patient wherein the cancer cell expresses α5β1 integrin on its surface, the method comprising administering to the patient a therapeutically effective amount of a pharmaceutical composition comprising a liquid formulation comprising:
   about 1.0 mg/mL to 15 mg/mL anti-α5β1 antibody;
   about 22 mM to 27 mM citrate;
   about 145 mM to 165 mM sodium chloride;
   about 0.04% to 0.06% polysorbate (TWEEN®) 80;
   and a pH of about 5.5 to 7.5.

2. The method of Claim 1, wherein the antibody neutralizes at least one biological activity of α5β1 integrin.

3. The method of claim 1, wherein the anti-α5β1 antibody binds to the same epitope of α5β1 integrin as the M200 antibody.

4. The method of claim 1, wherein the anti-α5β1 antibody competitively inhibits binding of M200 to α5β1 integrin expressed on the cell surface.

5. The method of claim 1, wherein the anti-α5β1 antibody is selected from the group consisting of M200, F200 and IIA1.

6. The method of Claim 1, wherein the antibody competitively inhibits binding of a second antibody to α5β1 integrin on the cancer cell surface wherein the second antibody comprises an amino acid sequence selected from SEQ ID NOs: 2, 4, 6 and 8.

7. The method of claim 1, wherein the therapeutically effective dose is about 10 mg/kg.

8. The method of Claim 1, wherein the cancer cell is selected from the group consisting of breast cancer cell, lung cancer cell, metastatic melanoma cell, pancreatic cancer cell, and renal cell carcinoma cell.

Samsung Bioepis Exhibit No. 1002
Page 2249

9. The method of claim 1, wherein the cancer is renal cell carcinoma or metastatic melanoma.

10. The method of Claim 1, further comprising administering a chemotherapeutic agent to the patient either serially or in combination with the antibody.

11. A method of treating a subject for a cancer that expresses $\alpha5\beta1$ wherein the subject has not yet developed a tumor comprising administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising a liquid formulation comprising:

about 1.0 mg/mL to 15 mg/mL anti-$\alpha5\beta1$ antibody;
about 22 mM to 27 mM citrate;
about 145 mM to 165 mM sodium chloride;
about 0.04% to 0.06% polysorbate (TWEEN®) 80;
and a pH of about 5.5 to 7.5.

12. The method of Claim 11, wherein the antibody binds to the same epitope of $\alpha5\beta1$ integrin as M200.

13. The method of Claim 11, wherein the antibody competitively inhibits binding of M200 to $\alpha5\beta1$ integrin expressed on the cancer cell surface.

14. The method of Claim 11, wherein the cancer is selected from the group consisting of bladder cancer, breast cancer, colon cancer, fibrosarcoma, lung cancer, metastatic melanoma, pancreatic cancer, prostate cancer, ovarian cancer, renal cell carcinoma and spleen cancer.

15. The method of claim 11, wherein the cancer is renal cell carcinoma or metastatic melanoma.

16. The method of claim 11, wherein the therapeutically effective dose is about 10 mg/kg.

17. The method of Claim 11, further comprising administering a chemotherapeutic agent to the patient either serially or in combination with the antibody.

18. A pharmaceutical composition comprising a liquid formulation comprising:

Samsung Bioepis Exhibit No. 1002
Page 2250

about 1.0 mg/mL to 15 mg/mL anti-α5β1 antibody;
about 22 mM to 27 mM citrate;
about 145 mM to 165 mM sodium chloride;
about 0.04% to 0.06% polysorbate (TWEEN®) 80;
and a pH of about 5.5 to 7.5.

19. The pharmaceutical composition of claim 18, wherein the concentration of anti-α5β1 antibody is about 10 mg/mL.

20. The pharmaceutical composition of claim 18, wherein the anti-α5β1 antibody is M200.

21. The pharmaceutical composition of claim 18, wherein the anti-α5β1 antibody is M200.

22. The pharmaceutical composition of claim 18, wherein the composition further comprises a chemotherapeutic agent.

Samsung Bioepis Exhibit No. 1002
Page 2251

A. IIA1 V_H sequences
[NA, SEQ ID NO: 1; AA, SEQ ID NO: 2]

```
1   ATGGCTGTCCTGGGGCTGCTTCTCTGCCTGGTGACTTTCCCAAGCTGTGTCCTGTCCCAG
     M  A  V  L  G  L  L  L  C  L  V  T  F  P  S  C  V  L  S  Q
61  GTGCAGCTGAAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCCTGTCCATCACA
     V  Q  L  K  E  S  G  P  G  L  V  A  P  S  Q  S  L  S  I  T
121 TGCACCATCTCAGGGTTCTCATTAACCGACTATGGTGTTCACTGGGTTCGCCAGCCTCCA
     C  T  I  S  G  F  S  L  T  D  Y  G  V  H  W  V  R  Q  P  P
181 GGAAAGGGTCTGGAGTGGCTGGTAGTGATTTGGAGTGATGGAAGCTCAACCTATAATTCA
     G  K  G  L  E  W  L  V  V  I  W  S  D  G  S  S  T  Y  N  S
241 GCTCTCAAATCCAGAATGACCATCAGGAAGGACAACTCCAAGAGCCAAGTTTTCTTAATA
     A  L  K  S  R  M  T  I  R  K  D  N  S  K  S  Q  V  F  L  I
301 ATGAACAGTCTCCAAACTGATGACTCAGCCATGTACTACTGTGCCAGACATGGAACTTAC
     M  N  S  L  Q  T  D  D  S  A  M  Y  Y  C  A  R  H  G  T  Y
361 TACGGTATGACTACGACGGGGGATGCTTTGGACTACTGGGGTCAAGGAACCTCAGTCACC
     Y  G  M  T  T  T  G  D  A  L  D  Y  W  G  Q  G  T  S  V  T
421 GTCTCCTCA
     V  S  S
```

B. IIA1 V_L sequences
[NA, SEQ ID NO: 3; AA, SEQ ID NO: 4]

```
1   ATGGATTTTCAGGTGCAGATTTTCAGCTTCCTGCTAATCAGTGCCTCAGTCATAATGTCC
     M  D  F  Q  V  Q  I  F  S  F  L  L  I  S  A  S  V  I  M  S
61  AGAGGACAAATTGTTCTCACCCAGTCTCCAGCAATCATGTCTGCATCTCTAGGGGAACGG
     R  G  Q  I  V  L  T  Q  S  P  A  I  M  S  A  S  L  G  E  R
121 GTCACCATGACCTGCACTGCCAGTTCAAGTGTAAGTTCCAATTACTTGCACTGGTACCAG
     V  T  M  T  C  T  A  S  S  S  V  S  S  N  Y  L  H  W  Y  Q
181 CAGAAGCCAGGATCCGCCCCCAATCTCTGGATTTATAGCACATCCAACCTGGCTTCTGGA
     Q  K  P  G  S  A  P  N  L  W  I  Y  S  T  S  N  L  A  S  G
241 GTCCCAGCTCGTTTCAGTGGCAGTGGGTCTGGGACCTCTTACTCTCTCACAATCAGCAGC
     V  P  A  R  F  S  G  S  G  S  G  T  S  Y  S  L  T  I  S  S
301 ATGGAGGCTGAAGATGCTGCCACTTATTACTGCCACCAGTATCTTCGTTCCCCACCGACG
     M  E  A  E  D  A  A  T  Y  Y  C  H  Q  Y  L  R  S  P  P  T
361 TTCGGTGGAGGCACCAAGCTGGAAATCAAA
     F  G  G  G  T  K  L  E  I  K
```

FIGURE 1

Samsung Bioepis Exhibit No. 1002
Page 2252

A. M200 V_H sequences
[NA, SEQ ID NO: 5; AA, SEQ ID NO: 6]

```
1   TCTAGACCACCATGGCTGTCCTGGGGCTGCTTCTCTGCCTGGTGACTTTCCCAAGCTGTG
                M  A  V  L  G  L  L  L  C  L  V  T  F  P  S  C
61  TCCTGTCCCAGGTGCAGCTGAAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCC
    V  L  S  Q  V  Q  L  K  E  S  G  P  G  L  V  A  P  S  Q  S
121 TGTCCATCACATGCACCATCTCAGGGTTCTCATTAACCGACTATGGTGTTCACTGGGTTC
    L  S  I  T  C  T  I  S  G  F  S  L  T  D  Y  G  V  H  W  V
181 GCCAGCCTCCAGGAAAGGGTCTGGAGTGGCTGGTAGTGATTTGGAGTGATGGAAGCTCAA
    R  Q  P  P  G  K  G  L  E  W  L  V  V  I  W  S  D  G  S  S
241 CCTATAATTCAGCTCTCAAATCCAGAATGACCATCAGGAAGGACAACTCCAAGAGCCAAG
    T  Y  N  S  A  L  K  S  R  M  T  I  R  K  D  N  S  K  S  Q
301 TTTTCTTAATAATGAACAGTCTCCAAACTGATGACTCAGCCATGTACTACTGTGCCAGAC
    V  F  L  I  M  N  S  L  Q  T  D  D  S  A  M  Y  Y  C  A  R
361 ATGGAACTTACTACGGAATGACTACGACGGGGGATGCTTTGGACTACTGGGGTCAAGGAA
    H  G  T  Y  Y  G  M  T  T  T  G  D  A  L  D  Y  W  G  Q  G
421 CCTCAGTCACCGTCTCCTCAG^GTAAGAATGGCCTCTAGA
    T  S  V  T  V  S  S
```

B. M200 V_L sequences
[NA, SEQ ID NO: 7; AA, SEQ ID NO: 8]

```
1   ACGCGTCCACCATGGATTTTCAGGTGCAGATTTTCAGCTTCCTGCTAATCAGTGCCTCAG
                M  D  F  Q  V  Q  I  F  S  F  L  L  I  S  A  S
61  TCATAATGTCCAGAGGACAAATTGTTCTCACCCAGTCTCCAGCAATCATGTCTGCATCTC
    V  I  M  S  R  G  Q  I  V  L  T  Q  S  P  A  I  M  S  A  S
121 TAGGGGAACGGGTCACCATGACCTGCACTGCCAGTTCAAGTGTCAGTTCCAATTACTTGC
    L  G  E  R  V  T  M  T  C  T  A  S  S  S  V  S  S  N  Y  L
181 ACTGGTACCAGCAGAAGCCAGGATCCGCCCCCAATCTCTGGATTTATAGCACATCCAACC
    H  W  Y  Q  Q  K  P  G  S  A  P  N  L  W  I  Y  S  T  S  N
241 TGGCTTCTGGAGTCCCAGCTCGTTTCAGTGGCAGTGGGTCTGGGACCTCTTACTCTCTCA
    L  A  S  G  V  P  A  R  F  S  G  S  G  S  G  T  S  Y  S  L
301 CAATCAGCAGCATGGAGGCTGAAGATGCTGCCACTTATTACTGCCACCAGTATCTTCGTT
    T  I  S  S  M  E  A  E  D  A  A  T  Y  Y  C  H  Q  Y  L  R
361 CCCCACCGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAAC^GTAAGTAGAATCCAAAGT
    S  P  P  T  F  G  G  G  T  K  L  E  I  K
421 CTAGA
```

FIGURE 2

Samsung Bioepis Exhibit No. 1002
Page 2253

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(71) Applicants *(for all designated States except US)*: **NOVO NORDISK A/S** [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK). **INNATE PHARMA** [FR/FR]; Immeuble Grand Pré, 121, Ancien Chemin de Cassis, F-13009 Marseille (FR). **UNIVERSITY OF GENOA** [IT/IT]; Via L.B. Alberti 2, I-16132 Genoa (IT).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **MORETTA, Alessandro** [IT/IT]; Via Nizza 18/8, I-16133 Genoa (IT). **DELLA CHIESA, Mariella** [IT/IT]; Via L.B. Alberti, I-16132 Genoa (IT). **ANDRE, Pascale** [FR/FR]; 38, rue de La Loubiere, F-13006 Marseille (FR). **GAUTHIER, Laurent** [FR/FR]; 139 Avenue de Hambourg, Residence la Sarette Bãt 5, F-13008 Marseille (FR). **ROMAGNÉ, Francois** [FR/FR]; Bat. A3, Les Hauts de Fontsainte, F-13600 La Ciotat (FR). **WAGTMANN, Peter, Andreas, Nicolai, Reumert** [DK/DK]; Vallerød Banevej 34, DK-2960 Rungsted Kyst (DK). **SVENDSEN, Ivan** [DK/DK]; Tuliphaven 76, DK-2765 Smørum (DK). **ZAHN, Stefan** [DK/DK]; Byager 23, DK-2750 Ballerup (DK). **SVENSSON, Anders** [SE/SE]; Roskildevägen 17 B, DK-217 46 Malmö (DK). **THÓRÓLFSSON, Matthias** [IS/DK]; Brannersvej 9, DK-2920 Charlottenlund (DK).

(74) Common Representative: **NOVO NORDISK A/S**; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).

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(54) Title: HUMAN ANTI-KIR ANTIBODIES

(57) Abstract: Compositions and methods for regulating an immune response in a subject are described. More particularly, described are human antibodies that regulate the activity of NK cells and allow a potentiation of NK cell cytotoxicity in mammalian subjects, and antibodies having antigen-binding properties similar to those of human monoclonal antibody 1-7F9 or 14F1. Described also are also fragments and derivatives of such antibodies, as well as pharmaceutical compositions comprising the same and their uses, particularly for use in therapy, to increase NK cell activity or cytotoxicity in subjects.

WO 2006/003179 A2

HUMAN ANTI-KIR ANTIBODIES

FIELD OF THE INVENTION

The present invention relates to human antibodies, as well as fragments and derivatives thereof, which cross-react with and/or block two or more inhibitory KIR receptors present on the surface of NK cells, and potentiate NK cell cytotoxicity in mammalian subjects or in a biological sample. The invention also relates to methods of making such antibodies, fragments, variants, and derivatives; pharmaceutical compositions comprising the same; and the use of such molecules and compositions, particularly in therapy, to increase NK cell activity or cytotoxicity in subjects.

BACKGROUND OF THE INVENTION

Natural killer (NK) cells are a sub-population of lymphocytes, involved in immunity and in the host immune surveillance system.

NK cells are mononuclear cell that develop in the bone marrow from lymphoid progenitors, and morphological features and biological properties typically include the expression of the cluster determinants (CDs) CD16, CD56, and/or CD57; the absence of the alpha/beta or gamma/delta TCR complex on the cell surface; the ability to bind to and kill target cells that fail to express "self" major histocompatibility complex (MHC)/human leukocyte antigen (HLA) proteins; and the ability to kill tumor cells or other diseased cells that express ligands for activating NK receptors. NK cells are characterized by their ability to bind and kill several types of tumor cell lines without the need for prior immunization or activation. NK cells can also release soluble proteins and cytokines that exert a regulatory effect on the immune system; and can undergo multiple rounds of cell division and produce daughter cells with similar biologic properties as the parent cell. Upon activation by interferons and/or cytokines, NK cells mediate the lysis of tumor cells and of cells infected with intracellular pathogens by mechanisms that require direct, physical contacts between the NK cell and the target cell. Lysis of target cells involves the release of cytotoxic granules from the NK cell onto the surface of the bound target, and effector proteins such as perforin and granzyme B that penetrate the target plasma membrane and induce apoptosis or programmed cell death. Normal, healthy cells are protected from lysis by NK cells.

Based on their biological properties, various therapeutic and vaccine strategies have been proposed in the art that rely on a modulation of NK cells. However, NK cell activity is regulated by a complex mechanism that involves both stimulating and inhibitory signals.

Briefly, the lytic activity of NK cells is regulated by various cell surface receptors that transduce either positive or negative intracellular signals upon interaction with ligands on the target cell. The balance between positive and negative signals transmitted via these receptors determines whether or not a target cell is lysed (killed) by a NK cell. NK cell stimulatory signals can be mediated by Natural Cytotoxicity Receptors (NCR) such as NKp30, NKp44, and NKp46; as well as NKG2C receptors, NKG2D receptors, certain activating Killer Ig-like Receptors (KIRs), and other activating NK receptors (Lanier, Annual Review of Immunology 2005;23:225-74). NK cell inhibitory signals can be mediated by receptors like Ly49, CD94/NKG2A, as well as certain inhibitory KIRs, which recognize major histocompatibility complex (MHC) class I-molecules (Kärre et al., Nature 1986;319:675-8; Öhlén et al, Science 1989;246:666-8). These inhibitory receptors bind to polymorphic determinants of MHC class I molecules (including HLA class I) present on other cells and inhibit NK cell-mediated lysis.

KIRs, sometimes also referred to as Killer Inhibitory Receptors, have been characterized in humans and non-human primates, and are polymorphic type 1 trans-membrane molecules present on certain subsets of lymphocytes, including NK cells and some T cells. KIRs interact with determinants in the alpha 1 and 2 domains of the MHC class I molecules and, as described above, distinct KIRs are either stimulatory or inhibitory for NK cells.

The nomenclature for KIRs is based upon the number of extracellular domains (KIR2D and KIR3D having two and three extracellular Ig-domains, respectively) and whether the cytoplasmic tail is long (KIR2DL or KIR3DL) or short (KIR2DS or KIR3DS). The presence or absence of a given KIR is variable from one NK cell to another within the NK population present in a single individual. Among humans, there is also a relatively high level of polymorphism of KIR genes, with certain KIR genes being present in some, but not all individuals. The expression of KIR alleles on NK cells is stochastically regulated, meaning that, in a given individual, a given lymphocyte may express one, two, or more different KIRs, depending on the genoptype of the individual. The NK cells of a single individual typically express different combinations of KIRs, providing a repertoire of NK cells with different specificities for MHC class I molecules.

Certain KIR gene products cause stimulation of lymphocyte activity when bound to an appropriate ligand. The activating KIRs all have a short cytoplasmic tail with a charged transmembrane residue that associates with an adapter molecule having an Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) which transduce stimulatory signals to the NK cell. By contrast, inhibitory KIRs have a long cytoplasmic tail containing Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM), which transduce inhibitory signals to the NK cell upon engagement of their MHC class I ligands. The known inhibitory KIRs include members of the

KIR2DL and KIR3DL subfamilies. Inhibitory KIRs having two Ig domains (KIR2DL) recognize HLA-C allotypes: KIR2DL2 (formerly designated p58.2) and the closely related, allelic gene product KIR2DL3 both recognize "group 1" HLA-C allotypes (including HLA-Cw1, -3, -7, and -8), whereas KIR2DL1 (p58.1) recognizes "group 2" HLA-C allotypes (such as HLA-Cw2, -4, -5, and -6). The recognition by KIR2DL1 is dictated by the presence of a Lys residue at position 80 of HLA-C alleles. KIR2DL2 and KIR2DL3 recognition is dictated by the presence of an Asn residue at position 80 in HLA-C. Importantly, the great majority of HLA-C alleles have either an Asn or a Lys residue at position 80. Therefore, KIR2DL1, -2, and -3 collectively recognize essentially all HLA-C allotypes found in humans. One KIR with three Ig domains, KIR3DL1 (p70), recognizes an epitope shared by HLA-Bw4 alleles. Finally, KIR3DL2 (p140), a homodimer of molecules with three Ig domains, recognizes HLA-A3 and -A11.

Although multiple inhibitory KIRs and/or other MHC class I-specific inhibitory receptors (Moretta et al, Eur J Immunogenet. 1997;24(6):455-68; Valiante et al, Immunol Rev 1997;155:155-64; Lanier, Annu Rev Immunol 1998;16:359-93) may be co-expressed by NK cells, in any given individual's NK repertoire there are cells that express only a single KIR, and thus are inhibited only by specific MHC class I alleles (or alleles belonging to the same group of MHC class I allotypes). Human MHC class I molecules often are referred to as Human Histocompatibility Antigen (HLA) class I.

NK cell populations or clones that are KIR-ligand mismatched with respect to their targets, *i.e.*, that express KIRs which do not recognize any HLA molecule of a host, have been shown to mediate potent, life-saving anti-tumor responses after allogeneic bone-marrow transplantation in leukemia patients (Ruggeri et al., Science 2002,295:2097-2100). The underlying mechanism is believed to be that HLA mismatched hematopoietic transplantation leads to the expansion of donor-derived NK cells expressing KIR which do not recognize any HLA ligands in the recipient, and thus are not inhibited via KIR. These allogeneic NK clones exert potent anti-tumor activity. This response is very strong in patients diagnosed with acute myeloid leukaemia (AML), and treated with KIR-MHC mismatched haploidentical transplants. One way of reproducing this effect by pharmacological treatment of a patient would be to administer reagents that block the KIR/HLA interaction to activate the patient's endogenous NK cells.

Certain monoclonal antibodies specific for KIR2DL1 have been shown to block the interaction of KIR2DL1 with "group 2" HLA-C allotypes, such as HLA-Cw4 (Moretta et al., J Exp Med 1993;178:597-604), and to promote NK-mediated lysis of target cells that express those HLA-C allotypes. Monoclonal antibodies against KIR2DL2/3 that block the interaction of KIR2DL2/3 with HLA-Cw3 or similar allotypes have also been described (Moretta et al., J

Exp Med 1993;178:597-604). Such antibodies are not ideal for use in clinical situations, as the development of two therapeutic monoclonal antibodies (mAbs) and administration of both of such antibodies or a selection of one of such antibodies (after appropriate diagnosis) would be required to treat all potential patients, depending on whether any given patient was expressing group 1 or group 2 HLA-C allotypes.

Watzl et al. (Tissue Antigens 2000;56:240-247) produced cross-reacting murine antibodies recognizing multiple isotypes of KIRs, but those antibodies did not potentiate the lytic activity of NK cells. Further, Spaggiari et al. (Blood 2002;99:1706-1714 and Blood 2002;100:4098-4107) carried out experiments utilizing numerous murine monoclonal antibodies against various KIRs. One of those antibodies, NKVSF1 (also known as Pan2D), was reported to recognize a common epitope of KIR2DL1 (CD158a), KIR2DL2 (CD158b) and KIR2DS4 (p50.3). Shin et al. (Hybridoma 1999;18:521-7) also reported the production of two monoclonal antibodies, denoted A210 and A803g, capable of binding to all of KIR2DL1, KIR2DL3, and KIR2DS4. However, for therapeutic use of an antibody in blocking the inhibitory KIRs of a patient's NK cells, the fewer activating KIR molecules an antibody cross-reacts with, the better, since blockade of activating receptors could impair the stimulation of NK cells. Thus, an antibody having the antigen-binding characteristics of NKVSF1, A210, or A803g would not be optimal in a clinical setting. Additionally, the use of murine monoclonal antibodies in the treatment of a human patient may result in a host immune-response against the antibodies, thus compromising the efficacy of the treatment.

Accordingly, practical and effective approaches for the therapeutic modulation of inhibitory KIRs have not been made available so far in the art.

SUMMARY OF THE INVENTION

This invention provides novel and useful human antibodies that specifically bind to KIR2DL1 and at least one of KIR2DL2 and KIR2DL3, or to all three of these KIRs, and/or human antibodies which potentiate NK-cell lytic activity by blocking the interactions between one or more such KIRs and HLA-C. Fragments and derivatives of such antibodies are also provided. The invention also pertains to novel and useful antibodies, antibody fragments, and derivatives of antibodies, which comprise VH and VL sequences substantially identical to those of human antibodies 1-7F9 and 1-4F1, as described herein. The invention also provides nucleic acids comprising nucleotide sequences encoding such antibodies; vectors comprising such nucleic acids; host cells and organisms comprising such nucleic acids and/or vectors; and compositions, such as pharmaceutically acceptable compositions and kits, comprising such proteins, nucleic acids, vectors, and/or cells and typically one or more

additional ingredients that can be active ingredients or inactive ingredients that promote formulation, delivery, stability, or other characteristics of the composition (*e.g.*, various carriers). The invention further provides various new and useful methods making and using such antibodies, nucleic acids, vectors, cells, organisms, and/or compositions, such as in the modulation of KIR-mediated biological activities, for example in the treatment of diseases related thereto.

In one aspect, the invention provides a human or humanized antibody that binds to each one of KIR2DL1, KIR2DL2, and KIR2DL3, but which does not bind to KIR2DS4. In one embodiment, the antibody further does not bind to KIR2DS3. In another embodiment, the human or humanized antibody blocks the binding of at least one of KIR2DL1, KIR2DL2, and KIR2DL3 to an HLA-C class I molecule. In a further embodiment, the antibody may block the binding of an HLA-Cw4 molecule to KIR2DL1, and the binding of an HLA-Cw3 molecule to at least one of KIR2DL2 or KIR2DL3. For example, the antibody may block the binding of an HLA-Cw4 molecule to residues M44, F45 and D72 of the extracellular portion of KIR2DL1 (SEQ ID NO:23). In yet another embodiment, the antibody potentiates the lytic activity of an NK cell against a human target cell expressing an HLA-C class I molecule.

In another aspect, the invention provides a human or humanized antibody that competes with an antibody comprising a light chain variable region comprising the amino acid sequence of SEQ ID NO:15, and a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:17, in the binding to at least one of KIR2DL1, KIR2DL2, and KIR2DL3. In one embodiment, the antibody competes with an antibody comprising a light chain variable region comprising the amino acid sequence of SEQ ID NO:15, and a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:17, in the binding to each of KIR2DL1, KIR2DL2, and KIR2DL3. In another embodiment, the antibody comprises a light chain variable region comprising the amino acid sequence of SEQ ID NO:15. In yet another embodiment, the antibody comprises (a) a heavy chain CDR1 amino acid sequence corresponding to residues 31-35 of SEQ ID NO:17; (b) a heavy chain CDR2 amino acid sequence corresponding to residues 50-65 of SEQ ID NO:17; and (c) a heavy chain CDR3 amino acid sequence corresponding to residues 99-112 of SEQ ID NO:17. For example, the antibody may comprise a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:17.

In another aspect, the invention provides an isolated human or humanized antibody that binds to a KIR2DL1 epitope substantially comprising the amino acid residues L38, R41, M44, F45, N46, D47, T48, L49, R50, I52, F64, D72, Y80, P87, and Y88.

In another aspect, the invention provides an isolated human or humanized antibody that has a dissociation constant (K_d) for KIR2DL1 of no more than about 0.45 nM and/or a K_d for KIR2DL3 of no more than about 0.025 nM.

The invention also provides a human or humanized antibody or antibody fragment, or a derivative thereof, which has any of the foregoing properties, alone or in any suitable combination. In one embodiment, the antibody is a monoclonal antibody. In another embodiment, the antibody is an IgG1, IgG2, IgG3, or IgG4 antibody. For example, the antibody may be an IgG4 antibody.

The invention also provides a nucleic acid encoding the human or humanized antibody or antibody fragment having any of the foregoing properties, a vector comprising such a nucleic acid, a cell comprising such a vector, and a method of producing a human anti-KIR antibody, comprising culturing such a cell under conditions suitable for expression of the anti-KIR antibody.

The invention also provides a pharmaceutical composition comprising a human or humanized antibody or antibody fragment having one or more of the foregoing properties or produced by any method, in an amount effective to detectably potentiate NK cell cytotoxicity in a patient, together with a pharmaceutically acceptable carrier or excipient. The composition may, for example, comprise Polysorbate 80, sucrose, or both Polysorbate 80 and sucrose.

The invention also provides a method of potentiating NK cell activity in a subject in need thereof, which method comprises administering to the subject an effective amount of any of the foregoing compositions. In one embodiment, the subject is a patient suffering from cancer. For example, the patient may be suffering from a cancer selected from acute myeloid leukaemia, chronic myeloid leukaemia, multiple myeloma, and non-Hodgkin's lymphoma. Alternatively, the patient may be suffering from a cancer selected from colorectal cancer, renal cancer, ovarian cancer, lung cancer, breast cancer, and malignant melanoma. In another embodiment, the subject is a patient suffering from an infectious disease. In yet another embodiment, the method further comprises administering a therapeutic agent selected from an immunomodulatory agent, a hormonal agent, a chemotherapeutic agent, an anti-angiogenic agent, an apoptotic agent, a second antibody that binds to an inhibitory KIR, an anti-infective agent, a targeting agent, and an adjunct compound.

It should be understood that description of antibodies "provided" by the invention or antibodies to which the invention "relates", etc., also, by implication, refers to antibodies that may be useful in practice of the inventive methods described herein, unless otherwise stated or clearly contradicted by context.

These and other aspects and features of the invention are described in further detail herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the murine monoclonal antibody DF200 binding to a common determinant of various human KIR2DL receptors. (A) Clone CP11, KIR2DL1+, DF200. (B) Clone CP502, KIR2DL3+. (C) Clone CP11, KIR2DL1+, Anti-KIR2DL1. (D) Clone CP502, KIR2DL3+, Anti-KIR2DL2/3.

Figure 2 depicts the murine monoclonal antibody DF200 neutralizing the KIR2DL1-mediated inhibition of NK cell cytotoxicity against Cw4-expressing target cells, showing the reconstitution of lysis with the anti-KIR mAb on C1R-Cw4 target at effector/target (E/T) ratios of 4/1.

Figure 3 depicts the murine monoclonal antibody DF200, a Fab fragment of DF200, and conventional murine antibodies specific for either KIR2DL1 (mAbs EB6 and XA-141) or KIR2DL2/3 (mAbs GL183 and Y249) neutralizing the KIR2DL1-mediated inhibition of cytotoxicity against Cw4-positive target cells, and the KIR2DL2/3-mediated inhibition of NK cell cytotoxicity against Cw3-positive target cells. (A) and (C) KIR2DL1+, target cells 721.221-Cw4+. (B) KIR2DL2+, target cells 721.221-Cw3+. (D) KIR2DL3+, target cells 721.221-Cw3+.

Figure 4 depicts reconstitution of cell lysis by NK cells that express KIR2DL1 against HLA-Cw4 positive target cells in the presence of F(ab')2 fragments of the DF200 and EB6 antibodies. (A) Target cells 721.221-Cw4; E/T ratio = 1. (B) Target cells B-EBV cells expressing Cw4, E/T ratio = 2.

Figure 5 depicts induction of NK-mediated killing by cross-reactive murine (DF200 and NKVSF1 (Pan2D)) and human (1-7F9, 1-4F1, 1-6F5 and 1-6F1) mAbs, and a murine conventional KIR2DL1-specific mAb (EB6). The mAbs induce (reconstitute) killing by KIR2DL1-expressing NK cells (YTS-KIR2DL1) of 721.221 target cells transfected with HLA-Cw4. E/T ratio = 1. (A) 30 µg/ml mAb. (B) 10 µg/ml mAb.

Figure 6 depicts induction of NK-mediated killing by the same antibodies as in Figure 5. The mAbs induce (reconstitute) killing by KIR2DL1-expressing NK cells (YTS-KIR2DL1) of B-EBV cells expressing endogenous HLA-Cw4. E/T ratio = 2. (A) 30 µg/ml mAb. (B) 10 µg/ml mAb.

Figure 7 depicts an epitope map showing results of competitive binding experiments obtained by surface plasmon resonance (BIAcore®) analysis with anti-KIR antibodies to KIR2DL1, where overlapping circles designate overlap among the mAbs in binding to

KIR2DL1. Results show that 1-7F9 is competitive with EB6 and 1-4F1, but not with NKVSF1 (Pan2D) and DF200, on KIR2DL1. Antibody 1-4F1 in turn is competitive with EB6, DF200, NKVSF1 (Pan2D), and 1-7F9. Antibody NKVSF1 (Pan2D) competes with DF200, 1-4F1, and EB6, but not 1-7F9, on KIR2DL1. DF200 competes with NKVSF1 (Pan2D), 1-4F1, and EB6, but not 1-7F9, on KIR2DL1.

Figure 8 depicts an epitope map showing results of competitive binding experiments obtained by BIAcore® analysis with anti-KIR antibodies to KIR2DL3, where overlapping circles designate overlap in binding to KIR2DL3. Results show that 1-4F1 is competitive with NKVSF1 (Pan2D), DF200, gl183, and 1-7F9 on KIR2DL3. 1-7F9 is competitive with DF200, gl183, and 1-4F1, but not with NKVSF1 (Pan2D), on KIR2DL3. NKVSF1 (Pan2D) competes with DF200, 1-4F1, and GL183, but not 1-7F9, on KIR2DL3. DF200 competes with NKVSF1 (Pan2D), 1-4F1, and 1-7F9, but not with GL183, on KIR2DL3.

Figure 9 depicts an epitope map showing results of competitive binding experiments obtained by BIAcore® analysis with anti-KIR antibodies to KIR2DS1, where overlapping circles designate overlap in binding to KIR2DS1. Results show that antibody 1-4F1 is competitive with NKVSF1 (Pan2D), DF200, and 1-7F9 on KIR2DS1. Antibody 1-7F9 is competitive with 1-4F1, but not competitive with DF200 and NKVSF1 (Pan2D) on KIR2DS1. NKVSF1 competes with DF200 and 1-4F1, but not with 1-7F9, on KIR2DS1. DF200 competes with NKVSF1 and 1-4F1, but not with 1-7F9, on KIR2DS1.

Figure 10 depicts Pan2D (NKVSF1) mAb titration demonstrating binding of the mAb to cynomolgus NK cells. Cynomolgus NK cells (NK bulk day 16) were incubated with different amount of NKVSF1 (Pan2D) mAb followed by PE-conjugated goat F(ab')2 fragments anti-mouse IgG (H+L) antibodies. The percentage of positive cells was determined with an isotypic control (purified mouse IgG1). Samples were done in duplicate. Mean fluorescence intensity = MFI.

Figure 11 shows the binding of soluble KIR2DL1 and KIR2DL1(R131W) mutant to cells. (A) Binding of increasing concentrations of soluble KIR2DL1-Fc, and a KIR2DL1(R131W)-hFc mutant, to cells expressing HLA-Cw3 or –Cw4. The bound KIR-Fc proteins were detected using a secondary fluorochrome-conjugated antibody, and revealed by flow-cytometry. Mean fluorescence is shown on the y-axis. (B) Binding of the indicated anti-KIR mAbs (GL183, EB6, DF200, and Pan2D (NKVSF1)) to KIR2DL1-Fc and the 2DL1(R131W) mutant protein, using human Ig as control. The figure shows that DF200 and Pan2D (NKVSF1) bind less well to the mutant than the wild-type KIR2DL1-Fc, indicating that both mAbs are affected by the R131W mutation. Therefore, R131 is one of the residues constituting the epitope for DF200 and Pan2D on KIR2DL1.

Figure 12 provides a comparative alignment of the amino acid sequences of the light chain variable regions, and light chain CDRs of antibodies DF200 and Pan2D (NKVSF1). (A) Alignment of anti-KIR variable light (VL) regions of DF200 (SEQ ID NO:1) and Pan-2D (SEQ ID NO:2). Numbers above amino acid sequences indicate position respective to initiation of translation Met (+1) in the immature (non-secreted) immunoglobulin. (B) Alignment of CDR-L1 sequences. Residue before: Normally Cys. Residues after: Trp. Typically Trp-Tyr-Leu.Length: 10-17 aa. (C) Alignment of CDR-L2 sequences. Residues before: Generally Ile-Tyr. Length: 7 aa. Start: approximately 16 aa after the end of CDR-L1. Start: approximately 24 aa from the beginning of secreted protein. (D) Alignment of CDR-L3 sequences. Residues before: Cys. Residues after: Phe-Gly-XXX-Gly. Length: 7-11 aa. Start: approximately 33 aa after the end of CDR-L2.

Figure 13 provides the heavy chain variable region, and the heavy-chain CDRs of antibody DF200. (A) DF-200 VH region, immature protein. The secreted, mature VH starts at position 20: residue Q. The VH region ends with residue S and thereafter the constant region (not shown) continues. (B) CDR-H1. Residues before: Cys-XXX-XXX-XXX. Residues after: Trp. Generally Trp-Val or Trp-Ile. Length: 10-14 aa. Start: Approximately 22-26 aa from the beginning of the secreted protein. (C) CDR-H2. Residues before: Leu-Glu-Trp-Ile-Gly but other variations possible. Residues after: Lys or Arg / Leu or Ile or Val or Phe or Thr or Ala / Thr or Ser or Ile or Ala. Length: 16-20 aa. Start: Approximately 15 aa after the end of CDR-H1. (D) CDR-H3. Residues before: Cys-XXX-XXX (Typically Cys-Ala-Arg). Residues after: Trp-Gly-XXX-Gly. Length: 3-25 aa. Start: Approximately 33 after the end of CDR-H2.

Figure 14 depicts the nucleotide and amino acid sequences of the VH and VL sequence of human antibody 1-7F9. (A) Translation of HuKIR 1-7F9 mature variable light chain. (B) Nucleotide sequence encoding HuKIR 1-7F9 mature variable light chain. (C) Translation of HuKIR 1-7F9 mature variable heavy chain. (D) Nucleotide sequence encoding HuKIR 1-7F9 mature heavy chain.

Figure 15 shows the amino acid sequences of the VH and VL sequences of monoclonal antibodies 1-7F9, DF200 (VH sequence: SEQ ID NO:19; VL sequence: SEQ ID NO:21), and Pan2D (NKVSF1; VH sequence: SEQ ID NO:20; VL sequence: SEQ ID NO:22). The CDRs are boxed.

Figure 16 depicts flow cytometry data, showing binding of the murine antibody, Pan2D (NKVSF1) and the human mAbs 1-7F9 and 1-4F1 to cells transfected with KIR2DL1, KIR2DL3, KIR2DS1, KIR2DS2, KIR2DS3 or KIR2DS4, as indicated (purified antibody: 1 µg/ml). (A) - (R) NKVSF1 (pan2D), 1-7F9, and 1-4F1 all bind to KIR2DL1, KIR2DL3,

KIR2DS1, and KIR2DS2. Neither antibody binds to KIR2DS3. NKVSF1, but not 1-7F9 or 1-4F1, binds to KIR2DS4.

Figure 17 depicts FACS analysis results. (A) Antibody-mediated neutralization of binding of soluble KIR-Fc to HLA-Cw4 on cells, detected by flow cytometry (FACS). FACS-measurement of KIR2DL1-mFc binding to LCL721.221-Cw4 cells, after pre-incubation with various concentrations of the cross-reactive mAb DF200, or the KIR2DL2/3-specific mAb GL183. DF200, but not GL183, reduces binding of KIR2DL1 to HLA-Cw4. Measurements are plotted as a percentage of KIR2DL1-hFc-binding in the absence of inhibitory antibodies. (B) FACS-measurement of KIR2DL1-mFc binding to LCL721.221-Cw4 cells, after pre-incubation with various concentrations of 1-1F4 and 1-7F9. Measurements are plotted as a percentage of KIR2DL1-mFc-binding in the absence of inhibitory antibodies.

Figure 18 depicts results of ^{51}Cr-release cytotoxicity assays. (A) LCL 721.221-Cw3 cells are efficiently killed by both YTS cells (diamonds) and YTS-2DL1 cells (triangles) at various E:T ratios. In contrast, LCL 721.221-Cw4 cells are efficiently killed by YTS cells (squares), but can not be killed by YTS-2DL1 cells (crosses) due to KIR-restrictions. (B) In the absence of antibody, YTS-2DL1 cells cannot kill LCL-721.221-Cw4 (E:T ratio 12:1). 1-7F9 induces killing of LCL 721.221-Cw4 cells by YTS-2DL1 cells in a dose-dependent fashion.

Figure 19 depicts a superposition of two crystal complex structures; the KIR2DL1/1-7F9 Fab' and the KIR2DL1/MHC class I, PDB-code 1IM9 (Fan et al., Nat. Immunol. 2001;2;452-460), using the C_α-atoms of the common KIR molecule (labeled 'KIR') as template. KIR2DL1/1-7F9 Fab' is indicated in grey line ribbon style while KIR2DL1/MHC class I in a dark tube style. 1-7F9 Fab' is labeled '1-7F9' while MHC class I is labeled 'MHC'. An overlap between the MHC and the Fab' is seen in the superposition, demonstrating the ability of 1-7F9 Fab' to obstruct MHC class I binding to the KIR2DL1 receptor. See Example 11.

Figure 20 shows the binding epitope of 1-7F9 on KIR2DL1, as indicated in the KIR2DL1 sequence. Amino acids within 4.0 Å distance from 1-7F9 are highlighted in grey and black background. Amino acids highlighted by a black background are involved in hydrogen-bonding to 1-7F9.

DEFINITIONS

For convenience, several terms are defined here. However, the list of defined terms provided here is not exclusive. Other terms may be defined throughout the description of the invention.

Within the context of this invention "active" NK cells designate biologically active NK cells, including NK cells having the capacity of lysing target cells or enhancing the immune function of other cells. For instance, an "active" NK cell can be able to kill cells that express a ligand for an activating NK receptor and/or fail to express MHC/HLA antigens recognized by a KIR on the NK cell. NK cells can be obtained by various techniques known in the art, such as isolation from blood samples, cytapheresis, tissue or cell collections, etc. Useful protocols for assays involving NK cells can be found in Natural Killer Cells Protocols (edited by Campbell KS and Colonna M). Human Press. pp. 219-238 (2000).

As used herein, a "Killer Ig-like Receptor", "Killer Inhibitory Receptor", or "KIR", refers to a protein or polypeptide encoded by a gene that is a member of the KIR gene family or by a cDNA prepared from such a gene. A detailed review of the KIR gene family, including the nomenclature of KIR genes and KIR gene products, and Genbank accession numbers for exemplary KIRs, is "The KIR Gene Cluster" by M. Carrington and P. Norman, available at the NCBI web-site called "Bookshelf" (accessible via the World-Wide Web (WWW) address ncbi.nlm.nih.gov/books). The sequences of human KIR genes and cDNAs, as well as their protein products, are available in public databases, including GenBank. Non-limiting exemplary GenBank entries of human KIRs have the following accession numbers: KIR2DL1: Genbank accession number U24076, NM_014218, , AAR16197, or L41267; KIR2DL2: Genbank accession number U24075 or L76669; KIR2DL3: Genbank accession number U24074 or L41268; KIR2DL4: Genbank accession number X97229; KIR2DS1: Genbank accession number X89892; KIR2DS2: Genbank accession number L76667; KIR2DS3: Genbank accession number NM_012312 or L76670 (splice variant); KIR3DL1: Genbank accession number L41269; and KIR2DS4: Genbank accession number AAR26325. A KIR may comprise from 1 to 3 extracellular domains, and may have a long (*i.e.*, more than 40 amino acids) or short (*i.e.*, less than 40 amino acids) cytoplasmic tail. As previously described herein, these features determine the nomenclature of a KIR. Exemplary KIR2DL1, KIR2DL2, KIR2DL3, and KIR2DS4 molecules comprise the following respective amino acid sequences:

KIR2DL1 extracellular domain:

HEGVHRKPSLLAHPGXLVKSEETVILQCWSDVMFEHFLLHREGMFNDTLRLIGEHH DGVSKANFSISRMTQDLAGTYRCYGSVTHSPYQVSAPSDPLDIVIIGLYEKPSLSAQXGPTVL AGENVTLSCSSRSSYDMYHLSREGEAHERRLPAGPKVNGTFQADFPLGPATHGGTYRCFG SFHDSPYEWSKSSDPLLVSVTGNPSNSWPSPTEPSSKTGNPRHLH (SEQ ID NO:23), where "X" at position 16 is P or R, and where "X" at position 114 is P or L, representing allelic variants.

KIR2DL2 extracellular domain:

HEGVHRKPSLLAHPGRLVKSEETVILQCWSDVRFEHFLLHREGKFKDTLHLIGEHH DGVSKANFSIGPMMQDLAGTYRCYGSVTHSPYQLSAPSDPLDIVITGLYEKPSLSAQPGPTV LAGESVTLSCSSRSSYDMYHLSREGEAHECRFSAGPKVNGTFQADFPLGPATHGGTYRCF GSFRDSPYEWSNSSDPLLVSVIGNPSNSWPSPTEPSSKTGNPRHLH (SEQ ID NO:24)

KIR2DL3 extracellular domain:

HEGVHRKPSLLAHPGPLVKSEETVILQCWSDVRFQHFLLHREGKFKDTLHLIGEHH DGVSKANFSIGPMMQDLAGTYRCYGSVTHSPYQLSAPSDPLDIVITGLYEKPSLSAQPGPTV LAGESVTLSCSSRSSYDMYHLSREGEAHERRFSAGPKVNGTFQADFPLGPATHGGTYRCF GSFRDSPYEWSNSSDPLLVSVTGNPSNSWPSPTEPSSETGNPRHLH (SEQ ID NO:25).

KIR2DS4 extracellular domain:

QEGVHRKPSFLALPGHLVKSEETVILQCWSDVMFEHFLLHREGKFNNTLHLIGEHH DGVSKANFSIGPMMPVLAGTYRCYGSVPHSPYQLSAPSDPLDMV (SEQ ID NO:38).

The term "KIR2DL2/3" refers to either or both of the KIR2DL2 and KIR2DL3 receptors. These two receptors have a very high homology, they are allelic forms of the same gene, and are considered by the art to be functionally similar.

Unless otherwise specified, term "MHC" encompasses MHC molecules in all mammals, whereas an "HLA" molecule refers to a human MHC molecule.

Within the context of this invention, stating that an antibody "binds" a determinant (*i.e.*, the word "bind" in the context of antibody:determinant interaction) designates that the antibody binds the determinant with specificity and/or affinity. For example, GL183 is a conventional monoclonal antibody that binds to KIR2DL2/3. EB6 is a conventional monoclonal antibody that binds to KIR2DL1. EB6 and GL183 are both commercially available (Beckman Coulter Inc., Fullerton, CA).

A "cross-reactive" anti-KIR antibody is an antibody that binds more than one KIR molecule with specificity and/or affinity. For example, DF200 and 1-7F9 are monoclonal antibodies cross-reactive with KIR2DL1, -2, and -3. The hybridoma producing antibody DF200 has been deposited at the CNCM culture collection, under identification no. "DF200", registration no. CNCM I-3224, registered 10 June 2004, Collection Nationale de Cultures de Microorganismes, Institut Pasteur, 25, Rue du Docteur Roux, F-75724 Paris Cedex 15, France. NKVSF1, also referred to as "Pan2D" herein, is cross-reactive with KIR2DL1, -2, and -3 and KIR2DS4. This antibody is commercially available from Serotec (Cergy Sainte-Christophe, France), Catalog ref no. MCA2243.

"Specific binding" or "specificity" refers to the ability of an antibody or other agent to detectably bind an epitope presented on an antigen, such as a KIR, while having relatively little detectable reactivity with other proteins or structures (such as other proteins presented

on NK cells, or on other cell types). Specificity can be relatively determined by binding or competitive binding assays, using, *e.g.*, Biacore instruments, as described elsewhere herein. Specificity can be exhibited by, *e.g.*, an about 10:1, about 20:1, about 50:1, about 100:1, 10.000:1 or greater ratio of affinity/avidity in binding to the specific antigen versus nonspecific binding to other irrelevant molecules (in this case the specific antigen is a KIR). A KIR-binding antibody, specific for a KIR presented on the NK cells of a particular organism, such as a human, can sometimes exhibit binding to similar KIRs of other species (*i.e.*, a KIR-binding antibody, or other KIR-binding agent, may cross-react with KIRs of various species).

"Selectivity" refers to the preferential binding of a protein to a particular region, target, or peptide as opposed to one or more other biological molecules, structures, cells, tissues, etc. A KIR-binding antibody can also be selective for a KIR produced in a particular organism (*e.g.*, in a human as opposed to in a primate), and/or for a particular type of KIR (*e.g.*, a KIR with a long cytoplasmic tail), particularly where the antibody cross-reacts with more than one type of KIR produced in a particular organism, and/or a particular portion of a KIR (such as a particular epitope or antigenic determinant region). For example, selectivity can be determined by competitive ELISA or Biacore assays. The difference in affinity/avidity that marks selectivity can be any detectable preference (*e.g.*, a ratio of more than 1:1.1, or more than about 1:5, if detectable, would be suitable, including 1:10, 1:100, 1:1000 or more). Unless otherwise specified, any quantitative data on "affinity" presented herein refers to measurements of bivalent (as opposed to monovalent) binding.

An "epitope" or "binding site" is an area or region on an antigen to which an antigen-binding peptide (such as an antibody) specifically binds. A protein epitope may comprise amino acid residues directly involved in the binding (also called immunodominant component of the epitope) and other amino acid residues, which are not directly involved in the binding, such as amino acid residues which are effectively blocked by the specifically antigen binding peptide (in other words, the amino acid residue is within the "footprint" of the specifically antigen binding peptide). The term epitope herein includes both types of amino acid binding sites in any particular region of a KIR that specifically binds to an anti-KIR antibody, or another KIR-specific agent according to the invention, unless otherwise stated (*e.g.*, in some contexts the invention relates to antibodies that bind directly to particular amino acid residues) . KIRs may comprise a number of different epitopes, which may include, without limitation, (1) linear peptide antigenic determinants, (2) conformational antigenic determinants which consist of one or more non-contiguous amino acids located near each other in a mature KIR conformation; and (3) post-translational antigenic determinants which consist, either

in whole or part, of molecular structures covalently attached to a KIR, such as carbohydrate groups.

The phrase that a first antibody binds "substantially" or "at least partially" the same epitope as a second antibody means that the epitope binding site for the first antibody comprises at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more of the amino acid residues on the antigen that constitutes the epitope binding site of the second antibody. Also, that a first antibody binds substantially or partially the same epitope as a second antibody means that the first and second antibodies compete in binding to the antigen, as described above. Thus, the term "binds to substantially the same epitope or determinant as" the monoclonal antibody 1-7F9 means that an antibody "competes" with 1-7F9. Generally, an antibody that "binds to substantially the same epitope or determinant as" the monoclonal antibody of interest (*e.g.* DF200, NKVSF1, 1-7F9) means that the antibody "competes" with said antibody of interest for binding to one or more KIR molecules, preferably a KIR molecule selected from the group consisting of KIR2DL1 and KIR2DL2/3. In other examples, an antibody that binds to substantially the same epitope or determinant on a KIR2DL1 molecule as the antibody of interest "competes" with the antibody of interest for binding to KIR2DL1. An antibody that binds to substantially the same epitope or determinant on a KIR2DL2/3 molecule as the antibody of interest "competes" with the antibody of interest for binding to KIR2DL2/3.

The phrase "binds to essentially the same epitope or determinant as" an antibody of interest means that an antibody "competes" with said antibody of interest for at least one, or for any and all KIR molecules to which said antibody of interest specifically binds. The phrase "binds to essentially the same epitope or determinant as" the monoclonal antibody 1-7F9 means that an antibody "competes" with 1-7F9 for at least one, preferably any and all KIR molecules, to which 1-7F9 specifically binds. For example, an antibody that binds to essentially the same epitope or determinant as the monoclonal antibodies 1-7F9 or NKVSF1 can "compete" with said 1-7F9 or NKVSF1 respectively for binding to KIR2DL1, KIR2DL2/3, KIR2DS1 and KIR2DS2.

The ability of an anti-KIR antibody to "block" the binding of a KIR molecule and an HLA molecule means that the antibody, in an assay using soluble or cell-surface associated KIR and HLA molecules, can detectably reduce the binding of a KIR-molecule to an HLA molecule in a dose-dependent fashion, where the KIR molecule detectably binds to the HLA molecule in the absence of the antibody. An exemplary assay for determining whether an anti-KIR antibody is capable of such blocking is provided in Example 8.

The ability of an anti-KIR antibody to "reduce the inhibitory activity of a KIR", "facilitate NK cell activity," "facilitate NK cell cytotoxicity," "facilitate NK cells," "potentiate NK cell activity," "potentiate NK cell cytotoxicity," or "potentiate NK cells" means that an NK cell expressing a KIR, when contacted with the antibody, is capable of lysing target cells that express on their surface a particular MHC or HLA class I which is a ligand for said KIR. For example, the term "potentiation in NK cytotoxicity" means any substantial potentiation, or at least 5%, 10%, 20%, 30% or greater potentiation in NK cytotoxicity, *e.g.* at least about 50% potentiation of NK cytotoxicity (*e.g.*, at least about 60%, at least about 70%, at least about 80%, at least about 85%, at least about 90%, or at least about 95% as compared to a control. This includes, for example, about 55-100%, about 65-100%, about 75-100%, about 80-100%, or about 90-100% potentiation of NK cell cytotoxicity, as compared to a control. It also includes more than about 100%, more than about 500%, more than about 1000%, more than about 2000%, or higher increase in the cytotoxicity. This can be measured in, *e.g.*, an assay, such as a standard cytotoxicity assay, where a higher number, amounting to a higher percentage, of target cells are lysed by NK cells in the presence of an NK potentiator (such as an anti-KIR mAb) than in the absence of the potentiator (*i.e.*, the control).

The term "neutralize KIR-mediated inhibition of NK cell cytotoxicity" or "neutralize the inhibitory activity of a KIR," as used herein means the ability to increase specific lysis to more than about 20%, preferably with at least about 30%, at least about 40%, at least about 50%, at least about 100%, or more of the specific lysis obtained at the same effector:target cell ratio with NK cells or NK cell lines that are not blocked by their KIR, as measured by a classical chromium release test of cytotoxicity. "Neutralize KIR mediated inhibition" can also mean that in a chromium-release assay, or other cytotoxicity assay, using an NK cell clone or transfectant expressing one or several inhibitory KIRs and a target cell expressing at least one HLA class I allele that is recognized by one of the KIRs on the NK cell, the specific lysis obtained with the antibody is more than about 100%, preferably at least about 101%, at least about 150%, at least about 200%, or more (*e.g.*, about 101-150%, about 120-150%, about 120-200%, about 150-200%, or about 200-1000%), or more of the specific lysis obtained with the same concentration of NKVSF1 (commercially available from Serotec), using the same NK cells and target cells, at the same effector:target cell ratio.

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions identical to, essentially identical to, or derived from human germline immunoglobulin sequences. Such human antibodies can include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). How-

ever, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

In the context of the present invention, unless otherwise stated, "treatment" or "treating" refers to preventing, alleviating, managing, curing or reducing one or more symptoms or clinically relevant manifestations of a disease or disorder, unless contradicted by context. For example, "treatment" of a patient in whom no symptoms or clinically relevant manifestations of a disease or disorder have been identified is preventive therapy, whereas "treatment" of a patient in whom symptoms or clinically relevant manifestations of a disease or disorder have been identified generally does not constitute preventive therapy. Nonetheless, it should be understood that the various therapeutic and prophylactic method and use facets of the invention are distinct from one in many respects (*e.g.*, dosage of compound(s) to be delivered to a subject, timing of application, impetus for application, etc.) and may each be considered a unique facet of the invention.

In the context of the present invention "cancer" refers to any neoplastic disorder, including, but not limited to, such cellular disorders as sarcoma, carcinoma, melanoma, leukaemia, and lymphoma, which may include cancers in the breast, head and neck, ovaries, bladder, lung, pharynx, larynx, oesophagus, stomach, small intestines, liver, pancreas, colon, female reproductive tract, male reproductive tract, prostate, kidneys and central nervous system.

The term "biological sample" as used herein includes but is not limited to a biological fluid (for example serum, lymph, and blood), cell sample or tissue sample (for example bone marrow or tumor tissue) derived from a human or non-human mammal.

The term "substantially identical" in the context of two amino acid sequences means that the sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least about 50, at least about 60, at least about 70, at least about 80, at least about 90, at least about 95, at least about 98, or at least about 99 percent sequence identity. In one embodiment, residue positions that are not identical differ by conservative amino acid substitutions (further described elsewhere herein). Sequence identity is typically measured using sequence analysis software. Protein analysis software matches similar sequences using measures of similarity assigned to various substitutions, deletions and other modifications, including conservative amino acid substitutions. For instance, the publicly available GCG software contains programs such as "Gap" and "BestFit" which can be used with default parameters to determine sequence homology or sequence identity between closely related polypeptides, such as homologous polypeptides from different species

of organisms or between a wild-type protein and a mutein thereof. See, *e.g.*, GCG Version 6.1. Polypeptide sequences can also be compared using FASTA, applying default or recommended parameters. A program in GCG Version 6.1., FASTA (*e.g.*, FASTA2 and FASTA3) provides alignments and percent sequence identity of the regions of the best overlap between the query and search sequences (Pearson, Methods Enzymol. 1990;183:63-98; Pearson, Methods Mol. Biol. 2000;132:185-219). Another preferred algorithm when comparing a sequence to a database containing a large number of sequences from various organisms is the computer program BLAST, especially blastp, using default parameters. See, *e.g.*, Altschul et al., J. Mol. Biol. 1990;215:403-410; Altschul et al., Nucleic Acids Res. 1997;25:3389-402 (1997); each herein incorporated by reference. "Corresponding" amino acid positions in two substantially identical amino acid sequences are those aligned by any of the protein analysis software mentioned herein, using default parameters.

A "1-7F9-like" or "1-4F1-like" antibody as used herein means (1) an antibody that binds substantially the same epitope as an antibody comprising the VH and VL sequences of 1-7F9 or 1-4F1, respectively, such as, *e.g.*, monoclonal antibody 1-7F9 or 1-4F1, respectively, and/or (2) an antibody comprising VH and VL sequences identical or substantially identical to the VH and VL sequences of 1-7F9 or 1-4F1, respectively.

A "conservative" amino acid substitution is one in which an amino acid residue is substituted by another amino acid residue having a side chain ("R-group") with similar chemical properties (*e.g.*, charge or hydrophobicity). In general, a conservative amino acid substitution will not substantially change the functional properties of a protein. In cases where amino acid sequences differ from each other by conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well-known to those of skill in the art. See, *e.g.*, Pearson, Methods Mol. Biol. 1994;243:307-31. Examples of groups of amino acids that have side chains with similar chemical properties include 1) aliphatic side chains: glycine, alanine, valine, leucine, and isoleucine; 2) aliphatic-hydroxyl side chains: serine and threonine; 3) amide-containing side chains: asparagine and glutamine; 4) aromatic side chains: phenylalanine, tyrosine, and tryptophan; 5) basic side chains: lysine, arginine, and histidine; 6) acidic side chains: aspartic acid and glutamic acid; and 7) sulfur-containing side chains: cysteine and methionine. Exemplary conservative amino acids substitution groups include: valine-leucine-isoleucine, phenylalanine- tyrosine, lysine-arginine, alanine-valine, glutamate-aspartate, and asparagine-glutamine.

DESCRIPTION OF THE INVENTION

This invention is based on the generation of novel cross-reactive and neutralizing antibodies binding to inhibitory KIRs, the antibodies allowing effective activation of NK cells in most or all individuals in human populations.

Described herein are, for example, antibodies that bind to all of KIR2DL1, KIR2DL2, and KIR2DL3 and potentiate NK-cell lytic activity by blocking the interactions between these KIRs and HLA-C. Such antibodies are referred to herein as "cross-reactive and neutralizing anti-KIR mAbs".

In a particular aspect, the present invention relates to novel cross-reactive, neutralizing, or both crossreacting and neutralizing, fully human anti-KIR antibodies, as well as compositions comprising such antibodies and methods of using such antibodies or compositions. These antibodies include human antibodies 1-7F9 and 1-4F1, described in PCT/DK2004/000470, filed on July 1, 2004, which is hereby incorporated by reference in its entirety,

Antibodies, compositions, and methods described herein may, among other things, overcome current limitations associated with therapeutic modulation of KIR, and with therapeutic NK cell activation, and provide additional advantageous features and benefits. For example, the antibodies can cross-react with multiple inhibitory KIRs and reduce or neutralize their inhibitory signals, resulting in potentiation of NK cell cytotoxicity by NK cells expressing such inhibitory KIR receptors. The ability to cross-react with multiple KIR gene products can allow the antibodies of the invention to be effectively used to increase NK cell activity in most or all human subjects, without the burden or expense of pre-determining the KIR or HLA type of the subject. In one embodiment, the antibodies do not bind KIR2DS4, thus avoiding the reduction in stimulatory potential that would be associated with neutralization of the activating KIR2DS4 receptor. Additionally or alternatively, the antibodies do not bind KIR2DS3.

From such antibodies, various antibody fragments and derivatives can also be generated and used for the same or similar purposes as are described herein with respect to the antibodies of the invention, and aspects of the invention described with respect to antibodies apply equally to antibody fragments and derivatives, unless otherwise stated or clearly contradicted by context. In other words, a feature of the invention described herein with respect to an antibody should, unless otherwise indicated, also implicitly be understood to describe a similar feature with respect to an antibody fragment or derivative having similar functionality in terms of specificity. It should be recognized, however, that "full-length" antibodies and an-

tibody "fragments" can be characterized as distinct aspects of the invention, inasmuch as their biological and physiochemical properties may differ significantly.

Some antibodies of the invention can be characterized as "human", which are typically associated with a lower risk for an immune response against the antibodies by a human subject to whom they are administered. In one exemplary aspect, an isolated antibody that facilitates the activation of human NK cells in virtually all humans is described. In an exemplary embodiment, the antibody is human antibody 1-7F9 or a human 1-7F9-like antibody.

Antibodies, fragments, or derivatives of either thereof can cross-react with at least two inhibitory KIR receptors at the surface of NK cells, reduce or neutralize the inhibitory signals of the NK cells, and potentiate the activity of the NK cells. For example, an antibody, antibody fragment, or derivative may bind a common determinant of human KIR2DL receptors, so that the antibody, antibody fragment, or derivative binds at least KIR2DL1, KIR2DL2, and KIR2DL3 receptors. For the purposes of this invention, the term "KIR2DL2/3" refers to either or both of the KIR2DL2 and KIR2DL3 receptors.

As described herein, the anti-KIR mAbs 1-7F9 and 1-4F1 have several advantages over previously produced anti-KIR antibodies. For example, 1-7F9 and 1-4F1 are fully human, thus reducing or minimizing any immune response against the antibody once administered to a subject. Furthermore, both 1-7F9 and 1-4F1 are of suitable isotypes for therapeutic anti-KIR antibodies (IgG4 and IgG2, respectively), as described below. 1-7F9 is also more more effective at inducing killing by NK cells that express either KIR2DL1, -2, and/or -3 than murine mAbs EB6, GL183, DF200, and NKVSF1 (Pan2D). For example, as shown in Figures 5 and 6, 1-7F9 induced higher levels of specific lysis by KIR2DL1-expressing NK cells of target cells that expressed HLA-Cw4 than did EB6, DF200 or NKVSF1 (Pan2D). 1-7F9 further has a higher affinity for KIR compared to previously known anti-KIR mAbs. For example, 1-7F9 binds to KIR2DL1 and KIR2DL3 with dissociation constants (K_d's) of 0,43 nM and 0.025 nM, respectively, representing a higher affinity for both antigens than, for example, DF200 (see Examples 3 and 8). As opposed to the murine antibodies NKVSF1 (Pan2D), A210, and A208g, none of 1-7F9 and 1-4F1 binds to KIR2DS4, making them better suited for therapeutic purposes. Like NKVSF1 (Pan2D) and DF200, 1-7F9 and 1-4F1 also bind to KIR2DS1 and KIR2DS2, but KIR2DS1 and KIR2DS2 are not believed to be important in anti-leukemia efficacy. Particular antibodies according to the invention therefore have the same or similar antigen-specificities as 1-7F9 and/or 1-4F1. For example, antibodies comprising the same or similar VH and VL regions as 1-7F9 can have the same or similar antigen-binding and/or NK-stimulatory properties as 1-7F9; and antibodies comprising the same or

similar VH and VL regions as 1-4F1 can have the same or similar antigen-binding properties as 1-4F1

As shown in Figure 14, the amino acid sequences of the VL and VH regions of 1-7F9 have been determined:

1-7F9 VL region (SEQ ID NO:15):

EIVLTQSPVTLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRAT GIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWMYTFGQGTKLEIKRT

1-7F9 VH region (SEQ ID NO:17):

QVQLVQSGAEVKKPGSSVKVSCKASGGTFSFYAISWVRQAPGQGLEWMGGFIPIF GAANYAQKFQGRVTITADESTSTAYMELSSLRSDDTAVYYCARIPSGSYYYDYDMDVWGQ GTTVTVSS

The amino acid sequences of the 1-4F1 VL and VH regions are provided in SEQ ID NOS: 39 and 41, respectively, and the nucleotide sequences encoding the 1-4F1 VL and VH regions are provided in SEQ ID NOS:40 and 42, respectively. In a particular embodiment, residues 3, 4, 9, 24, 32, 41, 47, 50, 55, 71, and 74 of SEQ ID NO:39 are Q, L, S, R, A, G, L, D, E, F, and A, respectively. In another particular embodiment, residues 3, 4, 9, 24, 32, 41, 47, 50, 55, 71, and 74 of SEQ ID NO:39 are R, M, F, W, Y, A, F, Y, Q, Y, and T, respectively.

As shown in Figure 15, the amino acid sequences of the 1-7F9 CDRs have been identified as follows: the light chain CDR1 amino acid sequence corresponds to residues 24-34 of SEQ ID NO:15; the light chain CDR2 amino acid sequence corresponds to residues 50-56 of SEQ ID NO:15; the light chain CDR3 amino acid sequence corresponds to residues 89-97 of SEQ ID NO:15; the heavy chain CDR1 amino acid sequence corresponds to residues 31-35 of SEQ ID NO:17; the heavy chain CDR2 amino acid sequence corresponds to residues 50-65 of SEQ ID NO:17; and the heavy chain CDR3 amino acid sequence corresponds to residues 99-112 of SEQ ID NO:17. The amino acid sequences of the 1-4F1 CDRs have been identified as follows: the light chain CDR1 amino acid sequence corresponds to residues 24-34 of SEQ ID NO:39; the light chain CDR2 amino acid sequence corresponds to residues 50-56 of SEQ ID NO:39; the light chain CDR3 amino acid sequence corresponds to residues 89-97 of SEQ ID NO:39; the heavy chain CDR1 amino acid sequence corresponds to residues 31-35 of SEQ ID NO:41; the heavy chain CDR2 amino acid sequence corresponds to residues 50-66 of SEQ ID NO:41; and the heavy chain CDR3 amino acid sequence corresponds to residues 99-113 of SEQ ID NO:41.

Amino acid sequences for the entire 1-7F9 light and heavy chains are provided in SEQ ID NOS:36 and 37, respectively.

Accordingly, additional antibodies of, for example, various human antibody subclasses; antibody fragments, antibody derivatives, and other KIR-binding peptides, can be readily produced by, *e.g.*, recombinant techniques, based upon this information. For example, in one aspect, the invention provides an antibody having a VL and a VH sequence consisting essentially of SEQ ID NO:15 and SEQ ID NO:17, respectively, and/or an antibody having a VL and a VH sequence consisting essentially of SEQ ID NO:39 and SEQ ID NO:41, respectively. In another aspect, the invention provides an antibody comprising CDR regions consisting essentially of the 1-7F9 or 1-4F1 VH CDR1-3 and VL CDR1-3 described above. In another aspect, the invention provides an antibody comprising CDR regions as follows: a light chain CDR1 amino acid sequence corresponding to about residues 24-34 of SEQ ID NO:15; the light chain CDR2 amino acid sequence corresponding to about residues 50-56 of SEQ ID NO:15; the light chain CDR3 amino acid sequence corresponding to about residues 89-97 of SEQ ID NO:15; the heavy chain CDR1 amino acid sequence corresponding to about residues 31-35 of SEQ ID NO:17; the heavy chain CDR2 amino acid sequence corresponding to about to residues 50-65 of SEQ ID NO:17; and the heavy chain CDR3 amino acid sequence corresponding to about residues 99-112 of SEQ ID NO:17. In another aspect, the invention provides an antibody comprising CDR regions as follows: a light chain CDR1 amino acid sequence corresponding to about residues 24-34 of SEQ ID NO:39; a light chain CDR2 amino acid sequence corresponding to about residues 50-56 of SEQ ID NO:39; a light chain CDR3 amino acid sequence corresponding to about residues 89-97 of SEQ ID NO:39; a heavy chain CDR1 amino acid sequence corresponding to about residues 31-35 of SEQ ID NO:41; a heavy chain CDR2 amino acid sequence corresponding to about residues 50-66 of SEQ ID NO:41; and a heavy chain CDR3 amino acid sequence corresponding to about residues 99-113 of SEQ ID NO:41. In another aspect, the invention provides an antibody comprising a light chain CDR1 amino acid sequence consisting essentially of residues 24-34 of SEQ ID NO:15; a light chain CDR2 amino acid sequence consisting essentially of residues 50-56 of SEQ ID NO:15; a light chain CDR3 amino acid sequence consisting essentially of residues 89-97 of SEQ ID NO:15; a heavy chain CDR1 amino acid sequence consisting essentially of residues 31-35 of SEQ ID NO:17; a heavy chain CDR2 amino acid sequence consisting essentially of residues 50-65 of SEQ ID NO:17; and a heavy chain CDR3 amino acid sequence consisting essentially of residues 99-112 of SEQ ID NO:17. In another aspect, the invention provides an antibody comprising CDR regions as follows: a light chain CDR1 amino acid sequence consisting essentially of residues 24-34 of SEQ ID NO:39; a light chain CDR2 amino acid sequence consisting essentially of residues 50-56 of SEQ ID NO:39; a light chain CDR3 amino acid sequence consisting essentially of residues 89-97 of

SEQ ID NO:39; a heavy chain CDR1 amino acid sequence consisting essentially of residues 31-35 of SEQ ID NO:41; a heavy chain CDR2 amino acid sequence consisting essentially of residues 50-66 of SEQ ID NO:41; and a heavy chain CDR3 amino acid sequence consisting essentially of residues 99-113 of SEQ ID NO:41.

The invention also encompasses an anti-KIR antibody, antibody fragment, or antibody derivative, or a KIR-binding polypeptide, comprising at least one variant amino acid sequence substantially identical to the 1-7F9 or 1-4F1 VH or VL sequence, or to a CDR-region therein. A variant amino acid sequence can comprise or consist essentially of an amino acid sequence that is at least about 50, 80, 90, 95, 98, or 99 (*e.g.*, about 50-99, about 65-99, about 75-99, or about 85-99) percent identical to a 1-7F9 or 1-4F1 CDR, VH, or VL region. A variant amino acid sequence can also or alternatively comprise 1, 2, or 3 CDRs that comprise or consist of amino acid sequences that are at least about 80%, at least about 90%, or at least about 95% identical to 1-7F9 or 1-4F1 CDRs. Thus, in one aspect, the invention provides a human antibody comprising a light chain CDR1 amino acid sequence at least about 80%, at least about 90%, or at least about 95% identical to residues 24-34 of SEQ ID NO:15 or SEQ ID NO:39; a light chain CDR2 amino acid sequence at least about 80%, at least about 90%, or at least about 95% identical to residues 50-56 of SEQ ID NO:15 or SEQ ID NO:39; a light chain CDR3 amino acid sequence at least about 80%, at least about 90%, or at least about 95% identical to residues 89-97 of SEQ ID NO:15 or SEQ ID NO:39; a heavy chain CDR1 amino acid sequence at least about 80%, at least about 90%, or at least about 95% identical to residues 31-35 of SEQ ID NO:17 or SEQ ID NO:41; a heavy chain CDR2 amino acid sequence at least about 80%, at least about 90%, or at least about 95% identical to residues 50-65 of SEQ ID NO:17 or to residues 50 to 66 of SEQ ID NO:41; and a heavy chain CDR3 amino acid sequence at least about 80%, at least about 90%, or at least about 95% identical to residues 99-112 of SEQ ID NO:17 or to residues 99 to 113 of SEQ ID NO:41. The basic properties of 1-7F9- or 1-4F1-derived KIR-binding amino acid sequences that are retained in such variant amino acid sequences desirably include the specificity and/or avidity of the 1-7F9 or 1-4F1 sequence for one or more KIRs, and may also or alternatively include the capability of 1-7F9 in blocking KIR/HLA-C interaction and potentiating the lytic activity of NK cells.

In another aspect, the invention provides an anti-KIR antibody, antibody fragment, or antibody derivative, or a KIR-binding polypeptide, that comprises a KIR-binding amino acid sequence that differs from a 1-7F9 or 1-4F1 KIR-binding sequence in one or more amino acid residues (*e.g.*, at least 2, 3, 5, at least about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35, at least about 40, at least about 50, or

more amino acid residues) by way of one or more residue insertions, deletions, and/or substitutions. In one embodiment, such a variant KIR-binding sequence confers greater affinity; greater or different specificity; less immunogenicity (in terms of host response to the sequence); greater *in vivo* stability; and/or other beneficial properties to the variant sequence over an essentially identical amino acid sequence comprising the native 1-7F9 or 1-4F1 sequence. Suitable sequence variations are further described elsewhere herein. A KIR-binding portion of an anti-KIR antibody, antibody fragment, or antibody derivative, or a KIR-binding polypeptide, can also comprise any suitable number of non-amino acid components or substituents, such as non-amino acid organic moieties, that facilitate KIR binding and/or provide other advantageous physiochemical or immunological properties.

Some antibodies of the invention can also or alternatively be characterized by their binding affinity to one or more KIRs. For example, as shown in Examples 3 and 8, in terms of bivalent binding, DF200 has a K_d for KIR2DL1 of about 11 nM, and a K_d for KIR2DL3 of about 2.0 nM, and 1-7F9 has a K_d for KIR2DL1 of about 0.43 nM, and a K_d for KIR2DL3 of about 0,025 nM. Accordingly, in one aspect, the invention provides human or non-human (*e.g.*, murine, chimeric, or humanized) antibodies having a K_d in bivalent binding to KIR2DL1 of no more than about 20 nM, no more than about 11 nM, no more than about 5 nM, no more than about 1 nM, no more than about 0.5 nM, or no more than about 0.43 nM. Additionally or alternatively, human or non-human (*e.g.*, murine, chimeric, or humanized) antibodies of the invention may have a K_d for KIR2DL3 of no more than about 20 nM, no more than about 2 nM, no more than about 1 nM, no more than about 0.1 nM, or no more than about 0.05 nM, or no more than about 0.025 nM. In a particular aspect, the antibodies have about the same K_d values for bivalent binding to KIR2DL1 and KIR2DL3 as 1-7F9. As shown in Example 13, in terms of monovalent binding, 1-7F9 and 1-4F1 have K_d-values for KIR2DL3 of about 3.5 and 7 nM, respectively. Accordingly, in one aspect, the invention provides human or non-human (*e.g.*, murine, chimeric, or humanized) antibodies having a K_d in monovalent binding to KIR2DL3 of no more than about 20 nM, no more than about 10 nM, no more than about 7 nM, or no more than about 3.5 nM.

An anti-KIR antibody, antibody fragment, or antibody derivative, or a KIR-binding polypeptide selectively and/or specifically (typically specifically) binds at least one KIR, and more particularly an antigenic determinant region or epitope of at least one KIR, under appropriate conditions (*e.g.*, with respect to temperature, pH, etc., which typically will reflect human physiological conditions in a normal or NK cell-associated disease state, and in the context of a suitable protein comprising the sequence or combination). For example, in one aspect, the invention relates to antibodies that can be characterized in (among other things)

the ability to compete with 1-7F9, 1-4F1 or a 1-7F9- or 1-4F1-like antibody, and various methods involving the same. Other antibodies of the invention (and/or that are useful in the practice of inventive methods described herein) also or alternatively can be characterized on having the ability to compete with one or more of antibody DF200, antibody NKVSF1, antibody EB6, and antibody GL183.

The cross-reactive and neutralizing anti-KIR antibodies, antibody fragments, or derivatives of the invention reduce or neutralize the inhibitory activity of KIR by specifically inhibiting binding of MHC and/or HLA molecules to at least two inhibitory KIR receptors and facilitating NK cell activity, meaning that such antibodies, fragments or derivatives permit NK cells expressing an inhibitory KIR receptor on their surface to be capable of lysing cells that express a corresponding HLA ligand for that particular inhibitory KIR receptor (*e.g.*, a particular HLA antigen). In one aspect, the invention provides antibodies that specifically inhibit the binding of HLA-C molecules to KIR2DL1 and KIR2DL2/3 receptors. In another aspect, the invention provides antibodies that inhibit the binding of KIR2DL1 and/or KIR2DL2/3 to HLA-C. In yet another aspect, the invention provides antibodies that facilitate NK cell activity *in vivo*, and/or *in vitro*.

At least one of KIR2DL1 or KID2DL2/3 is present in at least about 90% or more of the human population, and the more preferred antibodies of this invention are capable of potentiating the activity of NK cells expressing either or both of these KIR. Therefore, compositions of this invention may be used to effectively activate or potentiate NK cells in most human individuals, typically in about 90% of human individuals or more. Accordingly, a single antibody composition according to the invention may be used to treat most human subjects, and there is seldom need to determine KIR- or HLA-allelic groups or to use mixtures or cocktails of two or more anti-KIR mAbs.

In one aspect, the antibody specifically binds both KIR2DL1 and KIR2DL2/3 human receptors and reverses inhibition of NK cell cytotoxicity mediated by these KIRs. The antibody may also be human and compete with monoclonal antibody 1-7F9 and/or 1-4F1. The term "competes with" when referring to a particular pair of antibodies (*e.g.*, one or more antibodies selected from DF200, NKVSF1 (Pan2D), 1-7F9, EB6, and GL183), means that a first antibody detectably competes with a second antibody (or other molecule) in a binding assay using either recombinant KIR molecules or cell-surface expressed KIR molecules. For example, in one aspect, where the percentages of inhibition for a certain antibody pair are above about 20%, above about 30%, above about 40%, above about 50%, regardless of which antibody is used as first antibody, the antibodies compete. Alternatively, if the percentages of inhibition for a certain antibody pair average at least about 29%, at least about

30%, at least about 40%, or at least about 50%, the antibodies compete. The percentage of inhibition of a second antibody binding to KIR2D protein by a first antibody can be calculated as: 100*(1-(detected binding second antibody)/(detected binding of first antibody)). The same applies to antibody fragments and antibody derivatives. Unless otherwise specified, an antibody that "competes" with 1-7F9, 1-4F1 or a 1-7F9- or 1-4F1-like antibody may compete with 1-7F9, 1-4F1 or the 1-7F9- or 1-4F1-like antibody for binding to human KIR2DL1, human KIR2DL2/3, or both human KIR2DL1 and KIR2DL2/3. For example, the antibody DF200 competes with 1-7F9 and 1-4F1 for binding to KIR2DL3.

Optionally, an antibody which competes with 1-7F9 or 1-4F1 is not 1-7F9 or 1-4F1 themselves, respectively (*i.e.*, the invention provides antibodies other than 1-7F9 and 1-4F1 that are characterized by, among other things, the ability to compete with 1-7F9 and/or 1-4F1 in binding to one or both of these KIRs).

In another aspect, the antibody binds both KIR2DL1 and KIR2DL2/3 human receptors, reduces or neutralizes or reverses inhibition of NK cell cytotoxicity mediated by these KIRs, and competes with 1-7F9 for binding to the KIR2DL1 human receptor, the KIR2DL2/3 human receptor, or both KIR2DL1 and KIR2DL2/3 human receptors. Optionally, said antibody is a chimeric, human, or humanized antibody.

In another aspect, the antibody binds both KIR2DL1 and KIR2DL2/3 human receptors, reduces, neutralizes or reverses inhibition of NK cell cytotoxicity mediated by these KIRs, and competes with EB6 for binding to the KIR2DL1 human receptor, or competes with GL183 and/or DF200 for binding to the KIR2DL2/3 human receptor; or it competes with both EB6 for binding to the KIR2DL1 human receptor and GL183 and/or DF200 for binding to the KIR2DL2/3 human receptor. In one embodiment, the antibody is not NKVSF1 (Pan2D), not A210, not A803g, and/or not DF200. The antibody can be, *e.g.*, a murine, chimeric, human, or humanized antibody.

In another aspect, the antibody comprises VH, VL, or both VH and VL regions that are at least substantially identical to the VH and/or VL regions of 1-7F9 or 1-4F1. The antibody can be of any subclass, including IgG1, IgG2, IgG3, and IgG4. In a particular aspect, the antibody is a human IgG4 antibody. In another particular aspect, the antibody is a human IgG2 antibody. The antibody can be a chimeric, human, or humanized antibody.

In another aspect, the antibody is human, competes with 1-7F9 or 1-4F1, and recognizes, binds to, or has immunospecificity for at least partially the same, or the same, epitope or "epitopic site" on a KIR molecule as the monoclonal antibody 1-7F9 or 1-4F1. Preferably, said KIR molecule is a human KIR2DL1 or KIR2DL2/3 receptor.

In another aspect, the antibody binds a common determinant present in both KIR2DL1 and KIR2DL2/3 human receptors and reduces, neutralizes or reverses inhibition of NK cell cytotoxicity mediated by these KIRs. The antibody more specifically can bind at least partially the same, substantially the same, or the same epitope on KIR as monoclonal antibody 1-7F9.

In a particular aspect, the antibody is a monoclonal antibody that exhibits one or more of the above-described characteristics.

In another aspect, functional fragments and derivatives of the antibodies described herein can be prepared which have substantially similar antigen binding, specificity and/or activity, including, without limitation, Fab fragments, Fab'2 fragments, immunoadhesins, diabodies, camelized antibodies, Janusins, minibodies, CDRs, and ScFv fragments.

Unless otherwise specified, antibodies or bivalent fragments or derivatives thereof are monospecific, *i.e.*, both "arms" of the antibodies, fragments, or derivatives bind the same antigen(s).

In yet another aspect, antibody derivatives comprising an antibody of the invention conjugated or covalently bound to a toxin, a radionuclide, a detectable moiety (*e.g.*, a fluor), or a solid support, can be prepared.

The invention also encompasses pharmaceutical compositions comprising an antibody as disclosed above, a fragment thereof, or a derivative of either thereof. Accordingly, the invention also relates to use of an antibody as disclosed herein in a method for the manufacture of a medicament. In preferred embodiments, the medicament or pharmaceutical composition is for the treatment of a cancer or other proliferative disorder, an infection, or for use in transplantation.

In one aspect, the invention relates to a composition (*e.g.*, a composition formulated for pharmaceutical administration, a kit for preparing such a composition, an assay kit or media, purification media, etc.) that comprises an antibody that binds at least two different human inhibitory KIR receptor gene products and is capable of neutralizing KIR-mediated inhibition cytotoxicity by NK cells expressing at least one of said two different human inhibitory KIR receptors, wherein the antibody is incorporated into a liposome. The liposome may also comprise an additional substance such as, *e.g.*, a nucleic acid molecule for the delivery of genes for gene therapy; a nucleic acid molecule for the delivery of antisense RNA, RNAi, or siRNA for suppressing a gene in an NK cell; or a toxin or a drug for the targeted killing of NK cells.

Described herein are also methods of regulating human NK cell activity *in vitro*, *ex vivo*, or *in vivo*, comprising contacting human NK cells with an effective amount of an anti-

body of the invention, a fragment of such an antibody, a derivative of either thereof, or a pharmaceutical composition comprising at least one of any thereof. Preferred methods comprise administration of an effective amount of a pharmaceutical compositions of this invention and are directed at increasing the cytotoxic activity of human NK cells, most preferably *ex vivo* or *in vivo*, in a subject having a cancer, an infectious disease, or an immune disease. For example, administration can be effected by intravenous infusion of the antibody in a suitable buffer to a patient with cancer or an infectious disease (such as a viral disease).

The invention also provides a composition comprising an antibody that binds at least two different human inhibitory KIR receptor gene products, wherein the antibody is capable of neutralizing KIR-mediated inhibition of NK cell cytotoxicity in NK cells expressing at least one of the two different human inhibitory KIR receptors, the antibody being present in an amount effective to detectably potentiate NK cell cytotoxicity in a patient or in a biological sample comprising NK cells; and a pharmaceutically acceptable carrier or excipient. Preferably the antibody binds a common determinant present on KIR2DL1 and KIR2DL2/3. Compositions comprising an antibody of the invention may optionally further comprise a second therapeutic agent (an agent that induces, promotes, and/or enhances a therapeutic effect in a host that is related to a condition or disorder for which the antibody is administered – *e.g.*, a condition related to a cancer, transplantation, an infectious disease, a viral infection, etc.). In one aspect, a second therapeutic agent that can be co-administered with an antibody of the invention in such a combination composition may be selected from, for example, an immunomodulatory agent, a hormonal agent, a chemotherapeutic agent, an anti-angiogenic agent, an apoptotic agent, a second antibody specific for a non-KIR antigen, optionally a second antibody that binds to and inhibits and neutralizes an inhibitory KIR, or reduces the signalling from an inhibitory KIR, an anti-infective agent, a targeting agent, or an adjunct compound. Advantageous immunomodulatory agents may be selected from IL-1alpha, IL-1beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-21, TGF-beta, GM-CSF, M-CSF, G-CSF, TNF-alpha, TNF-beta, LAF, TCGF, BCGF, TRF, BAF, BDG, MP, LIF, OSM, TMF, PDGF, IFN-alpha, IFN-beta, or IFN-gamma. Examples of the chemotherapeutic agents include alkylating agents, antimetabolites, cytotoxic antibiotics, adriamycin, dactinomycin, mitomycin, carminomycin, daunomycin, doxorubicin, tamoxifen, taxol, taxotere, vincristine, vinblastine, vinorelbine, etoposide (VP-16), 5-fluorouracil (5FU), cytosine arabinoside, cyclophosphamide, thiotepa, methotrexate, camptothecin, actinomycin-D, mitomycin C, cisplatin (CDDP), aminopterin, combretastatin(s), other vinca alkyloids and derivatives or prodrugs thereof. Examples of hormonal agents include leuprorelin, goserelin, triptorelin, buserelin, tamoxifen, toremifene, flutamide, nilutamide, cyproterone bicalutamid anastrozole, exeme-

stane, letrozole, fadrozole medroxy, chlormadinone, megestrol, other LHRH agonists, other anti-estrogens, other anti-androgens, other aromatase inhibitors, and other progestagens. Preferably, the second antibody that binds to and inhibits an inhibitory KIR receptor is an antibody or a derivative or fragment thereof that binds to an epitope of an inhibitory KIR receptor that differs from the epitope bound by the antibody that binds a common determinant present on at least two different human inhibitory KIR receptor gene products. In another aspect, a second antibody may be directed to a target associated with the disease state that is at least partially treatable by administration of the antibody of the invention (*e.g.*, a cancer-associated antigen, a viral infection-associated antigen, etc.).

The invention further provides a method of detectably potentiating NK cell activity in a patient in need thereof, comprising the step of administering to the patient a composition according to the invention. A patient in need of NK cell activity potentiation can be any patient diagnosed as having a disease or disorder wherein such potentiation may promote, enhance, and/or induce a therapeutic effect (or promotes, enhances, and/or induces such an effect in at least a substantial proportion of patients with the disease or disorder and substantially similar characteristics as the patient – as may determined by, *e.g.*, clinical trials). A patient in need of such treatment may be suffering from, *e.g.*, cancer, another proliferative disorder, an infectious disease or an immune disorder. Preferably, the method comprises the additional step of administering to the patient an appropriate additional therapeutic agent selected from an immunomodulatory agent, a hormonal agent, a chemotherapeutic agent, an anti-angiogenic agent, an apoptotic agent, a second antibody specific for an antigen distinct from KIR, optionally a second antibody that binds to and inhibits and neutralizes an inhibitory KIR receptor, or reduces the signalling from an inhibitory KIR, an anti-infective agent, a targeting agent or an adjunct compound wherein said additional therapeutic agent is administered to said patient as a single dosage form together with said antibody, or as separate dosage form. The dosage of the antibody (or antibody fragment/derivative) and the dosage of the additional therapeutic agent collectively are sufficient to detectably induce, promote, and/or enhance a therapeutic response in the patient which comprises the potentiation of NK cell activity. Where administered separately, the antibody, fragment, or derivative and the additional therapeutic agent are desirably administered under conditions (*e.g.*, with respect to timing, number of doses, etc.) that result in a detectable combined therapeutic benefit to the patient.

Further encompassed by the present invention are antibodies which are capable of specifically binding NK cells and/or KIR receptors in a non-human primate, preferably a monkey. Also encompassed are methods for evaluating the toxicity, dosage and/or activity or

efficacy of antibodies of the invention which are candidate medicaments. In one aspect, the invention encompasses a method for determining a dose of an antibody that is toxic to an animal or target tissue by administering an antibody of the invention to an non-human primate recipient animal having NK cells, and assessing any toxic or deleterious or adverse effects of the agent on the animal, or preferably on a target tissue. In another aspect, the invention is a method for identifying an antibody that is toxic to an animal or target tissue by administering an antibody of the invention to an non-human primate recipient animal having NK cells, and assessing any toxic or deleterious or adverse effects of the agent on the animal, or preferably on a target tissue. In another aspect, the invention is a method for identifying an antibody that is efficacious as treatment of an infection, disease or tumor by administering an antibody of the invention to a non-human primate model of infection, disease or cancer, and identifying the antibody that ameliorates the infection, disease or cancer, or a symptom thereof. In one embodiment, said antibody of the invention is an antibody which (a) cross reacts with at least two inhibitory human KIR receptors at the surface of human NK cells, and (b) cross-reacts with NK cells or a KIR receptor of the non-human primate.

Further encompassed by the present invention is a method of detecting the presence of NK cells bearing an inhibitory KIR on their cell surface in a biological sample or a living organism, said method comprising the steps of:

a) contacting said biological sample or living organism with an antibody of the invention, wherein said antibody is conjugated or covalently bound to a detectable moiety; and

b) detecting the presence of said antibody in said biological sample or living organism.

The invention also provides a method of purifying from a sample NK cells bearing an inhibitory KIR on their cell surface comprising the steps of:

a) contacting said sample with an antibody of the invention under conditions that allow said NK cells bearing an inhibitory KIR on their cell surface to bind to said antibody, wherein said antibody is conjugated or covalently bound to a solid support (*e.g.*, a bead, a matrix, etc.); and

b) eluting said bound NK cells from said antibody conjugated or covalently bound to a solid support.

It has been found that antibody NKVSF1 (Pan2D) also binds to NK cells from cynomolgus monkeys, see Figure 10.

The invention therefore provides an antibody, as well as fragments and derivatives thereof, wherein said antibody, fragment or derivative cross-reacts with at least two inhibitory human KIR receptors at the surface of human NK cells, and which furthermore binds to NK

cells from cynomolgus monkeys. In one embodiment hereof, the antibody is not antibody NKVSF1, not A210, and/or not A802g. The invention also provides a method of testing the toxicity of an antibody, as well as fragments and derivatives thereof, wherein said antibody, fragment or derivative cross-reacts with at least two inhibitory human KIR receptors at the surface of human NK cells, wherein the method comprises testing the antibody in a cynomolgus monkey.

In a further aspect, the invention provides an antibody, antibody fragment, or derivative of either thereof, that comprises the light variable region or one or more light variable region CDRs of antibody 1-7F9 or 1-4F1. In still another aspect, the invention provides an antibody, antibody fragment, or derivative of either thereof that comprises a sequence that is highly similar to all or essentially all of the light chain variable region sequence of 1-7F9 or 1-4F1.

In a further aspect, the invention provides an antibody, antibody fragment, or derivative of either thereof, which comprises the heavy chain variable region or one or more heavy chain variable region CDRs of antibody 1-7F9 or 1-4F1. In still another aspect, the invention provides an antibody, antibody fragment, or derivative of either thereof that comprises a sequence that is highly similar to all or essentially all of the heavy chain variable region sequence of 1-7F9 or 1-4F1.

Antibodies

The present invention provides novel antibodies and fragments or derivatives thereof that bind common determinants conserved among human inhibitory KIR receptors, preferably including a determinant present on at least two different KIR2DL gene products, but not on KIR2DS4, and which cause potentiation of NK cells expressing at least one of those KIR receptors. The invention discloses, for the first time, that such cross-reacting and neutralizing antibodies can be produced, and effectively used in modulation of NK cell activity, which represents an unexpected result and opens an avenue towards novel and effective NK-based therapies, particularly in human subjects.

Within the context of this invention, a "common determinant" designates a determinant or epitope that is shared by several gene products of the human inhibitory KIR receptors. Preferably, the common determinant is shared by at least two members of the KIR2DL receptor group. More preferably, the determinant is shared by at least KIR2DL1 and KIR2DL2/3. Certain antibodies of this invention may, in addition to recognizing multiple gene products of the KIR2DL type, also recognize determinants present on other inhibitory KIRs, such as gene product of the KIR3DL receptor group; *e.g.*, KIR3DL1 and/or KIR3DL2. The

determinant or epitope may represent a peptide fragment or a conformational epitope shared by said members. In a more specific embodiment, the antibody of this invention specifically binds to substantially the same epitope recognized by monoclonal antibody DF200. This determinant is present on both KIR2DL1 and KIR2DL2/3. In another preferred embodiment, the antibody of this invention specifically binds to substantially the same epitope recognized by human mAb 1-7F9, or the epitope recognized by human mAb 1-4F1, said epitopes being present on KIR2DL1, -2 and -3, but not on KIR2DS4.

The term "antibodies," as used herein, refers to polyclonal and monoclonal antibodies, as well as to fragments and derivatives of said polyclonal and monoclonal antibodies unless otherwise stated or clearly contradicted by context. Depending on the type of constant domain in the heavy chains, full length antibodies typically are assigned to one of five major classes: IgA, IgD, IgE, IgG, and IgM. Several of these are further divided into subclasses or isotypes, such as IgG1, IgG2, IgG3, IgG4, and the like. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are termed "alpha," "delta," "epsilon," "gamma" and "mu," respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known. IgG and/or IgM are the preferred classes of antibodies employed in this invention because they are the most common antibodies in the physiological situation and because they are most easily made in a laboratory setting. Typically, an "antibody" in the context of this invention refers to a monoclonal antibody, more preferably a "human" monoclonal antibody.

One embodiment of the invention provides a method of blocking the interaction of an inhibitory KIR and its corresponding HLA ligand *in vivo*, for therapeutic purposes that involve activation of endogenous NK cells. In such a setting, it may be advantageous to avoid depleting the NK cells, since, if depleted, the NK cells could not exert their therapeutically beneficial effects. Therefore, antibody isotypes such as IgG4 and IgG2 which exhibit little binding to Fc-receptors, and which do not activate the complement system, are typically preferred. The isotype of 1-7F9 is IgG4. IgG2 antibodies are also generally considered to be non-depleting, and may also be more stable molecules than IgG4 antibodies, thereby conferring a longer half-life *in vivo*. 1-4F1 is of the IgG2 isotype.

Antibody Production

The antibodies of this invention may be produced by a variety of techniques known in the art. Typically, they are produced by immunization of a non-human animal, preferably a mouse, with an immunogen comprising an inhibitory KIR polypeptide, preferably a KIR2DL polypeptide, more preferably a human KIR2DL polypeptide. The inhibitory KIR polypeptide

may comprise the full length sequence of a human inhibitory KIR polypeptide, or a fragment or derivative thereof, typically an immunogenic fragment, *i.e.*, a portion of the polypeptide comprising an epitope exposed on the surface of the cell expressing an inhibitory KIR receptor. Such fragments typically contain at least about 7 consecutive amino acids of the mature polypeptide sequence, even more preferably at least about 10 consecutive amino acids thereof. Fragments typically are essentially derived from the extra-cellular domain of the receptor. Even more preferred is a human KIR2DL polypeptide which includes at least one, more preferably both, extracellular Ig domains, of the full length KIRDL polypeptide and is capable of mimicking at least one conformational epitope present in a KIR2DL receptor. In other embodiments, said polypeptide comprises at least about 8 consecutive amino acids of an extracellular Ig domain of amino acid positions 1-224 of the KIR2DL1 polypeptide (amino acid numbering of according to Wagtmann et al., Immunity 1995;2:439-449, which is incorporated by reference herein in its entirety, or according to the PROW internet website found at the World Wide Web (www) address ncbi.nlm.nih.gov/prow/guide/1326018082.htm).

In one embodiment, the immunogen comprises a wild-type human KIR2DL polypeptide in a lipid membrane, typically at the surface of a cell. In a specific embodiment, the immunogen comprises intact NK cells, particularly intact human NK cells. In another embodiment, the cells are lysed or otherwise treated so as to not be intact. The immunogen can be, for example, suspended or dissolved in a buffer, optionally with an adjuvant, such as complete Freund's adjuvant, prior to administration to a non-human mammal, such as a mouse, rabbit, goat, horse, dog, sheep, guinea pig, rat, hamster, etc.

The step of immunizing a non-human mammal with an antigen may be carried out in any manner well known in the art for stimulating the production of antibodies in a mouse (see, for example, E. Harlow and D. Lane, Antibodies: A Laboratory Manual., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1988)). The immunogen is then suspended or dissolved in a buffer, optionally with an adjuvant, such as complete Freund's adjuvant. Methods for determining the amount of immunogen, types of buffers and amounts of adjuvant are well known to those of skill in the art and are not limiting in any way on the present invention. These parameters may be different for different immunogens, but are easily elucidated.

Similarly, principles relevant to selection of the location and frequency of immunization sufficient to stimulate the production of antibodies are also well known in the art. In an exemplary immunization protocol, the non-human animals are injected intraperitoneally with antigen on day 1 and again about a week later. This is followed by recall injections of the antigen around day 20, optionally with adjuvant such as incomplete Freund's adjuvant. The

recall injections are performed intravenously and may be repeated for several consecutive days. This is followed by a booster injection at day 40, either intravenously or intraperitoneally, typically without adjuvant. This protocol results in the production of antigen-specific antibody-producing B cells after about 40 days. Other protocols may also be utilized as long as they result in the production of B cells expressing an antibody directed to the antigen used in immunization.

For polyclonal antibody preparation, serum is obtained from an immunized non-human animal and the antibodies present therein isolated by well-known techniques. The serum may be affinity purified using any of the immunogens set forth above linked to a solid support so as to obtain antibodies that react with inhibitory KIR receptors.

In an alternate embodiment, lymphocytes from an unimmunized non-human mammal are isolated, grown *in vitro*, and then exposed to the immunogen in cell culture. The lymphocytes are then harvested and the fusion step described below is carried out.

For monoclonal antibodies, the next step is the isolation of splenocytes from the immunized non-human mammal and the subsequent fusion of those splenocytes with an immortalized cell in order to form an antibody-producing hybridoma. The isolation of splenocytes from a non-human mammal is well-known in the art and typically involves removing the spleen from an anesthetized non-human mammal, cutting it into small pieces and squeezing the splenocytes from the splenic capsule and through a nylon mesh of a cell strainer into an appropriate buffer so as to produce a single cell suspension. The cells are washed, centrifuged and resuspended in a buffer that lyses any red blood cells. The solution is again centrifuged and remaining lymphocytes in the pellet are finally resuspended in fresh buffer.

Once isolated and present in single cell suspension, the lymphocytes can be fused to an immortal cell line. This is typically a mouse myeloma cell line, although many other immortal cell lines useful for creating hybridomas are known in the art. Preferred murine myeloma lines include, but are not limited to, those derived from MOPC-21 and MPC-11 mouse tumors (available from the Salk Institute Cell Distribution Center, San Diego, Calif. U.S.A.), or the X63 Ag8653 and SP-2 cell lines (available from the American Type Culture Collection, Rockville, Maryland U.S.A.). The cell fusion is effected using polyethylene glycol or the like. The resulting hybridomas are then grown in selective media that contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Hybridomas are typically grown on a feeder layer of macrophages. The macrophages are preferably from littermates of the non-human mammal used to isolate splenocytes and are typically primed with incomplete Freund's adjuvant or the like several days before plating the hybridomas. Fusion methods are described in Goding, "Monoclonal Antibodies: Principles and Practice," pp. 59-103 (Academic Press, 1986), the disclosure of which is herein incorporated by reference.

The cells are allowed to grow in the selection media for sufficient time for colony formation and antibody production. This is usually between about 7 and about 14 days. The hybridoma colonies are then assayed for the production of antibodies that cross-react with multiple inhibitory KIR receptor gene products. The assay is typically a colorimetric ELISA-type assay, although several other types of assays may be employed, including immunoprecipitation and radioimmunoassay, or FACS, Biacore, Scintillation-proximity assays (SPA), or other types of assays well known in the art. The wells containing antibodies of the desired specificity are examined to determine if one or more distinct hybridoma cell colonies are present. If more than one colony is present, the cells may be re-cloned and grown to ensure that only a single cell has given rise to the colony producing the desired antibody. Positive wells with a single apparent colony are typically re-cloned and re-assayed to insure that only one monoclonal antibody is being detected and produced.

In a preferred embodiment, the non-human animal used to produce antibodies according to applicable methods of the invention is a mammal, such as a rodent (*e.g.*, mouse, rat, etc.), bovine, porcine, horse, rabbit, goat, sheep, etc. Also, the non-human mammal may be genetically modified or engineered to produce "human" antibodies, such as the Xenomouse™ (Abgenix) or HuMAb-Mouse™ (Medarex), as described below.

Antibodies also may be produced transgenically through the generation of a chordate (such as a mammal or bird) or a plant that is transgenic for the immunoglobulin heavy and light chain sequences of interest and production of the antibody in a recoverable form therefrom (see, *e.g.*, Ma et al., Nature Rev. Genetics 4:794-805 (2003); Nolke et al., Expert Opin Biol Ther. 2003 Oct;3(7):1153-62; Schillberg et al., Cell Mol Life Sci. 2003 Mar;60(3):433-45; Tekoah et al., Arch Biochem Biophys. 2004 Jun 15;426(2):266-78; Fischer et al., Eur. J. of Biochem., 262(3):810 (1999); and US Patent Application 20030084482 regarding production of antibodies and antibody-like proteins in plants). In connection with the transgenic production in mammals, antibodies and other proteins can be produced in, and recovered from, the milk of goats, cows, or other mammals. See, *e.g.*, US Patents 5,827,690, 5,756,687, 5,750,172, and 5,741,957. Antibodies also may be produced in the

eggs of birds and recovered therefrom. See, *e.g.*, Tini et al., Comp Biochem Physiol A Mol Integr Physiol. 2002 Mar;131(3):569-74 and US Patent 4,550,019.

Antibodies may also be produced by selection of combinatorial libraries of immunoglobulins, as disclosed for instance in Ward et al., Nature, 341 (1989) p. 544.

According to another embodiment, the invention provides a hybridoma derived from a B cell from a non-human host, wherein said B-cell produces an antibody that binds a determinant present on at least two different human inhibitory KIR receptor gene products and said antibody is capable of neutralizing the inhibitory activity of said receptors. More preferably, the hybridoma of this aspect of the invention is not a hybridoma that produces the monoclonal antibody NKVSF1, not A210, and/or not A802g. The hybridoma according to this aspect of the invention can be created as described above by the fusion of splenocytes from the immunized non-human mammal with an immortal cell line. Hybridomas produced by this fusion can be screened for the presence of such a cross-reacting antibody as described elsewhere herein. Preferably, the hybridoma produces an antibody that recognizes a determinant present on at least two different KIR2DL gene products, and cause potentiation of NK cells expressing at least one of those KIR receptors. Even more preferably, the hybridoma produces an antibody that binds to substantially the same epitope or determinant as 1-7F9 and which potentiates NK cell activity, or which binds substantially the saem epitope as 1-4F1. Most preferably, the hybridoma is hybridoma 1-7F9 which produces monoclonal antibody 1-7F9, or hybridoma 1-4F1 which produces monoclonal antibody 1-4F1.

Hybridomas that are confirmed to produce a monoclonal antibody of this invention can be grown up in larger amounts in an appropriate medium, such as DMEM or RPMI-1640. Alternatively, the hybridoma cells can be grown *in vivo* as ascites tumors in an animal.

After sufficient growth to produce the desired monoclonal antibody, the growth media containing monoclonal antibody (or the ascites fluid) can be separated away from the cells, and the monoclonal antibody is purified. Purification is typically achieved by chromatography using protein A or protein G-Sepharose, or an anti-mouse Ig linked to a solid support such as agarose or Sepharose beads (all described, for example, in the Antibody Purification Handbook, Amersham Biosciences, publication No. 18-1037-46, Edition AC, the disclosure of which is hereby incorporated by reference), or by other known techniques such as electrophoresis or dialysis. The bound antibody is typically eluted from protein A/protein G columns by using low pH buffers (glycine or acetate buffers of pH 3.0 or less) with immediate neutralization of antibody-containing fractions. These fractions can be pooled, dialyzed, and concentrated as needed.

Human Antibodies

In one aspect, the invention provides human anti-KIR antibodies. "Human" antibodies are distinguishable from "humanized" antibodies (which are described separately below). Such "human" antibodies may include amino acid residues not encoded by human germ line immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*), for example in the CDRs, such as in CDR3. However, the term "human antibody", as used herein, is intended to not include humanized antibodies or human/mouse chimera antibodies in which CDR sequences derived from the germ line of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

Transgenic animals can and have been developed that harbour human Ig-genes and, upon immunization, produce a full repertoire of human antibodies in the absence of mouse immunoglobulin production. Such human Ig-transgenic mice can be employed to produce human antibodies. Such human antibodies can be generated in human Ig-transgenic animals (*e.g.*, mice, rats, sheep, pigs, goats, cattle, horses, etc.) comprising human immunoglobulin loci and native immunoglobulin gene deletions, such as in a XenoMouse™ (Abgenix – Fremont, CA, USA) (see, *e.g.*, Green et al. Nature Genetics 7:13-21 (1994); Mendez et al. Nature Genetics 15:146-156 (1997); Green and Jakobovits J. Exp. Med. 188:483-495 (1998); European Patent No., EP 0 463 151 B1; International Patent Application Nos. WO 94/02602, WO 96/34096; WO 98/24893, WO 99/45031, WO 99/53049, and WO 00/037504; and US Patents 5,916,771, 5,939,598, 5,985,615, 5,998,209, 5,994,619, 6,075,181, 6,091,001, 6,114,598 and 6,130,364) or transgenic animals comprising a minilocus of human Ig-encoding genes such as the HuMab-mouse™ (Medarex – Princeton, NJ, USA) (see, *e.g.*, EP 0546073, EP0546073; U.S. Pat. Nos. 5,545,807, 5,545,806, 5,625,825, 5,625,126, 5,633,425, 5,661,016, 5,770,429, 5,789,650, 5,814,318, 5,591,669, 5,612,205, 5,721,367, 5,789,215, 5,643,763; and International Patent Application Nos. WO 92/03918, WO 92/22645, WO 92/22647, WO 92/22670, WO 93/12227, WO 94/00569, WO 94/25585, WO 96/14436, WO 97/13852, and WO 98/24884). Splenocytes from such transgenic mice can be used to produce hybridomas that secrete human monoclonal antibodies according to well known techniques, as described herein. Similar techniques and principles are described in, *e.g.*, Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551-255 (1993); Jakobovits et al., Nature, 362:255-258 (1993); and Bruggemann et al., Year in Immuno., 7:33 (1993)).

Further, human antibodies or antibodies from other species can be generated through display-type technologies, including, without limitation, phage display, retroviral display, ribosomal display, and other related techniques, using methods well known in the art, and the resulting molecules can be subjected to additional maturation methods, such as af-

finity maturation, as such techniques also are well known (see, *e.g.*, (Hoogenboom et al ., J. Mol. Biol. 227: 381 (1991) (phage display); Vaughan, et al., Nature Biotech 14:309 (1996) (phage display); Hanes and Plucthau PNAS USA 94:4937-4942 (1997) (ribosomal display), Parmley and Smith Gene 73:305-318 (1988) (phage display), Marks et al., J. Mol. Biol., 222:581 (1991), Scott TIBS 17:241-245 (1992), Cwirla et al. PNAS USA 87:6378-6382 (1990), Russel et al. Nucl. Acids Research 21:1081-1085 (1993), Hoganboom et al. Immunol. Reviews 130:43-68 (1992), Chiswell and McCafferty TIBTECH 10:80-84 (1992), and US Patent 5,733,743). If display technologies are utilized to produce antibodies that are not human, such antibodies can be humanized, *e.g.*, as described elsewhere herein.

Accordingly, as described herein, anti-KIR mAbs are promising agents for the treatment of cancer and viral infections and other diseases and disorders. Anti-KIR mAbs may be generated by various approaches, such as humanization of murine mAbs or by fusion of splenocytes from human Ig-transgenic mice (XenoMouse, or HuMab mice), by phage-display, or by immortalization of human Ab-producing B cells, or by other methods. In either case, the antibody can be produced by cell lines and purified in quantities suitable for formulation, packaging and injection into patients in need thereof.

Recombinant Production

Anti-KIR antibodies can also be prepared by recombinant expression in single cell organisms, such as yeast; or in bacterial cell cultures (such as in *E. coli*); or in eukaryotic cell culture (*e.g.*, in a culture of a mammalian cells) using standard techniques.

Thus, according to an alternate embodiment, the DNA encoding heavy and light chains of a cross-reactive and neutralizing anti-KIR antibody, that binds a determinant present on at least two different human inhibitory KIRs, is isolated from the hybridoma of this invention and placed in an appropriate expression vector for transfection into an appropriate host. The host is then used for the recombinant production of the antibody, or variants thereof, such as a humanized version of that monoclonal antibody, active fragments of the antibody, or chimeric antibodies comprising the antigen recognition portion of the antibody. Preferably, the DNA used in this embodiment encodes an antibody that recognizes a determinant present on at least two different KIR2DL gene products, but not on KIR2DS3 or -4, and that cause potentiation of NK cells expressing at least one of those KIR2DL receptors. Even more preferably, the DNA encodes an antibody that binds to substantially the same epitope or determinant as 1-7F9 and which potentiates NK cell activity. Most preferably, that DNA encodes monoclonal antibody 1-7F9.

DNA encoding the monoclonal antibodies of the invention is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capa-

ble of binding specifically to genes encoding the heavy and light chains of murine or human antibodies). Once isolated, the DNA can be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Recombinant expression in bacteria of DNA encoding fragments of the antibody is well known in the art (see, for example, Skerra et al., Curr. Opinion in Immunol., 5, pp. 256 (1993); and Pluckthun, Immunol. Revs. 130, pp. 151 (1992).

Additionally, recombinant production of antibodies from known variable heavy (VH) and variable light (VL) chains, and human constant regions has been described by, for example, Ruker et al. (Annals of the New York Academy of Sciences. 1991;646:212-219), who reports the expression of a human monoclonal anti-HIV-1 antibody in CHO cells; Bianchi et al. (Biotechnology and Bioengineering. 2003;84:439-444), who describes high-level expression of full-Length antibodies using trans-complementing expression vectors, No Soo Kim et al. (Biotechnol. Prog. 2001;17:69-75), who describes key determinants in the occurrence of clonal variation in humanized antibody expression of CHO cells during dihydrofolate reductase mediated gene amplification; King et al. (Biochemical Journal. 1992;281:317-323), who reports expression, purification and characterization of a mouse-human chimeric antibody and chimeric Fab' fragment; WO 2003064606 which describes isolated human monoclonal antibodies comprising a human heavy and a human light chain variable regions, both comprising FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4 sequences; and WO 2003040170 which describes chimeric or human monoclonal antibodies and antigen-binding portions that specifically binds to and activates human CD40.

The entire cDNA sequences encoding the constant regions of human IgG can be found in the following GenBank entries, each of which incorporated by reference in its entirety, accessed on January 6, 2005:

Human IgG1 constant heavy chain region: GenBank accession #: J00228

Human IgG2 constant heavy chain region: GenBank accession #: J00230

Human IgG3 constant heavy chain region: GenBank accession #: X04646

Human IgG4 constant heavy chain region: GenBank accession #: K01316

Human kappa light chain constant region: GenBank accession #: J00241.

In an exemplary embodiment, to produce recombinant mAb production from 1-7F9 or 1-4F1 VH and VL sequences, the following protocol can be applied. Steps 1-3 describe retrieval of the VH and VL regions from a hybridoma or other cell producing 1-7F9 or 1-4F1. However, the cDNA encoding the 1-7F9 or 1-4F1 VH and VL sequences (or mutants or de-

rivatives thereof), to be used in step 4, can also be prepared from the sequence information provided in Figures 14 or 15, using well-established techniques for synthesizing cDNA fragments. Alternatively, the VH and VL fragments of 1-7F9 or 1-4F1, or mutants or derivatives thereof, may be cloned into any one of a number of expression vectors described in the scientific literature or commercially available expression vectors, containing a constant region of the desired Ig subclass, in order to express a full-length antibody. Additionally, VH and VL fragments of 1-7F9 or 1-4F1, or mutants or derivatives thereof can be cloned into vectors encoding truncated constant regions in order to express antibody fragments (*e.g.*, Fab fragments). One example of a commercially available vector is pASK84, available from the ATCC (American Type Culture Collection, catalog number 87094).

(1) Isolation of total RNA from hybridoma cells:

$4x10^6$ hybridoma cells (such as 1-7F9 or 1-4F1) secreting antibodies against human KIR are used for isolation of total RNA using RNeasy Mini Kit from Qiagen, according to manufacturers instructions, and briefly outlined here: The cells are pelleted by centrifugation for 5 min at 1000 rpm and disrupted by addition of 350 µl RLT buffer containing 10 µl/ml β-mercaptoethanol. The lysate is transferred onto a QIAshredder column from Qiagen and centrifuged for 2 min at maximum speed. The flow-through is mixed with an equal volume of 70% ethanol. Up to 700 µl sample is applied per RNeasy spin column (Qiagen) and centrifuged at 14000 rpm, and the flow-through discarded. 700µl RW1 buffer is applied per column which is centrifuged at 14000 rpm for 15s to wash the column. The column is washed twice with 500µl RPE buffer and centrifuged for 14000rpm for 15s. To dry the column it is centrifuged for additionally 2 min at 14000rpm. The column is transferred to a new collection tube and the RNA is eluted with 50µl of nuclease-free water and centrifuged for 1min at 14000rpm. The RNA concentration is measured by absorbance at OD=260nm. The RNA is stored at -80ºC until needed.

(2) cDNA synthesis:

1 µg RNA is used for first-strand cDNA synthesis using SMART RACE cDNA Amplification Kit from Clontech. For preparation of 5'-RACE-Ready cDNA, a reaction mixture is prepared containing RNA isolated as described above, the reverse-primer 5'-CDS primer back, and SMART II A oligo, and this mixture is incubated at 72ºC for about 2 min., and subsequently cooled on ice for about 2 min. before adding 1xFirst-Strand buffer, DTT (20mM), dNTP (10mM) and PowerScript Reverse Transcriptase. The reaction mixture is incubated at 42ºC for 1.5 hour and Tricine-EDTA buffer is added and incubated at 72ºC for 7 min. At this point samples can be stored at -20ºC.

(3) PCR amplification and cloning of human variable light (VL) and human variable heavy (VH) chains:

A PCR (Polymerase Chain Reaction) reaction mixture containing 1xAdvantage HF 2 PCR buffer, dNTP (10mM) and 1xAdvantage HF 2 polymerase mix is established for separate amplification of variable regions of both VL and VH from cDNA made as above.

For amplification of VL the following primers are used:

UPM (Universal Primer Mix):

5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3' (SEQ ID NO:26)

5'-CTAATACGACTCACTATAGGG-3' (SEQ ID NO:27)

VK RACE2:

5'-GCAGGCACACAACAGAGGCAGTTCCAGATTTC-3' (SEQ ID NO:28)

For amplification of VH the following primers are used:

UPM (Universal Primer Mix):

5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3' (SEQ ID NO:29)

5'-CTAATACGACTCACTATAGGG-3' (SEQ ID NO:30)

AB90RACE:

5'-GTGCCAGGGGGAAGACCGATGGG-3' (SEQ ID NO:31)

Three rounds of PCR are conducted. Round 1: PCR is run for 5 cycles at 94ºC for 5s and 72ºC for 3 min. Round 2: PCR is run for 5 cycles at 94ºC for 5s, 70ºC for 10s, and 72ºC for 1 min. Round 3: PCR is run for 28 cycles at 94ºC for 5s, 68ºC for 10s, and 72ºC for 1 min.

The PCR products are analyzed by electrophoresis on a 1% agarose gel and the DNA purified from the gel using QIAEX11 agarose gel extraction kit from Qiagen.

The purified PCR products are introduced into PCR4-TOPO vector using TOPO TA Cloning kit from Invitrogen and used for transformation of TOP10 competent cells.

A suitable amount of colonies are analyzed by colony PCR using Taq polymerase, 1xTaq polymerase buffer, dNTP (10mM) and the following primers and PCR program:

M13forward primer: 5'-GTAAAACGACGGCCAG-3' (SEQ ID NO:32)

M13reverse primer: 5'-CAGGAAACAGCTATGAC-3' (SEQ ID NO:33)

PCR Program:

25 cycles are run at 94ºC for 30s, 55ºC for 30s, and 72ºC for 1 min.

Plasmid DNA from clones comprising VL and VH inserts, respectively, is extracted and sequenced using primer M13forward and M13reverse listed above. In the case of the

human anti-KIR mAb 1-7F9, the sequences encoding the heavy and light chain variable regions are shown in Fig 15.

(4) Subcloning of antibody genes into mammalian expression vectors

Based on the sequence data for cDNAs encoding the heavy and light chain variable regions of the mAb, primers are designed for the amplification of the variable light (VL) and variable heavy (VH) chain genes, respectively. The variable regions are formatted by PCR to include a Kozak sequence, leader sequence and unique restriction enzyme sites. For the VL, this is achieved by designing 5' PCR primers to introduce a HindIII site, the Kozak sequence and to be homologous to the 5' end of the leader sequence of the variable light chain region. The 3' primer is homologous to the 3' end of the variable region and introduced a BsiWI site at the 3' boundary of the variable region. The VH region is generated in a similar fashion except that a NotI and a NheI site are introduced in the 5' and 3' end instead of HindIII and BsiWI, respectively.

The amplified gene products are each cloned into a eukaryotic expression vector containing the light and heavy chain constant regions, using standard techniques. The VL DNA fragments is digested with HindIII and BsiWI and ligated into a eukaryotic expression vector containing the beta-lactamase gene encoding resistance to ampicillin and an E. coli replication origin (pUC); the resulting plasmid is designated VLCL. The VH DNA fragments, is digested with NotI and NheI and introduced into the VLCL vector resulting from the introduction of VL fragment as described above. The resulting plasmid contains functional expression cassettes encoding both the heavy and light chains of the antibody on the same plasmid. The ligated plasmid is used to transform *E. coli*. Plasmid DNA is prepared from these ampicillin resistant bacterial populations and used for transfection into Chinese hamster Ovary cells, or other mammalian cell lines. Transfection and cell culture is done be standard methods, as described for example in "Molecular Cloning", Sambrook et al. The result is transfected cell lines that stably express and secrete the antibody molecule of interest, such as the 1-7F9 or 1-4F1 human anti-KIR mAb or a mAb comprising the VH and VL regions of 1-7F9 or 1-4F1, or another human anti-KIR mAb.

Variants of the antibody can easily be generated. For example, an antibody with the exact same specificity as 1-7F9 or 1-4F1 but of a different isotype than IgG4 or IgG2, respectively, can be obtained by sub-cloning the cDNA encoding VL and VH of 1-7F9 or 1-4F1 into plasmids containing cDNA encoding the kappa light chain constant regions and a heavy constant chain region selected from IgG1 or IgG2 or IgG3 or IgG4 constant heavy chain regions. Thus, an antibody as generated can possess any isotype and the antibody can then be isotype switched using conventional techniques in the art. Such techniques include the use of

direct recombinant techniques (see, *e.g.*, US Patent 4,816,397), cell-cell fusion techniques (see *e.g.*, US Patent 5,916,771), and other suitable techniques known in the art. Accordingly, the effector function of antibodies provided by the invention may be "changed" with respect to the isotype of a parent antibody by isotype switching to, *e.g.*, an IgG1, IgG2, IgG3, IgG4, IgD, IgA, IgE, or IgM antibody for various uses, including therapeutic ones.

Thus, in further aspects, the invention provides a hybridoma comprising: (a) a B cell from a mammalian host (typically a non-human mammalian host) that has been immunized with an antigen that comprises an epitope present on an inhibitory KIR polypeptide, fused to (b) an immortalized cell (*e.g.*, a myeloma cell), wherein said hybridoma produces a monoclonal antibody that binds at least two different human inhibitory KIR receptors and is capable of at least substantially neutralizing KIR-mediated inhibition of NK cell cytotoxicity in a population of NK cells expressing said at least two different human inhibitory KIR receptors. In one embodiment, the mammalian host is a transgenic animal capable of producing human antibodies. Optionally, said hybridoma does not produce monoclonal antibody NKVSF1, not A210, and/or not A802g. In various embodiments, the antibody binds KIR2DL1 and KIR2DL2/3 receptors or a common determinant present on both KIR2DL1 and KIR2DL2/3. The hybridoma may produce an antibody that inhibits the binding of a HLA-C allele molecule having a Lys residue at position 80 to a human KIR2DL1 receptor, and the binding of a HLA-C allele molecule having an Asn residue at position 80 to human KIR2DL2/3 receptors. For example, the hybridoma may produce an antibody that binds to substantially the same epitope as monoclonal antibody 1-7F9 or 1-4F1 on either KIR2DL1 or KIR2DL2/3 or both KIR2DL1 and KIR2DL2/3.

The invention also provides methods of producing an antibody which cross-reacts with multiple KIR2DL gene products and which reduces or neutralizes the inhibitory activity of such KIRs, said method comprising the steps of:

(a) immunizing a non-human mammal with an immunogen comprising a KIR2DL polypeptide;

(b) preparing antibodies from said immunized mammal, wherein said antibodies bind said KIR2DL polypeptide,

(c) selecting antibodies of (b) that cross-react with at least two different KIR2DL gene products, and

(d) selecting antibodies of (c) that potentiate NK cells. In one embodiment, said non-human mammal is a transgenic animal engineered to express a human antibody repertoire (*e.g.*, a non-human mammal comprising human immunoglobulin loci and native immunoglobulin gene deletions, such as a Xenomouse™ (Abgenix – Fremont, CA, USA) or non-

human mammal comprising a minilocus of human Ig-encoding genes, such as the HuMab-mouse™ (Medarex – Princeton, NJ, USA)). Optionally, step a and b may be replaced by alternative methods for obtaining anti-KIR mAbs, including, without limitation, the use of phage-display or viral transduction of human B cells, or other methods known in the art. Optionally, the method further comprises selecting an antibody that binds to KIR in a primate, preferably a cynomolgus monkey, NK cell or KIR polypeptide. Optionally, the invention further comprises a method of evaluating an anti-KIR antibody, wherein an antibody produced according to the above method is administered to a primate, preferably a cynomolgus monkey, preferably wherein the monkey is observed for the presence or absence of an indication of toxicity of the antibody.

The invention also provides a method of producing an antibody that binds at least two different human inhibitory KIR receptor gene products, wherein the antibody is capable of neutralizing KIR-mediated inhibition of cytotoxicity (or potentiating NK cytotoxicity) by a population of NK cells expressing the at least two different human inhibitory KIR receptor gene products, the method comprising the steps of:

a) immunizing a non-human mammal with an immunogen comprising an inhibitory KIR polypeptide;

b) preparing antibodies from the immunized animal, wherein the antibodies bind the KIR polypeptide,

c) selecting antibodies of (b) that cross-react with at least two different human inhibitory KIR receptor gene products, and

selecting antibodies of (c) that capable of neutralizing KIR-mediated inhibition of NK cell cytotoxicity on a population of NK cells expressing the at least two different human inhibitory KIR receptor gene products, wherein the order of steps (c) and (d) is optionally reversed and any number of the steps are optionally repeated 1 or more times. The inhibitory KIR polypeptide used for immunization can, in one embodiment, be one or more KIR2DL polypeptide(s) and the antibodies selected in step (c) cross-react with at least KIR2DL1 and KIR2DL2/3. Preferably the antibody recognizes a common determinant present on at least two different KIR receptor gene products; most preferably the KIRs are KIR2DL1 and KIR2DL2/3. In one embodiment, step (c) comprises selecting antibodies which react with at least one KIR2DL and one KIR3DL receptor gene products. For example, the selected antibody could react with KIR2DL1 and KIR2DL2/3 as well as KIR3DL1 and/or KIR3DL2. Optionally, the method further comprises selecting an antibody that binds a primate, preferably a cynomolgus monkey, NK cell or KIR polypeptide. Optionally, the invention further comprises a method of evaluating an antibody, wherein an antibody produced according to the

above method is administered to a primate, preferably a cynomolgus monkey, preferably wherein the monkey is observed for the presence or absence of an indication of toxicity of the antibody.

Optionally, in the above-described methods, the antibody selected in step c) or d) is not NKVSF1, not A210, and/or not A802g. Preferably, the antibody prepared in step (b) in the above methods is a monoclonal antibody. Preferably the antibody selected in step (c) in the above methods inhibits the binding of one or more HLA-C allotypes having a Lys residue at position 80 to a human KIR2DL1 receptor, and the binding of one or more HLA-C allotypes having an Asn residue at position 80 to human KIR2DL2/3 receptors. Preferably, the antibodies selected in step (d) in the above methods cause a potentiation in NK cytotoxicity, or a neutralization of KIR-mediated inhibition of NK cytotoxicity. Preferably, the antibody binds to substantially the same epitope as monoclonal antibody 1-7F9 on KIR2DL1 and/or KIR2DL2/3. Optionally the methods also or alternatively comprise the additional step of making fragments of the selected monoclonal antibodies, making derivatives of the selected monoclonal antibodies (*e.g.*, by conjugation with a radionuclide, cytotoxic agent, reporter molecule, or the like), or making derivatives of antibody fragments produced from, or that comprise sequences that correspond to, the sequences of such monoclonal antibodies. In another aspect, a variant of such an antibody can be produced by preparing an antibody that comprises a variant amino acid sequence from the antibody obtained by the above-described methods by use of known genetic engineering methods (*e.g.*, a sequence that is modified so as to change the binding properties of the antibody, increase or decrease the stability of the antibody, enhance purification of the antibody, enhance detection of the antibody, and/or change the immunological properties of the antibody with respect to a host to which it is to be administered).

The invention further provides a method of producing an antibody that binds at least two different human inhibitory KIR receptor gene products, wherein the antibody is capable of neutralizing KIR-mediated inhibition of NK cell cytotoxicity on (or potentiating NK cytotoxicity by) a population of NK cells expressing at least one of the different human inhibitory KIR receptor gene products, the method comprising the steps of:

(a) selecting, from a library or repertoire, a monoclonal antibody or an antibody fragment that cross-reacts with at least two different human inhibitory KIR2DL receptor gene products, and

(b) selecting an antibody of (a) that is capable of neutralizing KIR-mediated inhibition of NK cell cytotoxicity in a population of NK cells expressing the at least two different human inhibitory KIR2DL receptor gene products. Preferably the antibody binds a common determi-

nant present on KIR2DL1 and KIR2DL2/3. Optionally, the antibody selected in step (b) is not NKVSF1. Preferably, the antibody selected in step (b) inhibits the binding of a HLA-c allele molecule having a Lys residue at position 80 to a human KIR2DL1 receptor, and the binding of a HLA-C allele molecule having an Asn residue at position 80 to human KIR2DL2/3 receptors. Preferably, the antibody selected in step (b) causes a potentiation in NK cytotoxicity. Preferably, the antibody binds to substantially the same epitope as monoclonal antibody 1-7F9 on KIR2DL1 and/or KIR2DL2/3. Alternatively, the antibody binds substantially the same epitope as monoclonal antibody 1-4F1. Optionally the method comprises the additional step of making fragments of the selected monoclonal antibodies, making derivatives of the selected monoclonal antibodies, or making derivatives of selected monoclonal antibody fragments.

Additionally, the invention provides a method of producing an antibody that binds at least two different human inhibitory KIR receptor gene products, wherein the antibody is capable of neutralizing KIR-mediated inhibition of NK cell cytotoxicity in a population of NK cells expressing at least one of the two different human inhibitory KIR receptor gene products, the method comprising the steps of:

a) culturing a hybridoma of the invention under conditions permissive for the production of the monoclonal antibody; and

b) separating the monoclonal antibody from the hybridoma cells. Optionally the method comprises the additional step of making fragments of the the monoclonal antibody, making derivatives of the monoclonal antibody, or making derivatives of such monoclonal antibody fragments. Preferably the antibody binds a common determinant present on KIR2DL1 and KIR2DL2/3.

Also provided by the present invention is a method of producing an antibody that binds at least two different human inhibitory KIR receptor gene products, wherein the antibody is capable of neutralizing KIR-mediated inhibition of NK cell cytotoxicity in a population of NK cells expressing at least one of the two different human inhibitory KIR receptor gene products, the method comprising the steps of:

a) isolating from a hybridoma of the invention a nucleic acid encoding the monoclonal antibody;

b) optionally modifying the nucleic acid so as to obtain a modified nucleic acid that comprises a sequence that encodes a modified or derivatized antibody comprising an amino acid sequence that corresponds to a functional sequence of the monoclonal antibody or is substantially similar thereto (*e.g.*, is at least about 65%, at least about 75%, at least about 85%, at least about 90%, at least about 95% (such as about 70-99%) identical to such a se-

quence) selected from a humanized antibody, a chimeric antibody, a single chain antibody, an immunoreactive fragment of an antibody, or a fusion protein comprising such an immunoreactive fragment;

c) inserting the nucleic acid or modified nucleic acid (or related nucleic acid coding for the same amino acid sequence) into an expression vector, wherein the encoded antibody or antibody fragment is capable of being expressed when the expression vector is present in a host cell grown under appropriate conditions;

d) transfecting a host cell with the expression vector, wherein the host cell does not otherwise produce immunoglobulin protein;

e) culturing the transfected host cell under conditions which cause the expression of the antibody or antibody fragment; and

f) isolating the antibody or antibody fragment produced by the transfected host cell. Preferably the antibody binds a common determinant present on KIR2DL1 and KIR2DL2/3.

Antibody Screening and Selection

Particular antibodies of this invention are able to reduce or neutralize the KIR-mediated inhibition of NK cell cytotoxicity; particularly inhibition mediated by KIR2DL receptors and more particularly at least both KIR2DL1- and KIR2DL2/3-mediated inhibition. These antibodies are thus "neutralizing", "blocking", or "inhibitory" antibodies, in the sense that they reduce, neutralize and/or block, at least partially and detectably, the inhibitory signaling pathway mediated by KIR receptors when these interact with MHC class I molecules. More importantly, this neutralization activity is displayed with respect to several types of inhibitory KIR receptors, preferably several KIR2DL receptor gene products, and more preferably at least both KIR2DL1 and KIR2DL2/3 so that these antibodies may be used in most or all human subjects with high efficacy. Neutralization of KIR-mediated inhibition of NK cell cytotoxicity can be assessed by various assays or tests, such as standard *in vitro* cellular cytotoxicity assays, as described herein.

Once an antibody that cross-reacts with multiple inhibitor KIR receptors is identified it can be tested for its ability to reduce or neutralize the inhibitory effect of those KIR receptors in intact NK cells. In a specific variant, the neutralizing activity can be illustrated by the capacity of said antibody to reconstitute lysis by KIR2DL-expressing NK cells of targets expressing HLA-C. In another specific embodiment, the neutralizing activity of the antibody is defined by the ability of the antibody to block or reduce the binding of HLA-C molecules to KIR2DL1 and KIR2DL3 (or the closely related KIR2DL2) receptors, further preferably as it is the capacity of the antibody to alter the binding of KIR2DL2/3 to a HLA-C molecule selected

from Cw1, Cw3, Cw7, and Cw8 (or of another HLA-C molecule having an Asn residue at position 80), and/or the binding of KIR2DL1 to a HLA-C molecule selected from Cw2, Cw4, Cw5 and Cw6 (or of another HLA-C molecule having a Lys residue at position 80).

In another variant, the neutralizing activity of an antibody of this invention can be assessed in a cell-based cytotoxicity assay, as disclosed in the Examples provided herein.

In another variant, the neutralizing activity of an antibody of this invention can be assessed in a cytokine-release assay, wherein NK cells are incubated with the test antibody and a target cell line expressing one HLA-C allele recognized by a KIR molecule of the NK population, to stimulate NK cell cytokine production (for example IFN-γ and/or GM-CSF production). In an exemplary protocol, IFN-γ production from PBMC is assessed by cell surface and intracytoplasmic staining and analysis by flow cytometry after about 4 days in culture. Briefly, Brefeldin A (Sigma Aldrich) can be added at a final concentration of about 5 µg/ml for the least about 4 hours of culture. The cells can then incubated with anti-CD3 and anti-CD56 mAb prior to permeabilization (IntraPrep™; Beckman Coulter) and staining with PE-anti-IFN-γ or PE-IgG1 (Pharmingen). GM-CSF and IFN- production from polyclonal activated NK cells can be measured in supernatants using ELISA (GM-CSF: DuoSet Elisa, R&D Systems, Minneapolis, MN; IFN-γ: OptE1A set, Pharmingen).

Antibodies of this invention may partially (*i.e.*, reduce) or fully neutralize the KIR-mediated inhibition of NK cell cytotoxicity. For example, preferred antibodies of this invention are able to induce or augment the lysis of HLA-matched, or HLA-compatible, or autologous target cell populations, *i.e.*, cell populations that would not be effectively lysed by NK cells in the absence of said antibody. Accordingly, the antibodies of this invention may also be defined as facilitating NK cell activity *in vivo*, and/or *in vitro*.

In a specific embodiment, the antibody binds substantially the same epitope as monoclonal antibody DF200 (produced by hybridoma DF200), 1-7F9, or 1-4F1. Such antibodies can be referred to herein as "DF200 like antibodies", "1-7F9-like antibodies", and "1-4F1-like antibodies", respectively. In a further preferred embodiment, the antibody is a monoclonal antibody. More preferred "DF200 like antibodies" of this invention are antibodies other than the monoclonal antibody NKVSF1. Most preferred is monoclonal antibody 1-7F9 or 1-4F1, and antibodies comprising the VH and/or VL regions of 1-7F9 or 1-4F1.

The identification of one or more antibodies that bind(s) to substantially or essentially the same epitope as the monoclonal antibodies described herein can be readily determined using any one of variety of immunological screening assays in which antibody competition can be assessed. A number of such assays are routinely practiced and well known in the art (see, *e.g.*, U.S. Pat. No. 5,660,827, issued Aug. 26, 1997, which is specifically incor-

porated herein by reference). It will be understood that actually determining the epitope to which an antibody described herein binds is not in any way required to identify an antibody that binds to the same or substantially the same epitope as the monoclonal antibody described herein.

For example, where the test antibodies to be examined are obtained from different source animals, or are even of a different Ig isotype, a simple competition assay may be employed in which the control antibody (DF200, for example) is mixed with the test antibody and then applied to a sample containing either or both KIR2DL1 and/or KIR2DL2/3, each of which is known to be bound by DF200. Protocols based upon ELISAs, radioimmunoassays, Western blotting, and the use of BIACORE analysis (as described, for example, in the Examples section herein) are suitable for use in such simple competition studies.

In certain embodiments, one would pre-mix the control antibody (1-7F9 or 1-4F1, for example) with varying amounts of the test antibody (*e.g.*, in ratios of about 1:1, 1:2, 1:10 or about 1:100) for a period of time prior to applying to the KIR antigen sample. In other embodiments, the control and varying amounts of test antibody can simply be added separately and admixed during exposure to the KIR antigen sample. As long as one can distinguish bound from free antibodies (*e.g.*, by using separation or washing techniques to eliminate unbound antibodies) and control antibody from the test antibody (*e.g.*, by using species specific or isotype specific secondary antibodies or by specifically labelling the control antibody with a detectable label) one will be able to determine if the test antibody reduce the binding of the control antibody to the different KIR2DL antigens, indicating that the test antibody recognizes substantially the same epitope as 1-7F9. The binding of the (labeled) control antibody in the presence of a completely irrelevant antibody (that does not bind KIR) can serve as the control high value. The control low value can be obtained by incubating the labeled control antibody with the same but unlabelled control antibody, where competition would occur and reduce binding of the labeled antibody. In a test assay, a significant reduction in labeled antibody reactivity in the presence of a test antibody is indicative of a test antibody that recognizes substantially the same epitope, *i.e.*, one that competes with the labeled control antibody. For example, any test antibody that reduces the binding of 1-7F9 or 1-4F1 to both of KIR2DL1 and KIR2DL2/3 antigens by at least about 50%, such as at least about 60%, or more preferably at least about 70% (*e.g.*, about 65-100%), at any ratio of 1-7F9:test antibody or 1-4F1:test antibody between about 1:1 or 1:10 and about 1:100 is considered to be an antibody that binds to substantially the same epitope or determinant as 1-7F9 or 1-4F1, respectively. Preferably, such test antibody will reduce the binding of 1-7F9 to at least one other, preferably each of the KIR2DL antigens preferably at least about 50%, at least about 60%, at

least about 80% or at least about 90% (*e.g.*, about 95%) of the binding of 1-7F9 or 1-4F1 observed in the absence of the test antibody. Of course, such methods can be adapted to identify and/or evaluate antibodies that compete with other antibodies and/or KIR antigens.

Competition can also or alternatively be assessed by, for example, a flow cytometry test. In such a test, cells bearing a given KIR can be incubated first with a control antibody (1-7F9 or 1-4F1, for example), and then with the test antibody labeled with a fluorochrome or biotin. The antibody is said to compete with control antibody if the binding obtained upon preincubation with saturating amount of control antibody is about 80%, preferably about 50%, about 40% or less (*e.g.*, about 30%) of the binding (as measured by mean of fluorescence) obtained by the test antibody without preincubation with control antibody. Alternatively, an antibody is said to compete with the control antibody if the binding obtained with a labeled control antibody (by a fluorochrome or biotin) on cells preincubated with saturating amount of test antibody is about 80%, preferably about 50%, about 40%, or less (*e.g.*, about 30%) of the binding obtained without preincubation with the test antibody.

A simple competition assay in which a test antibody is pre-adsorbed and applied at saturating concentration to a surface onto which either KIR2DL1 or KIR2DL2/3, or both, are immobilized also may be advantageously employed. The surface in the simple competition assay is preferably a BIACORE chip (or other media suitable for surface plasmon resonance analysis). The binding of a control antibody (*e.g.*, 1-7F9 or 1-4F1) to the KIR-coated surface is measured. This binding to the KIR-containing surface of the control antibody alone is compared with the binding of the control antibody in the presence of a test antibody. A significant reduction in binding to the KIR2DL1 and KIR2DL2/3-containing surface by the control antibody in the presence of a test antibody indicates that the test antibody recognizes substantially the same epitope as the control antibody such that the test antibody "competes" with the control antibody. Any test antibody that reduces the binding of control antibody (such as 1-7F9 or 1-4F1) to both of KIR2DL1 and KIR2DL2/3 antigens by at least about 20% or more, at least about 40%, at least about 50%, at least about 70%, or more, can be considered to be an antibody that binds to substantially the same epitope or determinant as the control antibody (*e.g.*, 1-7F9 or 1-4F1). Preferably, such test antibody will reduce the binding of the control antibody (*e.g.*, 1-7F9 or 1-4F1) to each of the KIR2DL antigens by at least about 50% (*e.g.*, at least about 60%, at least about 70%, or more). It will be appreciated that the order of control and test antibodies can be reversed; *i.e.* the control antibody can be first bound to the surface and then the test antibody is brought into contact with the surface thereafter in a competition assay. Preferably, the antibody having higher affinity for KIR2DL1 and KIR2DL2/3 antigens is bound to the KIR2DL1 and KIR2DL2/3-containing surface first, as

it will be expected that the decrease in binding seen for the second antibody (assuming the antibodies are competing) will be of greater magnitude. Further examples of such assays are provided in the Examples herein, and in *e.g.*, Saunal and Regenmortel, (1995) J. Immunol. Methods 183: 33-41, the disclosure of which is incorporated herein by reference.

While described in the context of 1-7F9 or 1-4F1 for the purposes of exemplification, it will be appreciated that the above-described screening assays can also be used to identify antibodies that compete with one or more of NKVSF1, DF200, EB6, GL183, and other antibodies, antibody fragments, and antibody derivatives according to the invention.

Upon immunization and production of antibodies in a vertebrate or cell, particular selection steps may be performed to isolate antibodies as claimed. In this regard, in a specific embodiment, the invention also relates to methods of producing such antibodies, comprising:

(a) immunizing a non-human mammal with an immunogen comprising an inhibitory KIR polypeptide;

(b) preparing antibodies from said immunized animal, wherein said antibodies bind said KIR polypeptide,

(c) selecting antibodies of (b) that cross-react with at least two different inhibitory KIR gene products, and

(d) selecting antibodies of (c) that are capable of neutralizing KIR-mediated inhibition of NK cell cytotoxicity on a population of NK cells expressing at least one of said two different human inhibitory KIR receptor gene products.

In certain embodiments, an additional step is performed between steps (c) and (d) above, to select anti-KIR mAbs that do not react with KIR2DS4.

The selection of an antibody that cross-reacts with at least two different inhibitory KIR gene products may be achieved by screening the antibody against two or more different inhibitory KIR antigens. In a more preferred embodiment, the antibodies prepared in step (b) are monoclonal antibodies. Thus, the term "preparing antibodies from said immunized animal," as used herein, includes obtaining B-cells from an immunized animal and using those B cells to produce a hybridoma that expresses antibodies, as well as obtaining antibodies directly from the serum of an immunized animal. In another preferred embodiment, the antibodies selected in step (c) are those that cross-react with at least KIR2DL1 and KIR2DL2/3.

In yet another preferred embodiment, the antibodies selected in step (d) cause at least about 10% more specific lysis mediated by NK cells displaying at least one KIR recognized by the antibody, and preferably at least about 40% more specific lysis, at least about 50% higher specific lysis, or more preferably at least about 70% increased specific lysis (*e.g.*, about 60-100% increased specific lysis), as measured in a standard chromium release assay

towards a target cell expressing cognate HLA class I molecule, compared to the specific lysis in the absence of an anti-KIR mAb.. Alternatively, the antibodies selected in step (d) when used in a chromium-release assay employing an NK cell clone expressing one or several inhibitory KIRs and a target cells expressing at least one HLA allele that is recognized by one of the KIRs on the NK clone, the level of cytotoxicity obtained with the antibody should be at least about 20%, preferably at least about 30 %, or more of the specific cytotoxicity obtained with the same concentration of DF200 or with a blocking anti-MHC class I antibody, at the same effector:target cell ratio.

The order of steps (c) and (d) of the immediately above-described method can be changed. Optionally, the method also or alternatively may further comprise additional steps of making fragments of the monoclonal antibody or derivatives of the monoclonal antibody or such fragments, *e.g.*, as described elsewhere herein.

In another variant, the invention provides a method for obtaining an antibody that comprises:

(a) selecting, from a library or repertoire, a monoclonal antibody, a fragment of a monoclonal antibody, or a derivative of either thereof that cross-reacts with at least two different human inhibitory KIR2DL receptor gene products, and

(b) selecting an antibody, fragment, or derivative of (a) that is capable of neutralizing KIR-mediated inhibition of NK cell cytotoxicity on a population of NK cells expressing said at least two different human inhibitory KIR2DL receptor gene products.

The repertoire may be any (recombinant) repertoire of antibodies or fragments thereof, optionally displayed by any suitable structure (*e.g.*, phage, bacteria, synthetic complex, etc.). Selection of inhibitory antibodies may be performed as disclosed above and further illustrated in the examples.

Computer modelling of the extra-cellular domains of KIR2DL1, -2 and -3 (KIR2DL1-3), based on their published crystal-structures (Maenaka et al. Structure with Folding and design 1999;7:391-398; Fan et al., Nature immunology 2001;2:452-460; Boyington et al. Nature, 2000;405:537-543), revealed the involvement of certain regions or KIR2DL1, -2 and -3 in the interaction between these KIR and the anti-KIR2DL cross-reactive murine monoclonal antibodies DF200 and NKVSF1. Thus, in one embodiment, the present invention provides antibodies that exclusively bind to KIR2DL1 within a region defined by the amino acid residues (105, 106, 107, 108, 109, 110, 111, 127, 129, 130, 131, 132, 133, 134, 135, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 181, and 192). In another embodiment the invention provides antibodies that bind to KIR2DL1 and KIR 2DL2/3 without interacting with amino acid residues outside the region defined by the residues (105, 106, 107, 108, 109,

110, 111, 127, 129, 130, 131, 132, 133, 134, 135, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 181, and 192).

In another embodiment, the invention provides antibodies that bind to KIR2DL1 and which does not bind to a mutant of KIR2DL1 in which R131 (*i.e.*, the Arg residue at position 131 of the KIR2DL1 mutant) is (*i.e.*, is substituted with) an Ala residue.

In another embodiment, the invention provides antibodies that bind to KIR2DL1 and which does not bind to a mutant of KIR2DL1 in which R157 is Ala.

In another embodiment, the invention provides antibodies that bind to KIR2DL1 and which does not bind to a mutant of KIR2DL1 in which R158 is Ala.

In another embodiment, the invention provides antibodies that bind to KIR2DL1 residues 131, 157, and 158.

In another embodiment, the invention provides antibodies that bind to KIR2DS3(R131W), but not to wild type KIR2DS3.

In a particular embodiment, the antibodies exclusively bind to KIR2DL1 within a region defined by the amino acid residues L38, R41, M44, F45, N46, D47, T48, L49, R50, I52, F64, D72, Y80, P87, and Y88 (in which the letters designate amino acids in single-letter code, and the number is the position of that residue in KIR2DL1 (SEQ ID NO:23), using the numbering system described in Wagtmann et al., Immunity 1995;2:439-449). These residues have been identified as the 1-7F9 KIR2DL1 epitope (see Example 11).

In another embodiment, the antibodies bind to an antigenic epitope in a KIR2DL1 sequence consisting of the fragment L38 to Y88 of SEQ ID NO:23. In yet another embodiment, the antibodies bind to an antigenic epitope in a KIR2DL1 sequence comprising the fragment L38 to Y88 of SEQ ID NO:23.

In another embodiment, the invention provides antibodies that bind to KIR2DL1 and KIR 2DL2/3 without interacting with amino acid residues outside the region defined by the residues L38, R41, M44, F45, N46, D47, T48, L49, R50, I52, F64, D72, Y80, P87, and Y88.

In another embodiment, the antibodies bind to KIR2DL1 within a region comprising at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more of the amino acid residues L38, R41, M44, F45, N46, D47, T48, L49, R50, I52, F64, D72, Y80, P87, and Y88. In a particular embodiment, the KIR2DL1 epitope for the antibodies comprises the amino acid residues M44 and F45 (see Examples 9 and 11).

In another embodiment, the antibodies bind to KIR2DL1 within a region comprising at least 1, 2, 4, 6, 8, 10, or 12 or all of the amino acid residues L38, R41, M44, F45, N46, D47, T48, L49, R50, I52, F64, D72, Y80, P87, and Y88. In a particular embodiment, the region comprises amino acid residues M44 and F45, and optionally at least 1, 2, 4, 6, 8, 10, or

all of L38, R41, N46, D47, T48, L49, R50, I52, F64, D72, Y80, P87, and Y88 (see Examples 9 and 11). In another particular embodiment, the region comprises at least amino acid residues M44, F45, and D72, which are in the KIR2DL1 region where the 1-7F9 epitope and the HLA-C binding sites overlap.

In another embodiment, the invention provides antibodies that bind to KIR2DL1, KIR2DL2/3, and KIR2DS4.

In another embodiment, the invention provides antibodies that bind to both KIR2DL1 and KIR2DL2/3, but not to KIR2DS4.

In another embodiment, the invention provides antibodies that bind to both KIR2DL1 and KIR2DL2/3, but not to KIR2DS3 or to KIR2DS4.

Determination of whether an antibody, antibody fragment, or antibody derivative binds within one of the epitope regions defined above can be carried out in ways known to the person skilled in the art. See, for example, Examples 9 and 11. In another example of such mapping/characterization methods, an epitope region for an anti-KIR antibody may be determined by epitope "foot-printing" using chemical modification of the exposed amines/carboxyls in the KIR2DL1 or KIR2DL2/3 protein. One specific example of such a foot-printing technique is the use of HXMS (hydrogen-deuterium exchange detected by mass spectrometry) wherein a hydrogen/deuterium exchange of receptor and ligand protein amide protons, binding, and back exchange occurs, wherein the backbone amide groups participating in protein binding are protected from back exchange and therefore will remain deuterated. Relevant regions can be identified at this point by peptic proteolysis, fast microbore high-performance liquid chromatography separation, and/or electrospray ionization mass spectrometry. See, *e.g.*, Ehring H, Analytical Biochemistry, Vol. 267 (2) pp. 252-259 (1999) and/or Engen, J.R. and Smith, D.L. (2001) Anal. Chem. 73, 256A-265A. Another example of a suitable epitope identification technique is nuclear magnetic resonance epitope mapping (NMR), where typically the position of the signals in two-dimensional NMR spectres of the free antigen and the antigen complexed with the antigen binding peptide, such as an antibody, are compared. The antigen typically is selectively isotopically labeled with 15N so that only signals corresponding to the antigen and no signals from the antigen binding peptide are seen in the NMR-spectrum. Antigen signals originating from amino acids involved in the interaction with the antigen binding peptide typically will shift position in the spectres of the complex compared to the spectres of the free antigen, and the amino acids involved in the binding can be identified that way. See, *e.g.*, Ernst Schering Res Found Workshop. 2004;(44):149-67; Huang et al, Journal of Molecular Biology, Vol. 281 (1) pp. 61-67 (1998); and Saito and Patterson, Methods. 1996 Jun;9(3):516-24.

Epitope mapping/characterization also can be performed using mass spectrometry methods. See, *e.g.*, Downward, J Mass Spectrom. 2000 Apr;35(4):493-503 and Kiselar and Downard, Anal Chem. 1999 May 1;71(9):1792-801.

Protease digestion techniques also can be useful in the context of epitope mapping and identification. Antigenic determinant-relevant regions/sequences can be determined by protease digestion, *e.g.* by using trypsin in a ratio of about 1:50 to KIR2DL1 or KIR2DL2/3 o/n digestion at 37 °C and pH 7-8, followed by mass spectrometry (MS) analysis for peptide identification. The peptides protected from trypsin cleavage by the anti-KIR antibody can subsequently be identified by comparison of samples subjected to trypsin digestion and samples incubated with antibody and then subjected to digestion by *e.g.* trypsin (thereby revealing a foot print for the antibody). Other enzymes like chymotrypsin, pepsin, etc., also or alternatively can be used in a similar epitope characterization methods. Moreover, enzymatic digestion can provide a quick method for analyzing whether a potential antigenic determinant sequence is within a region of the KIR2DL1 in the context of a KIR-binding polypeptide. If the polypeptide is not surface exposed, it is most likely not relevant in terms of immunogenicity/antigenicity. See, *e.g.*, Manca, Ann Ist Super Sanita. 1991;27(1):15-9 for a discussion of similar techniques.

Site-directed mutagenesis is another technique useful for elucidation of a binding epitope. For example, in "alanine-scanning", each residue within a protein segment is replaced with an alanine residue, and the consequences for binding affinity measured. If the mutation leads to a significant resuction in binding affinity, it is most likely involved in binding. Monoclonal antibodies specific for structural epitopes (*i.e.*, antibodies which do not bind the unfolded protein) can be used to verify that the alanine-replacement does not influence overall fold of the protein. See, *e.g.*, Clackson and Wells, Science 1995;267:383–386; and Wells, Proc Natl Acad Sci USA 1996;93:1–6.

Electron microscopy can also be used for epitope "foot-printing". For example, Wang et al., Nature 1992;355:275-278 used coordinated application of cryoelectron microscopy, three-dimensional image reconstruction, and X-ray crystallography to determine the physical footprint of a Fab-fragment on the capsid surface of native cowpea mosaic virus.

Other forms of "label-free" assay for epitope evaluation include surface plasmon resonance (SPR, BIACORE) and reflectometric interference spectroscopy (RifS). See, *e.g.*, Fägerstam et al., Journal Of Molecular Recognition 1990;3:208-14; Nice et al., J. Chromatogr. 1993;646:159–168; Leipert et al., Angew. Chem. Int. Ed. 1998;37:3308–3311; Kröger et al., Biosensors and Bioelectronics 2002;17:937-944.

Antibody Fragments and Derivatives

Fragments and derivatives of antibodies of this invention (which are encompassed by the terms "antibody" and "antibodies" as used in this application, unless otherwise stated or clearly contradicted by context), preferably a 1-7F9- or 1-4F1-like antibody, can be produced by techniques that are known in the art. "Immunoreactive fragments" comprise a portion of the intact antibody, generally the antigen binding site or variable region. Examples of antibody fragments include "kappa bodies" (see, *e.g.*, Ill et al., Protein Eng 10: 949-57 (1997)) and "janusins" (described further elsewhere herein).Fab, Fab', Fab'-SH, F(ab)2, F(ab')2, dAb (Ward et al., (1989) Nature 341:544-546); consisting essentially of a VH domain), Fd (consisting essentially of the VH and CH1 domains), and Fv (consisting essentially of the VL and VH domains) fragments; diabodies; kappa bodies; and janusins. For example, antibody fragments include a polypeptide having a primary structure consisting of one uninterrupted sequence of contiguous amino acid residues (referred to herein as a "single-chain antibody fragment" or "single chain polypeptide"), including without limitation (1) single-chain Fv (scFv) molecules (2) single chain polypeptides containing only one light chain variable domain, or a fragment thereof that contains the three CDRs of the light chain variable domain, without an associated heavy chain moiety and (3) single chain polypeptides containing only one heavy chain variable region, or a fragment thereof containing the three CDRs of the heavy chain variable region, without an associated light chain moiety; and multispecific antibodies formed from antibody fragments. For instance, Fab or F(ab')2 fragments may be produced by protease digestion of the isolated antibodies, according to conventional techniques.

It will be appreciated that immunoreactive fragments can be modified using known methods, for example to slow clearance *in vivo* and obtain a more desirable pharmacokinetic profile. For example, the fragment may be modified with polyethylene glycol (PEG) or polyoxyethylated polyols. Methods for coupling and site-specifically conjugating PEG to a Fab' fragment are described in, for example, Leong et al, Cytokine 16(3):106-119 (2001) and Delgado et al, Br. J. Cancer 73(2):175-182 (1996), the disclosures of which are incorporated herein by reference.

In a particular aspect, the invention provides antibodies, antibody fragments, and antibody derivatives comprising the light and/or heavy chain variable region sequence of 1-7F9 or 1-4F1 as set forth in Figure 14. In another aspect, the invention provides antibodies, antibody fragments, and derivatives thereof that comprise one or more of the light and/or heavy chain variable region CDRs of 1-7F9 or 1-4F1 as set forth in Fig. 15 or described above. Functional variants/analogs of such sequences can be generated by making suitable substitutions, additions, and/or deletions in these disclosed amino acid sequences using

standard techniques, which may be aided by the comparison of the sequences. From superimposition of crystal structures of the 1-7F9/KIR2DL1 complex and the and the KIR2DL1/MHC class I complex (Fan et al., Nat. Immunol. 2001; 2: 452-460), PDB-code 1IM9, it can be concluded that a scFv derivative of 1-7F9 will be able to block MHC class I binding to KIR2DL1. Also the single L-chain of 1-7F9 or the single VL domain of 1-7F9 alone, when bound to KIR2DL1, would block MHC class I binding.

Functional variants/analogs of such sequences can be generated by making suitable substitutions, additions, and/or deletions in these disclosed amino acid sequences using standard techniques, which may be aided by the comparison of the sequences. In another aspect, positions where a residue is present in a sequence of one of these antibodies, but not another, may be suitable for deletions, substitutions, and/or insertions.

Alternatively, the DNA of a encoding an antibody of this invention, such as a 1-7F9- or 1-4F1-like antibody, may be modified so as to encode for a fragment of this invention. The modified DNA is then inserted into an expression vector and used to transform or transfect an appropriate cell, which then expresses the desired fragment, as described elsewhere herein.

In an alternate embodiment, the DNA of a hybridoma producing a murine antibody of this invention, such as a DF200-like antibody, can be modified prior to insertion into an expression vector, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous non-human sequences (as described by *e.g.*, Morrison et al., Proc. Natl. Acad. Sci. U.S.A., 81, pp. 6851 (1984)). In another embodiment, the variable regions encoding the antibody of this invention may be joined, by recombinant DNA engineering, to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In that manner, "chimeric" or "hybrid" antibodies are prepared that have the binding specificity of the original antibody. Typically, such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody of the invention.

Thus, according to another embodiment, the antibody of this invention, preferably a DF-200-like antibody is humanized. "Humanized" forms of antibodies according to this invention are specific chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab') 2, or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from the murine immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of the original antibody (donor antibody) while maintaining the desired specificity, affin-

ity, and capacity of the original antibody. In some instances, Fv framework residues of the human immunoglobulin may be replaced by corresponding non-human residues. Furthermore, humanized antibodies can comprise residues that are not found in either the recipient antibody or in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of the original antibody and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details see Jones et al., Nature, 321, pp. 522 (1986); Reichmann et al., Nature, 332, pp. 323 (1988); and Presta, Curr. Op. Struct. Biol., 2, pp. 593 (1992).

Methods for humanizing the antibodies of this invention are well known in the art. Generally, a humanized antibody according to the present invention has one or more amino acid residues introduced into it from the original antibody. These murine or other non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., Nature, 321, pp. 522 (1986); Riechmann et al., Nature, 332, pp. 323 (1988); Verhoeyen et al., Science, 239, pp. 1534 (1988)). Accordingly, such "humanized" antibodies are chimeric antibodies (Cabilly et al., U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from the original murine antibody. In practice, humanized antibodies according to this invention are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in the original murine antibody.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of an antibody of this invention is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the mouse is then accepted as the human framework (FR) for the humanized antibody (Sims et al., J. Immunol., 151, pp. 2296 (1993); Chothia and Lesk, J. Mol. Biol., 196, pp. 901 (1987)). Another method uses a particular framework from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework can be used for several different humanized antibodies (Carter

et al., Proc. Natl. Acad. Sci. U.S.A., 89, pp. 4285 (1992); Presta et al., J. Immunol., 51, pp. 1993)).

It is further important that antibodies be humanized with retention of high affinity for multiple inhibitory KIR receptors and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

As described above, a method of making human monoclonal antibodies is to use a XenoMouse® (Abgenix, Fremont, CA) as the mouse used for immunization. A XenoMouse is a murine host according to this invention that has had its immunoglobulin genes replaced by functional human immunoglobulin genes. Thus, antibodies produced by this mouse or in hybridomas made from the B cells of this mouse, are already humanized. The XenoMouse is described in United States Patent No. 6,162,963, which is herein incorporated in its entirety by reference. An analogous method can be achieved using a HuMAb-Mouse™ (Medarex).

Human antibodies may also be produced according to various other techniques, such as by using, for immunization, other transgenic animals that have been engineered to express a human antibody repertoire (Jakobovitz et al., Nature 362 (1993) 255), or by selection of antibody repertoires using phage display methods. Such techniques are known to the skilled person and can be implemented starting from monoclonal antibodies as disclosed in the present application.

The antibodies of the present invention, preferably a 1-7F9- or 1-4F1-like antibody, may also be derivatized to "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in the original antibody, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another

antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (Cabilly et al., supra; Morrison et al., Proc. Natl. Acad. Sci. U.S.A., 81, pp. 6851 (1984)).

Other derivatives within the scope of this invention include functionalized antibodies, *i.e.*, antibodies that are conjugated or covalently bound to a toxin, such as ricin, diphtheria toxin, abrin and *Pseudomonas exotoxin*, or a therapeutic radionuclide such as, *e.g.*, ^{90}Y or ^{131}I; to a detectable moiety, such as a fluorescent moiety, a diagnostic radioisotope or an imaging agent; or to a solid support, such as agarose beads or the like. Methods for conjugation or covalent bonding of these other agents to antibodies are well known in the art.

Conjugation to a toxin is useful for targeted killing of NK cells displaying one of the cross-reacting KIR receptors on its cell surface. Once the antibody of the invention binds to the cell surface of such cells, it is internalized and the toxin is released inside of the cell, selectively killing that cell. Such use is an alternate embodiment of the present invention.

Conjugation to a detectable moiety is useful when the antibody of this invention is used for diagnostic purposes. Such purposes include, but are not limited to, assaying biological samples for the presence of the NK cells bearing the cross-reacting KIR on their cell surface and detecting the presence of NK cells bearing the cross-reacting KIR in a living organism. Such assay and detection methods are also alternate embodiments of the present invention.

Conjugation of an antibody of this invention to a solid support is useful as a tool for affinity purification of NK cells bearing the cross-reacting KIR on their cell surface from a source, such as a biological fluid. This method of purification is another alternate embodiment of the present invention, as is the resulting purified population of NK cells.

PHARMACEUTICAL COMPOSITIONS AND APPLICATIONS

The invention also provides pharmaceutical compositions that comprise a human or humanized antibody, or fragments and derivatives thereof, wherein said antibody, fragment or derivative cross-reacts with at least two inhibitory KIR receptors at the surface of NK cells, reduces or neutralizes their inhibitory signals and potentiates the activity of those cells, in any suitable vehicle in an amount effective to detectably potentiate NK cell cytotoxicity in a patient or in a biological sample comprising NK cells. The pharmaceutical compositions comprising a human or humanized anti-KIR mAb, optionally together with another active agent, for use according to the present invention may be formulated with pharmaceutically acceptable carriers or diluents as well as any other known adjuvants and excipients in accordance with conventional techniques such as those disclosed in Remington: The Science and Prac-

tice of Pharmacy, 19th Edition, Gennaro, Ed., Mack Publishing Co., Easton, PA, 1995. The compositions may appear in conventional forms, for example, capsules, tablets, aerosols, solutions or suspensions, or as freeze-dried powder.

Accordingly, one object of the present invention is to provide a pharmaceutical formulation comprising a human or humanized antibody, or fragments or derivatives thereof, which is present in a concentration from 1 mg/ml to 500 mg/ml, and wherein said formulation has a pH from 2.0 to 10.0. The formulation may further comprise a buffer system, preservative(s), tonicity agent(s), chelating agent(s), stabilizers and surfactants. In one embodiment of the invention the pharmaceutical formulation is an aqueous formulation, *i.e.*, a formulation comprising water. Such formulation is typically a solution or a suspension. In a further embodiment of the invention the pharmaceutical formulation is an aqueous solution. As used herein, the term "aqueous formulation" is a formulation comprising at least 50 %w/w water. Likewise, the term "aqueous solution" is a solution comprising at least 50 %w/w water, and the term "aqueous suspension" a suspension comprising at least 50 %w/w water.

In another embodiment, the pharmaceutical formulation is a freeze-dried formulation, whereto the physician or the patient adds solvents and/or diluents prior to use.

In another embodiment, the pharmaceutical formulation is a dried formulation (*e.g.*, freeze-dried or spray-dried) ready for use without any prior dissolution.

In a further aspect, the invention relates to a pharmaceutical formulation comprising an aqueous solution of an antibody as described herein, and a buffer, wherein said the antibody is present in a concentration from 1 mg/ml or above, and wherein said formulation has a pH from about 2.0 to about 10.0.

In another embodiment, the pH of the formulation is selected from the list consisting of 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9.0, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, and 10.0.

In a further embodiment, the buffer is selected from the group consisting of sodium acetate, sodium carbonate, citrate, glycylglycine, histidine, glycine, lysine, arginine, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium phosphate, and tris(hydroxymethyl)-aminomethan, bicine, tricine, malic acid, succinate, maleic acid, fumaric acid, tartaric acid, aspartic acid or mixtures thereof. Each one of these specific buffers constitutes an alternative embodiment of the invention.

In a further embodiment, the formulation also comprises a pharmaceutically acceptable preservative. In another embodiment, the preservative is selected from the group con-

sisting of phenol, o-cresol, m-cresol, p-cresol, methyl p-hydroxybenzoate, propyl p-hydroxybenzoate, 2-phenoxyethanol, butyl p-hydroxybenzoate, 2-phenylethanol, benzyl alcohol, chlorobutanol, and thiomerosal, bronopol, benzoic acid, imidurea, chlorohexidine, sodium dehydroacetate, chlorocresol, ethyl p-hydroxybenzoate, benzethonium chloride, chlorphenesine (3p-chlorphenoxypropane-1,2-diol) or mixtures thereof. In one embodiment, the preservative is present in a concentration from 0.1 mg/ml to 20 mg/ml. In another embodiment, the preservative is present in a concentration from 0.1 mg/ml to 5 mg/ml. In yet another embodiment, the preservative is present in a concentration from 5 mg/ml to 10 mg/ml. In still another embodiment, the preservative is present in a concentration from 10 mg/ml to 20 mg/ml. Each one of the specific preservatives listed constitutes an alternative embodiment of the invention. While the use of a preservative in pharmaceutical compositions is well-known to the skilled person; for convenience, reference is made to Remington: The Science and Practice of Pharmacy, 19th edition, 1995.

In a further embodiment, the formulation also comprises an isotonic agent. In one embodiment, the isotonic agent is selected from the group consisting of a salt (*e.g.*, sodium chloride), a sugar, a sugar alcohol, an amino sugar, an amino acid (*e.g.*, L-glycine, L-histidine, arginine, lysine, isoleucine, aspartic acid, tryptophan, threonine), an alditol (*e.g.*, glycerol (glycerine), 1,2-propanediol (propyleneglycol), 1,3-propanediol, 1,3-butanediol), and polyethyleneglycol (*e.g.*, PEG400), or mixtures thereof. Any suitable sugar can be used, including, but not limited to mono-, di-, or polysaccharides; and water-soluble glucans, such as, for example, fructose, glucose, mannose, sorbose, xylose, maltose, lactose, sucrose, trehalose, dextran, pullulan, dextrin, cyclodextrin, soluble starch, hydroxyethyl starch and carboxymethylcellulose-Na. In one embodiment, the sugar additive is sucrose. Sugar alcohol is defined as a C4-C8 hydrocarbon having at least one --OH group and includes, for example, mannitol, sorbitol, inositol, galactitol, dulcitol, xylitol, and arabitol. In one embodiment the sugar alcohol additive is mannitol. The sugars or sugar alcohols mentioned above may be used individually or in combination. There is no fixed limit to the amount used, as long as the sugar or sugar alcohol is soluble in the liquid preparation and does not adversely effect the stabilizing effects achieved using the methods of the invention. In one embodiment, the sugar or sugar alcohol concentration is between about 1 mg/ml and about 150 mg/ml. In another embodiment, the isotonic agent is present in a concentration from 1 mg/ml to 50 mg/ml. In another embodiment, the isotonic agent is present in a concentration from 1 mg/ml to 7 mg/ml. In another embodiment, the isotonic agent is present in a concentration from 8 mg/ml to 24 mg/ml. In another embodiment, the isotonic agent is present in a concentration from 25 mg/ml to 50 mg/ml. Each one the specific isotonic agents listed above constitutes an alterna-

tive embodiment of the invention. While the use of an isotonic agent in pharmaceutical compositions is well-known to the skilled person; for convenience, reference is made to Remington: The Science and Practice of Pharmacy, 19th edition, 1995.

In a further embodiment, the formulation also comprises a chelating agent. In one embodiment, the chelating agent is selected from salts of ethylenediaminetetraacetic acid (EDTA), citric acid, and aspartic acid, and mixtures thereof. In another embodiment, the chelating agent is present in a concentration from 0.1mg/ml to 5mg/ml. In another embodiment, the chelating agent is present in a concentration from 0.1 mg/ml to 2 mg/ml. In a further embodiment of the invention the chelating agent is present in a concentration from 2 mg/ml to 5 mg/ml. Each one of these specific chelating agents constitutes an alternative embodiment of the invention. The use of a chelating agent in pharmaceutical compositions is well-known to the skilled person. For convenience reference is made to Remington: The Science and Practice of Pharmacy, 19th edition, 1995.

In a further embodiment, the formulation also comprises a stabilizer. While the use of a stabilizer in pharmaceutical compositions is well-known to the skilled person; for convenience, reference is made to Remington: The Science and Practice of Pharmacy, 19th edition, 1995.

Compositions of the invention may, for example, be stabilized liquid pharmaceutical compositions whose therapeutically active components include a polypeptide that possibly exhibits aggregate formation during storage in liquid pharmaceutical formulations. By "aggregate formation" is intended a physical interaction between the polypeptide molecules that results in formation of oligomers, which may remain soluble, or large visible aggregates that precipitate from the solution. By "during storage" is intended a liquid pharmaceutical composition or formulation once prepared, is not immediately administered to a subject. Rather, following preparation, it is packaged for storage, either in a liquid form, in a frozen state, or in a dried form for later reconstitution into a liquid form or other form suitable for administration to a subject. By "dried form" is intended the liquid pharmaceutical composition or formulation is dried either by freeze drying (*i.e.*, lyophilization; see, for example, Williams and Polli (1984) J. Parenteral Sci. Technol. 38:48-59), spray drying (see Masters (1991) in Spray-Drying Handbook (5th ed; Longman Scientific and Technical, Essez, U.K.), pp. 491-676; Broadhead et al. (1992) Drug Devel. Ind. Pharm. 18:1169-1206; and Mumenthaler et al. (1994) Pharm. Res. 11:12-20), or air drying (Carpenter and Crowe (1988) Cryobiology 25:459-470; and Roser (1991) Biopharm. 4:47-53). Aggregate formation by a polypeptide during storage of a liquid pharmaceutical composition can adversely affect biological activity of that polypeptide, resulting in loss of therapeutic efficacy of the pharmaceutical composition. Furthermore, aggregate

formation may cause other problems such as blockage of tubing, membranes, or pumps when the polypeptide-containing pharmaceutical composition is administered using an infusion system.

The pharmaceutical compositions of the invention may also comprise an amount of an amino acid base sufficient to decrease aggregate formation by the polypeptide during storage of the composition. By "amino acid base" is intended an amino acid or a combination of amino acids, where any given amino acid is present either in its free base form or in its salt form. Where a combination of amino acids is used, all of the amino acids may be present in their free base forms, all may be present in their salt forms, or some may be present in their free base forms while others are present in their salt forms. In one embodiment, amino acids to use in preparing the compositions of the invention are those carrying a charged side chain, such as arginine, lysine, aspartic acid, and glutamic acid. Any stereoisomer (*i.e.*, L, D, or a mixture thereof) of a particular amino acid (*e.g.* methionine, histidine, imidazole, arginine, lysine, isoleucine, aspartic acid, tryptophan, threonine and mixtures thereof) or combinations of these stereoisomers, may be present in the pharmaceutical compositions of the invention so long as the particular amino acid is present either in its free base form or its salt form. In one embodiment the L-stereoisomer is used. Compositions of the invention may also be formulated with analogues of these amino acids. By "amino acid analogue" is intended a derivative of the naturally occurring amino acid that brings about the desired effect of decreasing aggregate formation by the polypeptide during storage of the liquid pharmaceutical compositions of the invention. Suitable arginine analogues include, for example, aminoguanidine, ornithine and N-monoethyl L-arginine, suitable methionine analogues include ethionine and buthionine and suitable cysteine analogues include S-methyl-L cysteine. As with the other amino acids, the amino acid analogues are incorporated into the compositions in either their free base form or their salt form. In a further embodiment of the invention the amino acids or amino acid analogues are used in a concentration, which is sufficient to prevent or delay aggregation of the protein.

In a further embodiment, methionine (or other sulphuric amino acids or amino acid analogous) may be added to inhibit oxidation of methionine residues to methionine sulfoxide when the polypeptide acting as the therapeutic agent is a polypeptide comprising at least one methionine residue susceptible to such oxidation. By "inhibit" is intended minimal accumulation of methionine oxidized species over time. Inhibiting methionine oxidation results in greater retention of the polypeptide in its proper molecular form. Any stereoisomer of methionine (L or D) or combinations thereof can be used. The amount to be added should be an amount sufficient to inhibit oxidation of the methionine residues such that the amount of me-

thionine sulfoxide is acceptable to regulatory agencies. Typically, this means that the composition contains no more than about 10% to about 30% methionine sulfoxide. Generally, this can be achieved by adding methionine such that the ratio of methionine added to methionine residues ranges from about 1:1 to about 1000:1, such as 10:1 to about 100:1.

In a further embodiment, the formulation also comprises a stabilizer selected from the group of high molecular weight polymers or low molecular compounds. In one embodiment, the stabilizer is selected from polyethylene glycol (*e.g.* PEG 3350), polyvinyl alcohol (PVA), polyvinylpyrrolidone, carboxy-/hydroxycellulose or derivates thereof (*e.g.*, HPC, HPC-SL, HPC-L and HPMC), cyclodextrins, sulphur-containing substances as monothioglycerol, thioglycolic acid and 2-methylthioethanol, and different salts (*e.g.* sodium chloride). Each one of these specific stabilizers constitutes an alternative embodiment of the invention.

The pharmaceutical compositions may also comprise additional stabilizing agents, which further enhance stability of a therapeutically active polypeptide therein. Stabilizing agents of particular interest to the present invention include, but are not limited to, methionine and EDTA, which protect the polypeptide against methionine oxidation, and a nonionic surfactant, which protects the polypeptide against aggregation associated with freeze-thawing or mechanical shearing.

In a further embodiment, the formulation also comprises a surfactant. In one embodiment, the surfactant is selected from a detergent, ethoxylated castor oil, polyglycolyzed glycerides, acetylated monoglycerides, sorbitan fatty acid esters, polyoxypropylene-polyoxyethylene block polymers (eg. poloxamers such as Pluronic® F68, poloxamer 188 and 407, Triton X-100), polyoxyethylene sorbitan fatty acid esters, polyoxyethylene and polyethylene derivatives such as alkylated and alkoxylated derivatives (tweens, *e.g.* Tween-20, Tween-40, Tween-80 and Brij-35), monoglycerides or ethoxylated derivatives thereof, diglycerides or polyoxyethylene derivatives thereof, alcohols, glycerol, lectins and phospholipids (eg. phosphatidyl serine, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, diphosphatidyl glycerol and sphingomyelin), derivates of phospholipids (eg. dipalmitoyl phosphatidic acid) and lysophospholipids (eg. palmitoyl lysophosphatidyl-L-serine and 1-acyl-sn-glycero-3-phosphate esters of ethanolamine, choline, serine or threonine) and alkyl, alkoxyl (alkyl ester), alkoxy (alkyl ether)- derivatives of lysophosphatidyl and phosphatidylcholines, *e.g.* lauroyl and myristoyl derivatives of lysophosphatidylcholine, dipalmitoylphosphatidylcholine, and modifications of the polar head group, that is cholines, ethanolamines, phosphatidic acid, serines, threonines, glycerol, inositol, and the positively charged DODAC, DOTMA, DCP, BISHOP, lysophosphatidylserine and lysophosphatidylthreonine, and glycerophospholipids (eg. cephalins), glyceroglycolipids (eg. galactopyransoide), sphingoglycol-

ipids (eg. ceramides, gangliosides), dodecylphosphocholine, hen egg lysolecithin, fusidic acid derivatives- (*e.g.* sodium tauro-dihydrofusidate etc.), long-chain fatty acids and salts thereof C6-C12 (eg. oleic acid and caprylic acid), acylcarnitines and derivatives, N^{α}-acylated derivatives of lysine, arginine or histidine, or side-chain acylated derivatives of lysine or arginine, N^{α}-acylated derivatives of dipeptides comprising any combination of lysine, arginine or histidine and a neutral or acidic amino acid, N^{α}-acylated derivative of a tripeptide comprising any combination of a neutral amino acid and two charged amino acids, DSS (docusate sodium, CAS registry no [577-11-7]), docusate calcium, CAS registry no [128-49-4]), docusate potassium, CAS registry no [7491-09-0]), SDS (sodium dodecyl sulphate or sodium lauryl sulphate), sodium caprylate, cholic acid or derivatives thereof, bile acids and salts thereof and glycine or taurine conjugates, ursodeoxycholic acid, sodium cholate, sodium deoxycholate, sodium taurocholate, sodium glycocholate, N-Hexadecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, anionic (alkyl-aryl-sulphonates) monovalent surfactants, zwitterionic surfactants (*e.g.* N-alkyl-N,N-dimethylammonio-1-propanesulfonates, 3-cholamido-1-propyldimethylammonio-1-propanesulfonate, cationic surfactants (quaternary ammonium bases) (*e.g.* cetyl-trimethylammonium bromide, cetylpyridinium chloride), non-ionic surfactants (*e.g.* dodecyl β-D-glucopyranoside), poloxamines (*e.g.*, Tetronic's), which are tetrafunctional block copolymers derived from sequential addition of propylene oxide and ethylene oxide to ethylenediamine, or the surfactant may be selected from the group of imidazoline derivatives, or mixtures thereof. Each one of these specific surfactants constitutes an alternative embodiment of the invention.

While the use of a surfactant in pharmaceutical compositions is well-known to the skilled person; for convenience, reference is made to Remington: The Science and Practice of Pharmacy, 19th edition, 1995.

In a further embodiment, the formulation also comprises protease inhibitors such as EDTA (ethylenediamine tetraacetic acid) and benzamidineHCl, although other commercially available and suitable protease inhibitors may also be used. The use of a protease inhibitor is particular useful in pharmaceutical compositions comprising zymogens of proteases in order to inhibit autocatalysis.

Other ingredients may also be present in the pharmaceutical formulation of the present invention. Such additional ingredients may include wetting agents, emulsifiers, antioxidants, bulking agents, tonicity modifiers, chelating agents, metal ions, oleaginous vehicles, proteins (*e.g.*, human serum albumin, gelatine or proteins) and a zwitterion (*e.g.*, an amino acid such as betaine, taurine, arginine, glycine, lysine and histidine). Such additional ingredi-

ents preferably do not adversely affect the overall stability of the pharmaceutical formulation of the present invention.

Pharmaceutical compositions containing an antibody according to the present invention may be administered to a patient in need of such treatment at several sites, for example, at topical sites, for example, skin and mucosal sites, at sites which bypass absorption, for example, administration in an artery, in a vein, in the heart, and at sites which involve absorption, for example, administration in the skin, under the skin, in a muscle or in the abdomen.

Administration of pharmaceutical compositions according to the invention may be through several routes of administration, for example, lingual, sublingual, buccal, in the mouth, oral, in the stomach and intestine, nasal, pulmonary, for example, through the bronchioles and alveoli or a combination thereof, epidermal, dermal, transdermal, vaginal, rectal, ocular, for examples through the conjunctiva, uretal, and parenteral to patients in need of such a treatment.

Compositions of the current invention may be administered in several dosage forms, for example, as solutions, suspensions, emulsions, microemulsions, multiple emulsion, foams, salves, pastes, plasters, ointments, tablets, coated tablets, rinses, capsules, for example, hard gelatine capsules and soft gelatine capsules, suppositories, rectal capsules, drops, gels, sprays, powder, aerosols, inhalants, eye drops, ophthalmic ointments, ophthalmic rinses, vaginal pessaries, vaginal rings, vaginal ointments, injection solution, in situ transforming solutions, for example in situ gelling, in situ setting, in situ precipitating, in situ crystallization, infusion solution, and implants.

Compositions of the invention may further be compounded in, or attached to, for example through covalent, hydrophobic and electrostatic interactions, a drug carrier, drug delivery system and advanced drug delivery system in order to further enhance stability of the antibody, increase bioavailability, increase solubility, decrease adverse effects, achieve chronotherapy well known to those skilled in the art, and increase patient compliance or any combination thereof. Examples of carriers, drug delivery systems and advanced drug delivery systems include, but are not limited to, polymers, for example cellulose and derivatives, polysaccharides, for example dextran and derivatives, starch and derivatives, poly(vinyl alcohol), acrylate and methacrylate polymers, polylactic and polyglycolic acid and block copolymers thereof, polyethylene glycols, carrier proteins, for example albumin, gels, for example, thermogelling systems, for example block co-polymeric systems well known to those skilled in the art, micelles, liposomes, microspheres, nanoparticulates, liquid crystals and dispersions thereof, L2 phase and dispersions there of, well known to those skilled in the art

of phase behaviour in lipid-water systems, polymeric micelles, multiple emulsions, self-emulsifying, self-microemulsifying, cyclodextrins and derivatives thereof, and dendrimers.

Compositions of the current invention are useful in the formulation of solids, semisolids, powder and solutions for pulmonary administration of the antibody, using, for example a metered dose inhaler, dry powder inhaler and a nebulizer, all being devices well known to those skilled in the art.

Compositions of the current invention are specifically useful in the formulation of controlled, sustained, protracting, retarded, and slow release drug delivery systems. More specifically, but not limited to, compositions are useful in formulation of parenteral controlled release and sustained release systems (both systems leading to a many-fold reduction in number of administrations), well known to those skilled in the art. Even more preferably, are controlled release and sustained release systems administered subcutaneous. Without limiting the scope of the invention, examples of useful controlled release system and compositions are hydrogels, oleaginous gels, liquid crystals, polymeric micelles, microspheres, nanoparticles,

Methods to produce controlled release systems useful for compositions of the current invention include, but are not limited to, crystallization, condensation, co-crystallization, precipitation, co-precipitation, emulsification, dispersion, high pressure homogenisation, encapsulation, spray drying, microencapsulating, coacervation, phase separation, solvent evaporation to produce microspheres, extrusion and supercritical fluid processes. General reference is made to Handbook of Pharmaceutical Controlled Release (Wise, D.L., ed. Marcel Dekker, New York, 2000) and Drug and the Pharmaceutical Sciences vol. 99: Protein Formulation and Delivery (MacNally, E.J., ed. Marcel Dekker, New York, 2000).

Parenteral administration may be performed by subcutaneous, intramuscular, intraperitoneal or intravenous injection by means of a syringe, optionally a pen-like syringe. Alternatively, parenteral administration can be performed by means of an infusion pump. A further option is a composition which may be a solution or suspension for the administration of the antibody in the form of a nasal or pulmonal spray. As another option, the pharmaceutical compositions containing the antibody of the invention can also be adapted to transdermal administration, *e.g.* by needle-free injection or from a patch, optionally an iontophoretic patch, or transmucosal, *e.g.* buccal, administration.

The antibody can be administered via the pulmonary route in a vehicle, as a solution, suspension or dry powder using any of known types of devices suitable for pulmonary drug delivery. Examples of these comprise of, but are not limited to, the three general types of aerosol-generating for pulmonary drug delivery, and may include jet or ultrasonic nebuliz-

ers, metered-dose inhalers, or dry powder inhalers (Cf. Yu J, Chien YW. Pulmonary drug delivery: Physiologic and mechanistic aspects. Crit Rev Ther Drug Carr Sys 14(4) (1997) 395-453).

Based on standardised testing methodology, the aerodynamic diameter (d_a) of a particle is defined as the geometric equivalent diameter of a reference standard spherical particle of unit density (1 g/cm3). In the simplest case, for spherical particles, d_a is related to a reference diameter (d) as a function of the square root of the density ratio as described by:

$$d_a = \sqrt{\frac{\rho}{\rho_a}} d$$

Modifications to this relationship occur for non-spherical particles (cf. Edwards DA, Ben-Jebria A, Langer R. Recent advances in pulmonary drug delivery using large, porous inhaled particles. J Appl Physiol 84(2) (1998) 379-385). The terms "MMAD" and "MMEAD" are well-described and known to the art (cf. Edwards DA, Ben-Jebria A, Langer R and represents a measure of the median value of an aerodynamic particle size distribution. Recent advances in pulmonary drug delivery using large, porous inhaled particles. J Appl Physiol 84(2) (1998) 379-385). Mass median aerodynamic diameter (MMAD) and mass median effective aerodynamic diameter (MMEAD) are used inter-changeably, are statistical parameters, and empirically describe the size of aerosol particles in relation to their potential to deposit in the lungs, independent of actual shape, size, or density (cf. Edwards DA, Ben-Jebria A, Langer R. Recent advances in pulmonary drug delivery using large, porous inhaled particles. J Appl Physiol 84(2) (1998) 379-385). MMAD is normally calculated from the measurement made with impactors, an instrument that measures the particle inertial behaviour in air.

In a further embodiment, the formulation could be aerosolized by any known aerosolisation technology, such as nebulisation, to achieve a MMAD of aerosol particles less than 10 µm, more preferably between 1-5 µm, and most preferably between 1-3 µm. The preferred particle size is based on the most effective size for delivery of drug to the deep lung, where protein is optimally absorbed (cf. Edwards DA, Ben-Jebria A, Langer A, Recent advances in pulmonary drug delivery using large, porous inhaled particles. J Appl Physiol 84(2) (1998) 379-385).

Deep lung deposition of the pulmonal formulations comprising the antibody may optional be further optimized by using modifications of the inhalation techniques, for example, but not limited to: slow inhalation flow (eg. 30 L/min), breath holding and timing of actuation.

The term "stabilized formulation" refers to a formulation with increased physical stability, increased chemical stability or increased physical and chemical stability.

The term "physical stability" of the protein formulation as used herein refers to the tendency of the protein to form biologically inactive and/or insoluble aggregates of the protein as a result of exposure of the protein to thermo-mechanical stresses and/or interaction with interfaces and surfaces that are destabilizing, such as hydrophobic surfaces and interfaces. Physical stability of the aqueous protein formulations is evaluated by means of visual inspection and/or turbidity measurements after exposing the formulation filled in suitable containers (*e.g.* cartridges or vials) to mechanical/physical stress (*e.g.* agitation) at different temperatures for various time periods. Visual inspection of the formulations is performed in a sharp focused light with a dark background. The turbidity of the formulation is characterized by a visual score ranking the degree of turbidity for instance on a scale from 0 to 3 (a formulation showing no turbidity corresponds to a visual score 0, and a formulation showing visual turbidity in daylight corresponds to visual score 3). A formulation is classified physical unstable with respect to protein aggregation, when it shows visual turbidity in daylight. Alternatively, the turbidity of the formulation can be evaluated by simple turbidity measurements well-known to the skilled person. Physical stability of the aqueous protein formulations can also be evaluated by using a spectroscopic agent or probe of the conformational status of the protein. The probe is preferably a small molecule that preferentially binds to a non-native conformer of the protein. One example of a small molecular spectroscopic probe of protein structure is Thioflavin T. Thioflavin T is a fluorescent dye that has been widely used for the detection of amyloid fibrils. In the presence of fibrils, and perhaps other protein configurations as well, Thioflavin T gives rise to a new excitation maximum at about 450 nm and enhanced emission at about 482 nm when bound to a fibril protein form. Unbound Thioflavin T is essentially non-fluorescent at the wavelengths.

Other small molecules can be used as probes of the changes in protein structure from native to non-native states. For instance the "hydrophobic patch" probes that bind preferentially to exposed hydrophobic patches of a protein. The hydrophobic patches are generally buried within the tertiary structure of a protein in its native state, but become exposed as a protein begins to unfold or denature. Examples of these small molecular, spectroscopic probes are aromatic, hydrophobic dyes, such as antrhacene, acridine, phenanthroline or the like. Other spectroscopic probes are metal-amino acid complexes, such as cobalt metal complexes of hydrophobic amino acids, such as phenylalanine, leucine, isoleucine, methionine, and valine, or the like.

The term "chemical stability" of the protein formulation as used herein refers to chemical covalent changes in the protein structure leading to formation of chemical degradation products with potential less biological potency and/or potential increased immunogenic

properties compared to the native protein structure. Various chemical degradation products can be formed depending on the type and nature of the native protein and the environment to which the protein is exposed. Elimination of chemical degradation can most probably not be completely avoided and increasing amounts of chemical degradation products is often seen during storage and use of the protein formulation as well-known by the person skilled in the art. Most proteins are prone to deamidation, a process in which the side chain amide group in glutaminyl or asparaginyl residues is hydrolysed to form a free carboxylic acid. Other degradations pathways involves formation of high molecular weight transformation products where two or more protein molecules are covalently bound to each other through transamidation and/or disulfide interactions leading to formation of covalently bound dimer, oligomer and polymer degradation products (Stability of Protein Pharmaceuticals, Ahern. T.J. & Manning M.C., Plenum Press, New York 1992). Oxidation (of for instance methionine residues) can be mentioned as another variant of chemical degradation. The chemical stability of the protein formulation can be evaluated by measuring the amount of the chemical degradation products at various time-points after exposure to different environmental conditions (the formation of degradation products can often be accelerated by for instance increasing temperature). The amount of each individual degradation product is often determined by separation of the degradation products depending on molecule size and/or charge using various chromatography techniques (*e.g.* SEC-HPLC and/or RP-HPLC).

Hence, as outlined above, a "stabilized formulation" refers to a formulation with increased physical stability, increased chemical stability or increased physical and chemical stability. In general, a formulation must be stable during use and storage (in compliance with recommended use and storage conditions) until the expiration date is reached.

In one embodiment of the invention the pharmaceutical formulation comprising the antibody is stable for more than 6 weeks of usage and for more than 3 years of storage.

In another embodiment of the invention the pharmaceutical formulation comprising the antibody is stable for more than 4 weeks of usage and for more than 3 years of storage.

In a further embodiment of the invention the pharmaceutical formulation comprising the antibody is stable for more than 4 weeks of usage and for more than two years of storage.

In an even further embodiment of the invention the pharmaceutical formulation comprising the antibody is stable for more than 2 weeks of usage and for more than two years of storage.

Thus, as described above, pharmaceutically acceptable carriers that may be used in these compositions include, but are not limited to, ion exchangers; alumina; aluminium

stearate; lecithin; serum proteins such as human serum albumin; buffer substances such as phosphates and glycine; sorbic acid; potassium sorbate; partial glyceride mixtures of saturated vegetable fatty acids; water; salts or electrolytes such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, and zinc salts; colloidal silica; magnesium trisilicate; polyvinyl pyrrolidone; cellulose-based substances; polyethylene glycol; sodium carboxymethylcellulose; polyacrylates; waxes; polyethylene-polyoxypropylene-block polymers; polyethylene glycol; and wool fat.

The compositions of this invention may be employed in a method of potentiating the activity of NK cells in a patient or a biological sample. This method comprises the step of contacting said composition with said patient or biological sample. Such method will be useful for both diagnostic and therapeutic purposes.

For use in conjunction with a biological sample, the antibody composition can be administered by simply mixing with or applying directly to the sample, depending upon the nature of the sample (fluid or solid). The biological sample may be contacted directly with the antibody in any suitable device (plate, pouch, flask, etc.). For use in conjunction with a patient, the composition must be formulated for administration to the patient.

As described above, the compositions of the present invention may be administered orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally, vaginally or via an implanted reservoir. The term "parenteral" as used herein includes subcutaneous, intravenous, intramuscular, intra-articular, intra-synovial, intrasternal, intrathecal, intrahepatic, intralesional and intracranial injection or infusion techniques. Preferably, the compositions are administered orally, intraperitoneally or intravenously.

Sterile injectable forms of the compositions of this invention may be aqueous or an oleaginous suspension. These suspensions may be formulated according to techniques known in the art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or diglycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically-acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions may also contain a long-chain alcohol diluent or dispersant, such as carboxymethyl cellulose or similar dispersing agents that are commonly

used in the formulation of pharmaceutically acceptable dosage forms including emulsions and suspensions. Other commonly used surfactants, such as Tweens, Spans and other emulsifying agents or bioavailability enhancers which are commonly used in the manufacture of pharmaceutically acceptable solid, liquid, or other dosage forms may also be used for the purposes of formulation.

The compositions of this invention may be orally administered in any orally acceptable dosage form including, but not limited to, capsules, tablets, aqueous suspensions or solutions. In the case of tablets for oral use, carriers commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents include lactose and dried cornstarch. When aqueous suspensions are required for oral use, the active ingredient is combined with emulsifying and suspending agents. If desired, certain sweetening, flavoring or coloring agents may also be added.

Alternatively, the compositions of this invention may be administered in the form of suppositories for rectal administration. These can be prepared by mixing the agent with a suitable non-irritating excipient that is solid at room temperature but liquid at rectal temperature and therefore will melt in the rectum to release the drug. Such materials include cocoa butter, beeswax and polyethylene glycols.

The compositions of this invention may also be administered topically, especially when the target of treatment includes areas or organs readily accessible by topical application, including diseases of the eye, the skin, or the lower intestinal tract. Suitable topical formulations are readily prepared for each of these areas or organs.

Topical application for the lower intestinal tract can be effected in a rectal suppository formulation (see above) or in a suitable enema formulation. Topically-transdermal patches may also be used.

For topical applications, the compositions may be formulated in a suitable ointment containing the active component suspended or dissolved in one or more carriers. Carriers for topical administration of the compounds of this invention include, but are not limited to, mineral oil, liquid petrolatum, white petrolatum, propylene glycol, polyoxyethylene, polyoxypropylene compound, emulsifying wax and water. Alternatively, the compositions can be formulated in a suitable lotion or cream containing the active components suspended or dissolved in one or more pharmaceutically acceptable carriers. Suitable carriers include, but are not limited to, mineral oil, sorbitan monostearate, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water.

For ophthalmic use, the compositions may be formulated as micronized suspensions in isotonic, pH adjusted sterile saline, or, preferably, as solutions in isotonic, pH adjusted sterile saline, either with or without a preservative such as benzylalkonium chloride. Alternatively, for ophthalmic uses, the compositions may be formulated in an ointment such as petrolatum.

The compositions of this invention may also be administered by nasal aerosol or inhalation. Such compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other conventional solubilizing or dispersing agents.

Several monoclonal antibodies have been shown to be efficient in clinical situations, such as Rituxan (Rituximab), Herceptin (Trastuzumab) or Xolair (Omalizumab), and similar administration regimens (*i.e.*, formulations and/or doses and/or administration protocols) may be used with the antibodies of this invention. Schedules and dosages for administration of the antibody in the pharmaceutical compositions of the present invention can be determined in accordance with known methods for these products, for example using the manufacturers' instructions. For example, an antibody present in a pharmaceutical composition of this invention can be supplied at a concentration of 10 mg/mL in either 100 mg (10 mL) or 500 mg (50 mL) single-use vials. The product is formulated for IV administration in 9.0 mg/mL sodium chloride, 7.35 mg/mL sodium citrate dihydrate, 0.7 mg/mL polysorbate 80, and Sterile Water for Injection. The pH is adjusted to 6.5. An exemplary suitable dosage range for an antibody in a pharmaceutical composition of this invention may between about 10 mg/m2 and 500 mg/m2. However, it will be appreciated that these schedules are exemplary and that an optimal schedule and regimen can be adapted taking into account the affinity and tolerability of the particular antibody in the pharmaceutical composition that must be determined in clinical trials. Quantities and schedule of injection of an antibody in a pharmaceutical composition of this invention that saturate NK cells for 24 hours, 48 hours 72 hours or a week or a month will be determined considering the affinity of the antibody and its pharmacokinetic parameters in humans and non-human mammals.

According to another embodiment, the antibody compositions of this invention may further comprise another therapeutic agent, including agents normally utilized for the particular therapeutic purpose for which the antibody is being administered. The additional therapeutic agent will normally be present in the composition in amounts typically used for that agent in a monotherapy for the particular disease or condition being treated. Such therapeutic agents include, but are not limited to, therapeutic agents used in the treatment of cancers,

therapeutic agents used to treat infectious disease, therapeutic agents used in other immunotherapies, cytokines (such as IL-2 or IL-15), other antibodies and fragments of other antibodies.

In an alternate embodiment, an antibody that binds a common determinant present on at least two different human inhibitory KIR receptor gene products, wherein said antibody is capable of neutralizing KIR-mediated inhibition of NK cell cytotoxicity on NK cells expressing at least one of said two different human inhibitory KIR receptors of this invention, may be incorporated into liposomes ("immunoliposomes"), alone or together with another substance for targeted delivery to a tumor, or the site of an infection, in a human or other non-human mammal. Such other substances include nucleic acids for the delivery of genes for gene therapy or for the delivery of antisense RNA, RNAi or siRNA for suppressing a gene in an NK cell, or toxins or drugs for the targeted killing of NK cells.

For example, a number of therapeutic agents are available for the treatment of cancers. The antibody compositions and methods of the present invention may be combined with any other methods generally employed in the treatment of the particular disease, particularly a tumor, cancer disease, or other disease or disorder that the patient exhibits. So long as a particular therapeutic approach is not known to be detrimental to the patient's condition in itself, and does not significantly counteract the activity of the antibody in a pharmaceutical composition of this invention, its combination with the present invention is contemplated.

In connection with solid tumor treatment, the pharmaceutical compositions of the present invention may be used in combination with classical approaches, such as surgery, radiotherapy, chemotherapy, and the like. The invention therefore provides combined therapies in which a pharmaceutical composition of this invention is used simultaneously with, before, or after surgery or radiation treatment; or is administered to patients with, before, or after conventional chemotherapeutic, radiotherapeutic or anti-angiogenic agents, or targeted immunotoxins or coaguligands.

When one or more agents are used in combination with an antibody-containing composition of this invention in a therapeutic regimen, there is no requirement for the combined results to be additive of the effects observed when each treatment is conducted separately. Although at least additive effects are generally desirable, any increased anti-cancer effect above one of the single therapies would be of benefit. Also, there is no particular requirement for the combined treatment to exhibit synergistic effects, although this is possible and advantageous.

To practice combined anti-cancer therapy, one would simply administer to a patient an antibody composition of this invention in combination with another anti-cancer agent in a manner effective to result in their combined anti-cancer actions within the animal. The agents would therefore be provided in amounts effective and for periods of time effective to result in their combined presence within the tumor vasculature and their combined actions in the tumor environment. To achieve this goal, an antibody composition of this invention and anti-cancer agents may be administered to the patient simultaneously, either in a single combined composition, or as two distinct compositions using the same or different administration routes.

Alternatively, the administration of an antibody composition of this invention may precede, or follow, the anti-cancer agent treatment by, *e.g.*, intervals ranging from minutes to weeks and months. One would ensure that the anti-cancer agent and an antibody in the antibody composition of this invention exert an advantageously combined effect on the cancer. As an example, mAbs of the present invention may be administered to patients with Non-Hodgkin's Lymphoma (NHL). Such patients are typically treated with a combination of Rituximab and a combination of chemotherapy agents known as CHOP. Accordingly, anti-KIR antibodies of this invention may be used to treat NHL patients who are undergoing treatment with Rituximab and CHOP, by combining the administration of all the agents in a treatment schedule where the agents are given on the same day, or on different days, with a longer treatment-period.

Other anti-cancer agents may be given prior to, at the same time as, or following, administration of an anti-KIR antibody composition of this invention. However, when immunoconjugates of an antibody are used in the antibody composition of this invention, various anti-cancer agents may be simultaneously or subsequently administered.

In some situations, it may even be desirable to extend the time period for treatment significantly, where several days (2, 3, 4, 5, 6 or 7), several weeks (1, 2, 3, 4, 5, 6, 7 or 8) or even several months (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administration of the anti-cancer agent or anti-cancer treatment and the administration of an antibody composition of this invention. This might be advantageous in circumstances where the anti-cancer treatment was intended to substantially destroy the tumor, such as surgery or chemotherapy, and administration of an antibody composition of this invention was intended to prevent micrometastasis or tumor re-growth.

It also is envisioned that more than one administration of either an anti-KIR antibody-based composition of this invention or the anti-cancer agent will be utilized. These agents may be administered interchangeably, on alternate days or weeks; or a cycle of

treatment with an anti-KIR antibody composition of this invention, followed by a cycle of anti-cancer agent therapy. In any event, to achieve tumor regression using a combined therapy, all that is required is to deliver both agents in a combined amount effective to exert an anti-tumor effect, irrespective of the times for administration.

In terms of surgery, any surgical intervention may be practiced in combination with the present invention. In connection with radiotherapy, any mechanism for inducing DNA damage locally within cancer cells is contemplated, such as gamma-irradiation, X-rays, UV-irradiation, microwaves and even electronic emissions and the like. The directed delivery of radioisotopes to cancer cells is also contemplated, and this may be used in connection with a targeting antibody or other targeting means.

In other aspects, immunomodulatory compounds or regimens may be administered in combination with or as part of the antibody compositions of the present invention. Preferred examples of immunomodulatory compounds include cytokines. Various cytokines may be employed in such combined approaches. Examples of cytokines useful in the combinations contemplated by this invention include IL-1alpha IL-1beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-21, TGF-beta, GM-CSF, M-CSF, G-CSF, TNF-alpha, TNF-beta, LAF, TCGF, BCGF, TRF, BAF, BDG, MP, LIF, OSM, TMF, PDGF, IFN-alpha, IFN-beta, IFN-gamma. Cytokines used in the combination treatment or compositions of this invention are administered according to standard regimens, consistent with clinical indications such as the condition of the patient and relative toxicity of the cytokine. Other immunomodulatory compounds that may be administered in combination with, or as part of, the antibody compositions of the present invention include antibodies that bind specifically to other inhibitory receptors on lymphocytes, including without limitation antibodies such as anti-CTLA4 antibodies, or anti-CD94/NKG2A antibodies (see, for example, U.S. published patent application 20030095965). Variants and derivatives of these molecules that are known in the art also or alternatively can be used in such methods, and incorporated into compositions of the invention, as appropriate.

In certain embodiments, the cross-reacting, blocking, and/or inhibitory anti-KIR antibody-comprising therapeutic compositions of the present invention may be administered in combination with or may further comprise a chemotherapeutic or hormonal therapy agent. A variety of hormonal therapy and chemotherapeutic agents may be used in the combined treatment methods disclosed herein. Chemotherapeutic agents contemplated as exemplary include, but are not limited to, alkylating agents, antimetabolites, cytotoxic antibiotics, vinca alkaloids, for example adriamycin, dactinomycin, mitomycin, carminomycin, daunomycin, doxorubicin, tamoxifen, taxol, taxotere, vincristine, vinblastine, vinorelbine, etoposide (VP-

16), 5-fluorouracil (5FU), cytosine arabinoside, cyclophosphamide, thiotepa, methotrexate, camptothecin, actinomycin-D, mitomycin C, cisplatin (CDDP), aminopterin, combretastatin(s) and derivatives and prodrugs thereof.

Hormonal agents include, but are not limited to, for example LHRH agonists such as leuprorelin, goserelin, triptorelin, and buserelin; anti-estrogens such as tamoxifen and toremifene; anti-androgens such as flutamide, nilutamide, cyproterone and bicalutamide; aromatase inhibitors such as anastrozole, exemestane, letrozole and fadrozole; and progestagens such as medroxy, chlormadinone and megestrol.

As will be understood by those of ordinary skill in the art, the appropriate doses of chemotherapeutic agents will approximate those already employed in clinical therapies wherein the chemotherapeutics are administered alone or in combination with other chemotherapeutics. By way of example only, agents such as cisplatin, and other DNA alkylating may be used. Cisplatin has been widely used to treat cancer, with efficacious doses used in clinical applications of 20 mg/m2 for 5 days every three weeks for a total of three courses. Cisplatin is not absorbed orally and must therefore be delivered via injection intravenously, subcutaneously, intratumorally or intraperitoneally.

Further useful chemotherapeutic agents include compounds that interfere with DNA replication, mitosis and chromosomal segregation, and agents that disrupt the synthesis and fidelity of polynucleotide precursors. A number of exemplary chemotherapeutic agents for combined therapy are listed in Table C of U.S. Patent No. 6,524,583, the disclosure of which agents and indications are specifically incorporated herein by reference. Each of the agents listed are exemplary and not limiting. The skilled artisan is directed to "Remington's Pharmaceutical Sciences" 15th Edition, chapter 33, in particular pages 624-652. Variation in dosage will likely occur depending on the condition being treated. The physician administering treatment will be able to determine the appropriate dose for the individual subject.

The present cross-reacting, blocking, and/or inhibitory anti-KIR antibody compositions of this invention may be used in combination with any one or more anti-angiogenic therapies or may further comprise anti-angiogenic agents. Examples of such agents include neutralizing antibodies, antisense RNA, siRNA, RNAi, RNA aptamers and ribozymes each directed against VEGF or VEGF receptors (U.S. Patent No. 6,524,583, the disclosure of which is incorporated herein by reference). Variants of VEGF with antagonistic properties may also be employed, as described in WO 98/16551, specifically incorporated herein by reference. Further exemplary anti-angiogenic agents that are useful in connection with combined therapy are listed in Table D of U.S. Patent No. 6,524,583, the disclosure of which agents and indications are specifically incorporated herein by reference.

The anti-KIR antibody compositions of this invention may also be advantageously used in combination with methods to induce apoptosis or may comprise apoptotic agents. For example, a number of oncogenes have been identified that inhibit apoptosis, or programmed cell death. Exemplary oncogenes in this category include, but are not limited to, bcr-abl, bcl-2 (distinct from bcl-1, cyclin D1; GenBank accession numbers M14745, X06487; U.S. Pat. Nos. 5,650,491; and 5,539,094; each incorporated herein by reference) and family members including Bcl-x1, Mcl-1, Bak, A1, and A20. Overexpression of bcl-2 was first discovered in T cell lymphomas. The oncogene bcl-2 functions by binding and inactivating Bax, a protein in the apoptotic pathway. Inhibition of bcl-2 function prevents inactivation of Bax, and allows the apoptotic pathway to proceed. Inhibition of this class of oncogenes, *e.g.*, using antisense nucleotide sequences, RNAi, siRNA or small molecule chemical compounds, is contemplated for use in the present invention to give enhancement of apoptosis (U.S. Pat. Nos. 5,650,491; 5,539,094; and 5,583,034; each incorporated herein by reference).

The anti-KIR antibody compositions of this invention may also comprise or be used in combination with molecules that comprise a targeting portion, *e.g.*, antibody, ligand, or conjugate thereof, directed to a specific marker on a target cell ("targeting agent"), for example a target tumor cell. Generally speaking, targeting agents for use in these additional aspects of the invention will preferably recognize accessible tumor antigens that are preferentially, or specifically, expressed in the tumor site. The targeting agents will generally bind to a surface-expressed, surface-accessible or surface-localized component of a tumor cell. The targeting agents will also preferably exhibit properties of high affinity; and will not exert significant *in vivo* side effects against life-sustaining normal tissues, such as one or more tissues selected from heart, kidney, brain, liver, bone marrow, colon, breast, prostate, thyroid, gall bladder, lung, adrenals, muscle, nerve fibers, pancreas, skin, or other life-sustaining organ or tissue in the human body. The term "not exert significant side effects," as used herein, refers to the fact that a targeting agent, when administered *in vivo*, will produce only negligible or clinically manageable side effects, such as those normally encountered during chemotherapy.

In the treatment of tumors, an antibody composition of this invention may additionally comprise or may be used in combination with adjunct compounds. Adjunct compounds may include by way of example anti-emetics such as serotonin antagonists and therapies such as phenothiazines, substituted benzamides, antihistamines, butyrophenones, corticosteroids, benzodiazepines and cannabinoids; bisphosphonates such as zoledronic acid and pamidronic acid; and hematopoietic growth factors such as erythropoietin and G-CSF, for example filgrastim, lenograstim and darbepoietin.

In another embodiment, two or more antibodies of this invention recognizing different epitopes or determinants, including NKVSF1, DF200, 1-4F1 and/or 1-7F9, may be combined in a single composition so as to reduce or neutralize the inhibitory effects of as many KIR gene products as possible. Compositions comprising combinations of cross-reactive inhibitory KIR antibodies of this invention, or fragments or derivatives thereof, will allow even wider utility because there likely exists a small percentage of the human population that may lack each of the inhibitory KIR gene products recognized by a single cross-reacting antibody. Similarly, an antibody composition of this invention may further comprise one or more antibodies that recognize single inhibitory KIR subtypes. Such combinations would again provide wider utility in a therapeutic setting. Accordingly, an antibody of this invention can be combined with another anti-KIR antibody binding to one or more of, *e.g.*, KIR2DL1, KIR2DLK2, KIR2DL3, KIR3DL1, KIR3DL2, and KIR3DL3.

The invention also provides a method of potentiating NK cell activity in a patient in need thereof, comprising the step of administering a composition according to this invention to said patient. The method is more specifically directed at increasing NK cell activity in patients having a disease in which increased NK cell activity is beneficial, which involves, affects or is caused by cells susceptible to lysis by NK cells, or which is caused or characterized by insufficient NK cell activity, such as a cancer, another proliferative disorder, an infectious disease or an immune disorder. More specifically, the methods of the present invention are utilized for the treatment of a variety of cancers and other proliferative diseases including, but not limited to, carcinoma, including that of the bladder, breast, colon, kidney, liver, lung, ovary, prostate, pancreas, stomach, cervix, thyroid and skin, including squamous cell carcinoma; hematopoietic tumors of lymphoid lineage, including leukemia, acute lymphocytic leukemia, acute lymphoblastic leukemia, B-cell lymphoma, T-cell lymphoma, Hodgkins lymphoma, non-Hodgkins lymphoma, hairy cell lymphoma and Burketts lymphoma; hematopoietic tumors of myeloid lineage, including acute and chronic myelogenous leukemias and promyelocytic leukemia; tumors of mesenchymal origin, including fibrosarcoma and rhabdomyoscarcoma; other tumors, including melanoma, seminoma, teratocarcinoma, neuroblastoma and glioma; tumors of the central and peripheral nervous system, including astrocytoma, neuroblastoma, glioma, and schwannomas; tumors of mesenchymal origin, including fibrosarcoma, rhabdomyoscaroma, and osteosarcoma; and other tumors, including melanoma, xeroderma pigmentosum, keratoacanthoma, seminoma, thyroid follicular cancer and teratocarcinoma.

Other preferred disorders that can be treated according to the invention include hematopoietic tumors of lymphoid lineage, for example T-cell and B-cell tumors, including but

not limited to T-cell disorders such as T-prolymphocytic leukemia (T-PLL), including of the small cell and cerebriform cell type; large granular lymphocyte leukemia (LGL) preferably of the T-cell type; Sezary syndrome (SS); Adult T-cell leukemia lymphoma (ATLL); a/d T-NHL hepatosplenic lymphoma; peripheral/post-thymic T cell lymphoma (pleomorphic and immunoblastic subtypes); angio immunoblastic T-cell lymphoma; angiocentric (nasal) T-cell lymphoma; anaplastic (Ki 1+) large cell lymphoma; intestinal T-cell lymphoma; T-lymphoblastic; and lymphoma/leukaemia (T-Lbly/T-ALL).

Other proliferative disorders can also be treated according to the invention, including for example hyperplasias, fibrosis (especially pulmonary, but also other types of fibrosis, such as renal fibrosis), angiogenesis, psoriasis, atherosclerosis and smooth muscle proliferation in the blood vessels, such as stenosis or restenosis following angioplasty. The cross-reacting inhibitory KIR antibody of this invention can be used to treat or prevent infectious diseases, including preferably any infections caused by viruses, bacteria, protozoa, molds or fungi. Such viral infectious organisms include, but are not limited to, hepatitis type A, hepatitis type B, hepatitis type C, influenza, varicella, adenovirus, herpes simplex type I (HSV-1), herpes simplex type 2 (HSV-2), rinderpest, rhinovirus, echovirus, rotavirus, respiratory syncytial virus, papilloma virus, papilloma virus, cytomegalovirus, echinovirus, arbovirus, huntavirus, coxsackie virus, mumps virus, measles virus, rubella virus, polio virus, Ebola-virus, and human immunodeficiency virus type I or type 2 (HIV-1, HIV-2).

Bacterial infections that can be treated according to this invention include, but are not limited to, infections caused by the following: *Staphylococcus; Streptococcus,* including *S. pyogenes; Enterococcl; Bacillus,* including *Bacillus anthracis,* and *Lactobacillus; Listeria; Corynebacterium diphtheriae; Gardnerella* including *G. vaginalis; Nocardia; Streptomyces; Thermoactinomyces vulgaris; Treponerna; Camplyobacter, Pseudomonas* including *Raeruginosa; Legionella; Neisseria including N.gonorrhoeae* and *N.meningitides; Flavobacterium* including *F. meningosepticum and F. odoraturn; Brucella; Bordetella* including *B. pertussis and B. bronchiseptica; Escherichia* including *E. coli, Klebsiella; Enterobacter, Serratia* including *S. marcescens* and *S. liquefaciens; Edwardsiella; Proteus* including *P. mirabilis* and *P. vulgaris; Streptobacillus; Rickettsiaceae* including *R. fickettsfi, Chlamydia* including *C. psittaci* and *C. trachornatis; Mycobacterium* including *M. tuberculosis, M. intracellulare, M. folluiturn, M. laprae, M. avium, M. bovis, M. africanum, M. kansasii, M. intracellulare,* and *M. lepraernurium;* and *Nocardia.*

Protozoa infections that may be treated according to this invention include, but are not limited to, infections caused by *leishmania, kokzidioa,* and *trypanosoma.* A complete list of infectious diseases can be found on the website of the National Center for Infectious Dis-

ease (NCID) at the Center for Disease Control (CDC) (World Wide Web (www) at cdc.gov/ncidod/diseases/), which list is incorporated herein by reference. All of said diseases are candidates for treatment using the cross-reacting inhibitory KIR antibodies of the invention.

Such methods of treating various infectious diseases may employ the antibody composition of this invention, either alone or in combination with other treatments and/or therapeutic agents known for treating such diseases, including anti-viral agents, anti-fungal agents, antibacterial agents, antibiotics, anti-parasitic agents and anti-protozoal agents. When these methods involve additional treatments with additional therapeutic agents, those agents may be administered together with the antibodies of this invention as either a single dosage form or as separate, multiple dosage forms. When administered as a separate dosage form, the additional agent may be administered prior to, simultaneously with, of following administration of the antibody of this invention.

Further aspects and advantages of this invention will be disclosed in the following experimental section, which should be regarded as illustrative and not limiting the scope of this application.

EXAMPLES

Example 1: Purification of PBLs and generation of polyclonal or clonal NK cell lines

PBLs were derived from healthy donors by Ficoll-Hypaque gradients and depletion of plastic adherent cells. To obtain enriched NK cells, PBLs were incubated with anti-CD3, anti-CD4 and anti-HLA-DR mAbs for 30 minutes at 4°C, followed by incubation with goat anti-mouse magnetic beads (Dynal) (30 minutes at 4°C) and immunomagnetic selection by methods known in the art (Pende et al., J Exp Med 1999;190:1505-1516). $CD3^-$, $CD4^-$, DR^- cells were cultivated on irradiated feeder cells and 100 U/ml Interleukin 2 (Proleukin, Chiron Corporation) and 1.5 ng/ml Phytohemagglutinin A (Gibco BRL) to obtain polyclonal NK cell populations. NK cells were cloned by limiting dilution and clones of NK cells were characterized by flow cytometry for expression of cell surface receptors.

The mAbs used were JT3A (IgG2a, anti-CD3), EB6 and GL183 (IgG1, anti-KIR2DL1 and KIR2DL3, respectively), XA-141 (IgM, anti-KIR2DL1 with the same specificity as EB6), anti-CD4 (HP2.6), and anti-DR (D1.12, IgG2a). Instead of JT3A, HP2.6, and DR1.12, other commercially available mAbs of the same specificities can be used. EB6 and GL183 are commercially available (Beckman Coulter Inc., Fullerton, CA). XA-141 is not commercially available, but mAb EB6 can equally well be used for control reconstitution of lysis, as shown

in the examples and Figures herein, as described by Moretta et al., J Exp Med 1993;178:597-604.

Cells were stained with the appropriate antibodies (30mns at 4°C) followed by PE- or FITC-conjugated polyclonal anti-mouse antibodies (Southern Biotechnology Associates Inc). Samples were analyzed by cytofluorometric analysis (flow cytometry) on a FACScan apparatus (Becton Dickinson, Mountain View, CA).

The following NK cell clones were used in this study. CP11, CN5 and CN505 are KIR2DL1 positive clones and are stained by EB6 (IgG1, anti-KIR2DL1) or XA-141 (IgM, anti KIR2DL1 with same specificity as compared to EB6 antibodies). CN12 and CP502 are KIR2DL3-expressing NK clones and are stained by GL183 antibody (IgG1, anti-KIR2DL2/3).

The cytolytic activity of NK clones was assessed by a standard 4-hour ^{51}Cr-release assay in which effector NK cells were tested for their ability to kill target cells expressing HLA-Cw3 or -Cw4, HLA positive cell lines known for their sensitivity to NK cell lysis. All the targets were used at 5000 cells per well in microtitration 96-well plates and the effector:target ratio is indicated in the Figures (usually 4 effectors per target cells). The cytolytic assay was performed with or without hybridoma supernatant of the indicated monoclonal antibodies at a ½ (1:1) dilution. The procedure was essentially the same as described in Moretta et al., J Exp Med 1993;178:597-604.

Example 2: Generation of new mAbs

mAbs were generated by immunizing 5 week old Balb/C mice with activated poly-clonal or monoclonal human NK cell lines as described in (Moretta et al., J Exp Med 1990;172:1589-1598). After different cell fusions, the mAbs were first selected for their ability to cross-react with EB6- and GL183-positive NK cell lines and clones. Positive monoclonal antibodies were further screened for their ability to reconstitute lysis by EB6- positive or GL183-positive NK clones of Cw4 or Cw3 positive targets, respectively.

Cell staining was carried out as follows. Cells were stained with a panel of antibodies (1µg/ml or 50µl supernatant, 30mns at 4°C) followed by PE-conjugated goat F(ab')2 fragments anti-mouse IgG (H+L) or PE-conjugated goat F(ab')2 fragment anti-human IgG (Fc gamma) antibodies (Beckman Coulter). Cytofluorometric analysis was performed on an Epics XL.MCL apparatus (Beckman Coulter).

One of the monoclonal antibodies, the DF200 mAb, was found to react with various members of the KIR family including KIR2DL1, and KIR2DL2/3. Both KIR2DL1-positive and KIR2DL2/3-positive NK cells were stained brightly with DF200mAb (Figure 1).

NK clones expressing one or another (or even both) of these HLA class I-specific inhibitory receptors were used as effectors cells against target cells expressing one or more HLA-C alleles. Cytotoxicity assays were carried out as follows. The cytolytic activity of YTS-KIR2DL1 or YTS-Eco (KIR-negative) cell lines was assessed by a standard 4 hours Cr-51 release assay. The target cells were HLA-Cw4-positive or -negative B-EBV cell lines, or HLA-Cw4-transfected 721.221 cells. All targets were used at 3000 cells per well in microtitration plate. The effector/target ratio is indicated in the figures. The cytolytic assay was performed with or without the indicated full length or F(ab')2 fragments of monoclonal mouse or human antibodies. As expected, KIR2DL1-positive (KIR2DL1$^+$) NK clones displayed little if any cytolytic activity against target cells expressing HLA-Cw4 and KIR2DL3+ NK clones displayed little or no activity on Cw3 positive targets. However, in the presence of DF200mAb (used to mask their KIR2DL receptors) NK clones became unable to recognize their HLA-C ligands and displayed strong cytolytic activity against Cw3- or Cw4-expressing targets (examples of results are shown in Figures 2-6) .

For example, the C1R cell line (CW4+ EBV cell line, ATCC No. CRL-1993), which expresses HLA-Cw4 (but no group 1 HLA-C allotypes) was not killed by KIR2DL1-positive NK clones (CN5/CN505), but the inhibition could be efficiently reversed by the use of either DF200 or a conventional anti KIR2DL1 mAb. On the other hand, NK clones expressing the KIR2DL2/3$^+$ KIR2DL1$^-$ phenotype (CN12) efficiently killed C1R cells and this killing was unaffected by the DF200mAb (Figure 2). Similar results are obtained with KIR2DL2- or KIR2DL3-positive NK clones on Cw3-positive targets.

Similarly, the Cw4+ 221 EBV cell line was not killed by KIR2DL1+transfected NK cells, but the inhibition could be efficiently reversed by the use of the DF200 mAb, a Fab fragment of DF200, or the a conventional anti-KIR2DL1 mAbs EB6 or XA141. Also, a Cw3-positive 721.221-transfectant was not killed by KIR2DL2$^+$ NK cells, but this inhibition could be reversed by the use of either DF200 or a DF200 Fab fragment. Finally, the Cw3+ 721.221-transfectant was not killed by KIR2DL3$^+$ NK cells, but this inhibition could be reversed by the use of either a DF200 Fab fragment or the conventional anti-KIR2DL3 mAb GL183 or Y249. The results are shown in Figure 3.

F(ab')2 fragments were also tested for their ability to reconstite lysis of Cw4 positive targets. F(ab')2 fragments of the DF200 and EB6 Abs were both able to reverse inhibition of lysis by KIR2DL1-transfected NK cells of the Cw4 transfected 221 cell line and the Cw4+ TUBO EBV cell line. Results are shown in Figure 4.

Example 3: Biacore analysis of DF200 mAb/KIR2DL1 and DF200 mAb/KIR2DL3 interactions

Production and purification of recombinant proteins. The KIR2DL1 and KIR2DL3 recombinant proteins were produced in *E. coli*. cDNA encoding the entire extracellular domain of KIR2DL1 (SEQ ID NO:23) and KIR2DL3 (SEQ ID NO:25) were amplified by PCR from pCDM8 clone 47.11 vector (Biassoni et al, Eur J Immunol. 1993;23:1083-7) and RSV.5(gpt)183 clone 6 vector (Wagtmann et al, Immunity 1995;2:439-49 and 1995;3:801-809) respectively, using the following primers:

Sense: 5'-GGAATTCCAGGAGGAATTTAAAATGCATGAGGGAGTCCACAG-3' (SEQ ID NO:13)

Anti-sense: 5'- CGGGATCCCAGGTGTCTGGGGTTACC -3' (SEQ ID NO:14)

They were cloned into the pML1 expression vector in frame with a sequence encoding a biotinylation signal (Saulquin et al, J Exp Med. 2003;197:933-8).

Protein expression was performed in the BL21(DE3) bacterial strain (Invitrogen). Transfected bacteria were grown to OD600=0.6 at 37°C in medium supplemented with ampicillin (100 µg/ml) and expression was induced with 1 mM IPTG.

Proteins were recovered from inclusion bodies under denaturing conditions (8 M urea). Refolding of the recombinant proteins was performed in 20 mM Tris, pH 7.8, NaCl 150 mM buffer containing L-arginine (400 mM, Sigma) and β-mercaptoethanol (1 mM), at room temperature, by decreasing the urea concentration in a six step dialysis (4, 3, 2, 1 0.5 and 0 M urea, respectively). Reduced and oxidized glutathione (5 mM and 0.5 mM respectively, Sigma) were added during the 0.5 and 0 M urea dialysis steps. Finally, the proteins were dialyzed extensively against 10 mM Tris, pH 7.5, NaCl 150 mM buffer. Soluble, refolded proteins were concentrated and then purified on a Superdex 200 size-exclusion column (Pharmacia; ÄKTA system).

Surface plasmon resonance measurements were performed on a Biacore apparatus (Biacore). In all Biacore experiments HBS buffer supplemented with 0.05% surfactant P20 served as running buffer.

Protein immobilisation. Recombinant KIR2DL1 and KIR2DL3 proteins produced as described above were immobilized covalently to carboxyl groups in the dextran layer on a Sensor Chip CM5 (Biacore). The sensor chip surface was activated with EDC/NHS (N-ethyl-N'-(3-dimethylaminopropyl)carbodiimidehydrochloride and N-hydroxysuccinimide, Biacore). Proteins, in coupling buffer (10 mM acetate, pH 4.5) were injected. Deactivation of the remaining activated groups was performed using 100 mM ethanolamine pH 8 (Biacore).

Affinity measurements. For kinetic measurements, various concentrations of the soluble antibody (1 x 10-7 to 4 x 10-10 M) were applied onto the immobilized sample. Measurements were performed at a 20 µl/min continuous flow rate. For each cycle, the surface of the sensor chip was regenerated by 5 µl injection of 10 mM NaOH pH 11. The BIAlogue Kinetics Evaluation program (BIAevaluation 3.1, Biacore) was used for data analysis. The soluble analyte (40 µl at various concentrations) was injected at a flow rate of 20 µl/min in HBS buffer, on dextran layers containing 500 or 540 reflectance units (RU), and 1000 or 700 RU of KIR2DL1 and KIR2DL3, respectively. Data are representative of 6 independent experiments. The results are shown in Table 1, below.

Table 1

BIAcore analysis of DF200 mAb binding to immobilized KIR2DL1 and KIR2DL3. K_D: Dissociation constant.

Protein	K_D (10^{-9} M)
KIR2DL1	10.9 +/- 3.8
KIR2DL3	2.0 +/- 1.9

Example 4: Generation of new human anti-KIR mAbs

Human monoclonal anti-KIR mAbs were generated by immunizing transgenic mice, engineered to express a human antibody repertoire, with recombinant, soluble KIR proteins. After different cell fusions to establish hybridomas, mAbs were first selected for their ability to cross-react with immobilized KIR2DL1 and KIR2DL3 protein (produced as described in Example 3, above). Several human monoclonal antibodies, including 1-7F9, 1-4F1, 1-6F5 and 1-6F1, were found to be cross-reactive with KIR2DL1 and KIR2DL2/3.

Positive monoclonal antibodies were further screened for their ability to reconstitute lysis by KIR2DL1-positive NK transfectants against Cw4-positive target cells. The NK cells expressing the HLA class I-specific inhibitory receptors were used as effectors cells against target cells expressing one or more HLA-C alleles (Figures 5 and 6). Cytotoxicity assays were carried out as described above. The effector/target ratio is indicated in the figure legends, and antibodies were used at either 10 µg/ml or 30 µg/ml.

As expected, KIR2DL1+ NK cells displayed little if any cytolytic activity against target cells expressing HLA-Cw4. However, in the presence of 1-7F9 mAb, NK cells became unable to recognize their HLA-C (*i.e.*, were no longer inhibited by HLA-C molecules), and now displayed strong cytolytic activity against the Cw4-positive targets. For example, the two cell lines tested (the HLA-Cw4 transfected 721.221 and the CW4+ EBV cell lines) were

not killed by KIR2DL1+ NK cells, but the inhibition could be efficiently reversed by the use of either mAb 1-7F9 or the conventional anti-KIR2DL1 mAb EB6. The murine mAbs DF200 and Pan2D (NKVSF1) were compared to the human mAb 1-7F9 and in all experiments 1-7F9 was more potent than any of the other mAbs tested in terms of inducing cytotoxicity by the NK cells. Antibodies 1-4F1, 1-6F5 and 1-6F1 on the other hand were not able to reconstitute cell lysis by NK cells against Cw4-positive targets.

Example 5: Biacore competitive binding analysis of murine and human anti-KIR antibodies

Epitope mapping analysis was performed according to a previously described method (Gauthier et al., Journal of Immunology 1999;162:41-50; Saunal and van Regenmortel, Journal of Immunology 1995;183:33-41) on immobilized KIR2DL1 (900 RU), KIR 2DL3 (2000 RU) and KIR2DS1 (1000 RU) with mouse anti-KIR 2D antibodies DF200, NKVSF1 (Pan2D), gl183 and EB6, and human anti-KIR2D antibodies 1-4F1, 1-6F1, 1-6F5 and 1-7F9.

All experiments were done at a flow rate of 5 µl/min in HBS buffer with 2 min injection of the different antibodies at 15 µg/ml. For each pair of antibodies, competitive binding analysis was performed in two steps. In the first step the first monoclonal antibody (mAb) was injected on KIR2D target protein followed by the second mAb (without removing the first mAb) and second mAb RU value (RU2) was monitored. In the second step the second mAb was injected first, directly on nude KIR2D protein, and mAb RU value (RU1) was monitored. Percent inhibition of second mAb binding to KIR2D protein by first mab was calculated by: 100*(1-RU2/RU1).

Results are shown in Tables 2, 3 and 4, where the antibodies designated "first antibody" are listed on vertical column and the 'second antibody' are listed on the horizontal column. For each antibody combination tested, the values for direct binding level (RU) of the antibodies to the chip are listed in the table, where direct binding of the second antibody to the KIR2D chip is listed in the upper portion of the field and the value for binding of the second antibody to the KIR2D chip when the first antibody is present is listed in the lower portion of the field. Listed in the right of each field is the percentage inhibition of second antibody binding. Table 2 shows binding on a KIR2DL1 chip, Table 3 shows binding of antibodies to a KIR2DL3 chip, and Table 4 shows binding of antibodies to a KIR2DS1 chip.

Competitive binding of murine antibodies DF200, NKVSF1 and EB6, and human antibodies 1-4F1, 1-7F9 and 1-6F1 to immobilized KIR2DL1, KIR2DL2/3 and KIR2DS1 was assessed. Epitope mapping (Figure 7) from experiments with anti-KIR antibodies' binding to KIR2DL1 showed that (a) antibody 1-7F9 is competitive with EB6 and 1-4F1, but not with

NKVSF1 and DF200; (b) antibody 1-4F1 in turn is competitive with EB6, DF200, NKVSF1 and 1-7 F9; (c) NKVSF1 competes with DF200, 1-4F1 and EB6, but not 1-7F9; and (d) DF200 competes with NKVSF1, 1-4F1 and EB6, but not 1-7F9. Epitope mapping (Figure 8) from experiments with anti-KIR antibodies' binding to KIR2DL3 showed that (a) 1-4F1 is competitive with NKVSF1, DF200, gl183 and 1-7F9; (b) 1-7F9 is competitive with DF200, gl183 and 1-4F1, but not with NKVSF1; (c) NKVSF1 competes with DF200, 1-4F1 and GL183, but not 1-7F9; and (d) DF200 competes with NKVSF1, 1-4F1 and 1-7F9, but not with GL183. Epitope mapping (Figure 9) from experiments with anti-KIR antibodies' binding to KIR2DS1 showed that (a) 1-4F1 is competitive with NKVSF1, DF200 and 1-7F9; (b) 1-7F9 is competitive with 1-4F1 but not competitive with DF200 and NKVSF1; (c) NKVSF1 competes with DF200 and 1-4F1, but not 1-7F9; and (d) DF200 competes with NKVSF1 and 1-4F1, but not with 1-7F9.

Table 2

KIR2DL1 epitope mapping using competitive binding between first and second antibodies (ND = not determined).

First Ab (below)	Second Ab						
	DF200	Pan2D	EB6	1-4F1	1-7F9	1-6F1	1-6F5
DF200		80%	90%	490 92% 40	480 27% 350	540 15% 460	400 15% 340
Pan2D	90%		90%	900 95% 50	860 2% 840	750 12% 660	600 13% 520
EB6	60%	40%	460 57% 200	370 48% 190	490 65% 170	260 23% 200	Nd
1-4F1							
1-7F9	600 10% 545	545 2% 534	460 60% 180	360 95% 16		330 9% 300	Nd
1-6F1	350 11% 310	475 7% 440	260 18% 320	360 23% 275	490 10% 440		Nd
1-6F5	350 17% 290	475 7% 440	Nd	360 17% 300	Nd	290 40% 170	

Table 3

KIR2DL3 epitope mapping using competitive binding between first and second antibodies (ND = not determined)

First Ab (below)	Second Ab						
	DF200	Pan2D	GI183	1-4F1	1-7F9	1-6F1	1-6F5
DF200		75%	20%	1270 75% 320	520 62% 200	550 16% 460	440 4% 420
Pan2D	95%		85%	2250 68% 730	880 15% 750	840 8% 770	560 18% 460
gl183	8%	40%		1300 75% 330	670 76% 160	530 18% 430	Nd
1-4F1	1140 82% 210	2400 63% 890	1240 73% 330		1050 87% 140		
1-7F9	770 42% 450	870 5% 830	800 75% 200	1000 63% 270			
1-6F1	790 4% 760	990 0% 1090	620 8% 570				
1-6F5	800 5% 760	990 4% 950	Nd				

Table 4

KIR2DS1 epitope mapping using competitive binding between first and second antibodies (ND = not determined)

First Ab (below)	Second Ab			
	DF200	Pan2D	1-4F1	1-7F9
DF200		70%	660 87% 80	975 15% 825
Pan2D	100%		650 100% -8	920 45% 500
1-7F9	900 17% 1090	1350 11% 1200	660 96% 23	

Example 6: Anti-KIR mAb titration with cynomolgus NK cells

Anti-KIR antibody NKVSF1 was tested for its ability to bind to NK cells from cynomolgus monkeys.

Purification of monkey PBMC and generation of polyclonal NK cell bulk. Cynomolgus Macaque PBMC were prepared from Sodium citrate CPT tube (Becton Dickinson). NK

cells purification was performed by negative depletion (Macaque NK cell enrichment kit, Stem Cell Technology). NK cells were cultivated on irradiated human feeder cells with 300 U/ml Interleukin 2 (Proleukin, Chiron Corporation) and 1ng/ml Phytohemagglutinin A (Invitrogen, Gibco) to obtain polyclonal NK cell populations.

Pan2D mAb titration with cynomolgus NK cells. Cynomolgus NK cells (NK bulk day 16) were incubated with different amount of Pan2D mAb followed by PE-conjugated goat F(ab')2 fragments anti-mouse IgG (H+L) antibodies. The percentage of positive cells was determined with an isotypic control (purified mouse IgG1). Samples were done in duplicate. Mean fluorescence intensity = MFI.

Binding of the antibody to monkey NK cells is shown in Figure 10.

Example 7: Screening of human anti-KIR mAbs

Human monoclonal anti-KIR Abs were generated by immunizing transgenic mice engineered to express a human antibody repertoire with recombinant KIR protein, as described in Example 4. To generate cross-reactive anti-KIR antibodies, animals were sequentially immunized with different KIRs, either in soluble form as described in Example 3, or expressed at the surface of cells.

Next, after different cell fusions, the mAbs were first selected for their ability to cross-react with immobilized KIR2DL1 and KIR2DL3 protein, by ELISA, using standard methods. Several human monoclonal antibodies, including 1-7F9, 1-4F1, 1-6F5 and 1-6F1, were found to react with all of KIR2DL1, KIR2DL2 and KIR2DL3, by ELISA. The mAbs that reacted with both KIR2DL1 and KIR2DL2/3 were designated "KIR2DL-crossreactive mAbs."

The KIR2DL cross-reactive mAbs were then tested for their ability to react with KIR at the surface of cells by flow cytometry. Briefly, the binding of the human anti-KIR mAbs was tested by incubating them with various cell lines stably over-expressing KIRs (*e.g.*, YTS-2DL1, BWZ-KIR2DL2 and BWZ-KIR2DS4) or not expressing KIRs (*e.g.*, YTS and BWZ). Briefly, cells were incubated with various concentrations (typically 0-50 µg/ml) of human anti-KIR mAb1-7F9 in DMEM-medium containing 2% FCS. After incubation, cells were washed and incubated with APC-conjugated secondary antibodies specific for human IgG. All incubations and washing steps were performed at 0-4ºC. Subsequently, cells were washed, resuspended in PBS, and analyzed by flow cytometry on a FACScalibur or a FACScanto (both from Beckton Dickinson). A typical example is shown in Figure 16. For example, 1-7F9 and 1-4F1 were found to not bind to cells transfected with KIR2DS4, whereas NKVSF1 (Pan2D) did (Figure 16).

Next, the ability of the human anti-KIR mAbs to block the interaction between KIR and its HLA-C ligand was tested by 1) biochemical, direct binding experiments and 2) functional reconsitution of lysis assays.

In the biochemical binding assay, the capacity of human anti-KIR mAbs, including 1-7F9, to block the interaction between HLA-C and KIR-molecules was assessed by competing the binding of soluble, recombinant KIR-Fc fusion proteins to cells expressing HLA-C. The KIR-Fc proteins were produced as described (Wagtmann et al., Immunity 1995;3(6):801-9), except that the human Fc was replaced with murine IgG1 Fc. Soluble KIR-Fc binds to cells expressing the specific HLA-C allotypes that are recognized by KIR2DL1, and this binding can be visualized by flow-cytometry using a secondary fluorochrome-conjugated Ab specific for the murine Fc part of the KIR-Fc protein. For example, KIR2DL1-Fc binds to cells transfected with HLA-Cw*0402 (LCL 721.221-Cw4 transfectants) (Litwin et al., J Exp Med. 1993;178:1321-36) but not to untransfected LCL 721.221 cells (see Figure 11A). To test whether anti-KIR mAbs could prevent this interaction between KIR2DL1-Fc and HLA-Cw4, the KIR2DL1-FC proteins were pre-incubated with increasing concentrations of anti-KIR mAbs, and then added to LCL 721.221-Cw4 cells, incubated at 4 ºC, washed, incubated with an APC-conjugated anti-murine IgG1 Fc, washed, and analyzed by flow cytometry on a FACScalibur, or a FACScanto (Beckton Dickinson), by standard methods. Some murine anti-KIR mAbs, such as DF200, and some human anti-KIR mAbs, namely 1-7F9 and 1-4F1, prevented the binding of KIR2DL1-Fc to the cells expressing HLA-Cw4, showing that these mAbs block the interaction between KIR2DL1 and HLA-Cw4. As an example, Figure 17A shows that DF200 prevents binding of KIR2DL1-Fc to cells expressing HLA-Cw4. Figure 17B shows that the 1-7F9 mAbs prevented KIR2DL1 from binding to HLA-Cw4. In parallel, the anti-KIR mAbs were tested for their ability to prevent the binding of KIR2DL2 to HLA-Cw3. Antibodies that prevented the binding of KIR2DL-Fc to HLA-C-expressing cells in a dose-dependent fashion, as exemplified in Figure 17, were designated "blocking mAbs" and were further tested in functional cytotoxicity assays, as follows.

The therapeutic utility of the human anti-KIR mAbs is linked to their ability to induce KIR-expressing NK cells to kill tumor cells, by preventing inhibitory signaling via KIR. Therefore, it was important to assess whether human KIR2DL cross-reactive and blocking mAbs were able to induce lysis of target cells expressing HLA-C by NK cells expressing KIR2DL. For this purpose, the following experiments were performed:

In ^{51}Cr-release cytotoxicity assays, KIR2DL1-expressing YTS cells (YTS-2DL1) can kill LCL 721.221-Cw3, but not LCL 721.221-Cw4 cells (Figure 18A). In contrast, YTS effector-cells that lack KIRs (YTS) kill both cell-lines efficiently. Thus, YTS-2DL1 effector cells

cannot kill LCL 721.221-Cw4 cells due to HLA-Cw4-induced inhibitory signaling via KIR2DL1. When YTS-2DL1 cells were pre-incubated with 1-7F9 in ^{51}Cr-release cytotoxicity assays, LCL 721.221-Cw4 cells were killed in a 1-7F9 concentration-dependent fashion (Figure 18B). 3-1F13, a human cross-reactive anti-KIR mAb that binds KIR2DL1 with high affinity, but that can not prevent the interaction between KIR2DL1 and HLA-Cw4, can not induce killing of LCL 721.221-Cw4 cells by YTS-2DL1 cells. Thus, 1-7F9 induces NK-mediated killing of target cells by preventing KIR-HLA-C interactions between NK- and target-cells.

Example 8: Affinity measurements of 1-7F9

The affinity of 1-7F9 for binding to KIR2DL1 and KIR2DL3 was measured by Biacore analysis, using soluble recombinant KIR proteins produced as described in Example 3. The Biacore experiments were performed as described in Example 3, except that the antibody used was the human mAb 1-7F9.

The results of these affinity determinations of binding by 1-7F9 to KIR2DL1 and -3 are shown in Table 5.

Table 5

BIAcore analysis of 1-7F9 mAb binding to immobilized KIR2DL1 and KIR2DL3.
K_D: Dissociation constant.

Protein	K_D (10^{-9} M)
KIR2DL1	0,43
KIR2DL3	0,025

Example 9: Epitope mapping of 1-7F9 onto KIR2DL1

Epitope mapping of a given monoclonal antibody onto a given antigen can be obtained by *in silico* methods when suitable X-ray structures/homology models exist of both the antibody and the antigen by performing protein-protein docking.

A homology-model of an antibody can be obtained by aligning the sequence of the Light and Heavy Chain, respectively, with a selection of antibodies for which a 3D structure exists. The length of the 6 Complementarity Determining Regions (CDRs) can be calculated and the best template selected from antibodies with the same CDR lengths. Using standard techniques a homology model can be build from the selected template.

In the protein-protein docking approach, recently reviewed (Schneidman-Duhovny et al., Curr Med Chem 2004;11:91-107), the two surfaces are represented by features on surfaces. Features include hydrogen bonding capabilities, charges and hydrophobicity. In a grid based method, space is divided into cubes and each cube is given a value according to its

position relative to the surface (interior, surface, exterior) and assigned the relevant feature set. Brute force matching of the surfaces by a scoring function can now be employed by searching the entire 3 translational and 3 rotational degrees of freedom. Translations are handled by Fast Fourier Transform and rotation is treated as individual calculations within a standard discretization of rotational space. From the top scoring complexes the results can be filtered and scored, by a range of methods, *e.g.*, shape complementarity, Van der Waals interactions, hydrophobicity, electrostatics, desolvation, hydrogen bonding, atomic contact energy, residue-residue pairing statistics and hydrophilic group pairing. The top-scoring complexes can be evaluated in detail and interacting residues and thereby the epitope can be identified.

Methods and Analysis

Residue and domain nomenclature for the KIRs were according to Fan et al. (Nature 1997;389:96-100), so that Domain1 comprised residues 6-101 and Domain 2 comprised amino acids residues 105-200).

A homology-model of 1-7F9 was constructed using 1OM3 as a template. The 1OM3 structure, generated by Calarese et al (Science 2003;300:2065, *et seq.*), was retrieved from the PDB (Protein Data Bank, at World Wide Web (WWW) address rcsb.org/pdb/; 1OM3 VL sequence: SEQ ID NO:34; 1OM3 VH sequence: SEQ ID NO:35). Kabat Numbering and CDR assignment were used. The overall alignment was good and the CDR lengths were identical, so the homology model would be accurate enough to be used in a protein-protein docking experiment.

From the protein-protein docking of the 1-7F9 antibody homology model onto the KIR2DL1 structure, 1NKR.pdb 2000 solutions were obtained. They were filtered for their closeness to D183 (atoms < 6 Å from CD) and R131 (atoms < 12 Å from CZ) and 133 resulting solutions were analyzed in detail.

The highest scoring complex formed was analyzed and a possible interaction between R131 (KIR) and D100C (Heavy chain) was identified and restrained. A new rotamer for F181 was selected and the energy was minimized. This model predicted that the antibody interacted with the following residues on KIR2DL1 (SEQ ID NO:23): S20 (Hyd, CB) (HydAcceptor, OG), E21 (Hyd, CB, CG), M44 (Hyd, SD, CE), F45 (Hyd, CD1, CD1, CZ, CE2), R68 (HydDonor, NH2), T70 (Hyd, CB, CG2), Q71 (Hyd, CG), L104 (Hyd, CB, CG, CD1, CD2), Y105 (Hyd, CG, CD2, CE2), R131 (Ion, NH2) (HydDonor, NH2), S133 (Hyd, CA, CB) (HydDonor/Acceptor, OG), Y134 (Hyd, CD1, CE1), F181 (Hyd, CB, CG, CD1, CD2, CE1, CE2, CZ), and D183 (Hyd, CB).

Example 10: Biacore competitive binding analysis of murine and human anti-KIR antibodies

Competitive binding of 1-7F9, Pan2D (NKVSF1), and DF200 was evaluated according to the same method described in Example 5.

Results are shown in Tables 6 and 7, where the antibodies designated "first antibody" are listed on vertical column and the 'second antibody' are listed on the horizontal column. For each antibody combination tested, the percentage inhibition of second antibody binding is listed. Where the percentages of inhibition for a certain antibody pair were above 40%, regardless of which antibody was used as first antibody, the antibodies were considered competitive. Table 6 shows binding on a KIR2DL1 chip, and Table 7 shows binding of antibodies to a KIR2DL3 chip.

Briefly, for KIR2DL1 binding, the results show that DF200 and Pan2D are competitive, whereas 1-7F9 is not competitive with either one of DF200 or Pan2D. For KIR2DL3 binding, DF200 and Pan2D were competitive, whereas 1-7F9 was not competitive with Pan2D, but was competitive with DF200.

Table 6

KIR2DL1 competitive binding between first and second antibodies.

First antibody (below)	Second antibody		
	1-7F9	Pan2D	DF200
1-7F9	98 %	4 %	15 %
Pan2D	17 %	92 %	90 %
DF200	17 %	85 %	80 %

Table 7

KIR2DL1 competitive binding between first and second antibodies.

First antibody (below)	Second antibody		
First mAb	1-7F9	Pan2D	DF200
1-7F9	95 %	4 %	60 %
Pan2D	0 %	95 %	95 %
DF200	80 %	50 %	85 %

Example 11: Crystal structure of KIR2DL1 in complex with HuKIR1-7F9-Fab'

The crystal structure of KIR2DL1 in complex with the Fab' fragment of 1-7F9 was solved and refined to 2.35 Å resolution with X-ray crystallography. The results confirmed that the antibody, when bound to KIR2DL1, will be able to block binding to an MHC Class I molecule (Figure 19).

Materials and Methods

Extracellular KIR2DL1 (amino acid 1-223 of SEQ ID NO:23, with residue 16 being arginine (R) and residue 114 being leucine (L), and including an additional N-terminal methionine (M) residue) and HuKIR1-7F9 Fab' (with the light chain sequence of SEQ ID NO:36 and heavy chain sequence of residues 1-221 of SEQ ID NO:37) were mixed, with a slight excess of KIR2DL1, and the complex was purified on a gel-filtration column. The complex was then concentrated to about 13.5 mg/ml. Crystals were grown with the hanging drop-technique in 10 % PEG6000 and 500 mM citrate buffer with a pH of 4.2. Crystals were flash frozen in liquid N_2 and crystallographic data to 2.35 Å resolution were collected at 100 K using the beam-line BL711I, MAX-lab, Lund, Sweden. Data were integrated by the XDS program (Kabsch, J. Appl. Crystallogr. 1993;26:795-800). For structure determination molecular replacement, using the MOLREP program of the CCP4 suite (Bailey, Acta Crystallogr. Sect. D-Biol. Crystallogr. 1994;50:760-763) and the PDB-deposited structures 1RZJ (the Fab part1) and 1NKR (KIR), were used. Phase improvements were made with the ARP/WARP program (Lamzin and Wilson, Acta Crystallogr. Sect. D-Biol. Crystallogr. 1993;49:129-147) and manual modifications to the X-ray derived structure model were made with the QUANTA program (available from Accelrys Inc., San Diego, CA, USA). Refinement was carried out in the REFMAC5 computer program of the CCP4 suite. Water molecules were added by the ARP/WARP program. The model comprised residues 6-114 and 124-200 of KIR2DL1, 1-212 of the 1-7F9 light chain and 1-136 together with 143-224 of the 1-7F9 heavy chain. In addition, 330 water molecules were placed. R- and R-free for the model were 0.191 and 0.253, respectively.

Results

The contacts were identified by the CONTACT computer program of the CCP4 suite using a cut-off distance of 4.0 Å between the Fab' and KIR2DL1 molecules. The resulting KIR2DL1 epitope for human 1-7F9 was found to comprise the following residues of KIR2DL1 (SEQ ID NO:23): L38, R41, M44, F45, N46, D47, T48, L49, R50, I52, F64, D72, Y80, P87, and Y88 (Tables 8 and 9). Residues of 1-7F9 involved in interactions with KIR2DL1 included S28, V29, S30, Y32, S92, N93, and W94 of the 1-7F9 variable light (VL) chain (SEQ ID NO:15, Table 8), and T28, F29, S30, F31, I54, F55, E74, S77, G102, S103, Y105, Y106,

D107, and Y108 of the variable heavy (VH) chain (SEQ ID NO:17). The KIR2DL1 epitope, and the residues involved in hydrogen-binding, are also indicated in the amino-acid sequence of KIR2DL1 in Figure 20. The isotropic displacement factors (also called "temperature factors" or "B-values") obtained from the coordinate refinement showed relatively lower values for the N-terminal domain of KIR2DL1 and the variable domains of the 1-7F9 antibody Fab' fragment, all domains directly involved in the intermolecular binding. This showed that the binding forces between the two molecules in the complex of the crystal were strong and stable, also supporting that the crystal structure depicted a stable KIR2DL1/1-7F9 Fab' complex.

The 1-7F9 Fab' molecule bound to the KIR2DL1 molecule on one side of the C'CFGA' β-sheet of domain D1 but, in addition, touched also one E β-strand residue side-chain of the same domain. Connections to loop residues of the D1 domain are also important for binding (for topology naming conventions, see Fan et al., Nature 1997;389:96-100). More specifically, connections are made to the following topological parts of KIR2DL1; β-strand C (amino acids L38 and R41), the loop between β-strands C and C', L2 (M44, F45 and N46), β-strand C' (D47, T48, L49, R50 and I52), β-strand E (F64), the loop between the E and F β-strands, L3 (D72), β-stand F (Y80) and the loop between the F and G β-strands (P87 and Y88).

While the HLA-Cw4 molecule binds both to the D1 and D2 domain of the KIR2DL1 molecule (Fan et al. Nat. Immunol. 2001; 2: 452-460) the 1-7F9 antibody binds to the KIR2DL1 D1 domain only. There is, however, a spatial overlap between a bound 1-7F9 and a bound HLA-Cw4 molecule, large enough for the 1-7F9 antibody to successfully displace HLA-Cw4. Using the published structure of KIR2DL1 in complex with HLA-Cw4 (PDB accession code 1IM9) contact residues between KIR2DL1 and HLA-Cw4 can be calculated with the CONTACT program. In a calculation using a distance cut-off of 4.0 Å it can be seen that three KIR2DL1 residues involved in contacts with HLA-Cw4 residues are common to contact residues in the 1-7F9/KIR2DL1 complex (Table 8 and 9). Those three KIR2DL1 residues are M44, F45 and D72.

Without being limited to any specific theory, the crystallographic results also indicated that 1-7F9 does not bind the activating NK receptor KIR2DS4 because of amino acids N47 and V72 in the extracellular portion of the KIR2DS4 sequence (SEQ ID NO:38).

Table 8

KIR2DL1 – 1-7F9 Fab' VL chain interactions. A cut-off of 4.0 Å was used. The contacts were identified by the CONTACT computer program of the CCP4 suite. In the last col-

umn "***" indicates a strong possibility for a hydrogen bond at this contact (distance < 3.3 Å) as calculated by CONTACT, " *" indicates a weak possibility (distance > 3.3 Å). Blank indicates that the program considered there to be no possibility of a hydrogen bond.

KIR2DL1 Atoms		1-7F9 Fab' VL Atoms		Distance (Å)
Arg	41A NH2	Tyr	32L OH	2.73 ***
Met	44A O	Asn	93L CG	3.65
		Asn	93L OD1	3.54 *
		Asn	93L ND2	2.96 ***
Phe	45A CA	Asn	93L OD1	3.50
Phe	45A CB	Tyr	32L CE1	3.59
Phe	45A CG	Tyr	32L CE1	3.93
Phe	45A CE1	Ser	30L CB	3.82
Phe	45A CZ	Ser	28L O	3.77
		Ser	30L CB	3.74
		Ser	28L OG	3.89
		Ser	30L N	3.78
Phe	45A CE2	Ser	28L O	3.76
		Val	29L O	3.98
		Val	29L CA	3.94
		Ser	30L CB	3.87
		Val	29L C	3.72
		Ser	30L N	3.89
Phe	45A CD2	Asn	93L ND2	3.75
		Ser	92L CB	3.85
Phe	45A C	Asn	93L OD1	3.70
Asn	46A N	Asn	93L OD1	2.92 ***
Asn	46A CA	Asn	93L OD1	3.82
Asn	46A CB	Asn	93L OD1	3.52
		Trp	94L CH2	3.86
		Trp	94L CZ2	3.52
Asn	46A CG	Asn	93L OD1	3.31
		Trp	94L CH2	3.41
		Trp	94L CZ2	3.65
Asn	46A OD1	Trp	94L CH2	3.85
Asn	46A ND2	Asn	93L CB	3.99
		Asn	93L CG	3.48
		Asn	93L OD1	2.56 ***
		Trp	94L CZ3	3.52
		Trp	94L CH2	3.25
		Trp	94L CZ2	3.76
Asp	47A CB	Tyr	32L OH	3.63
Asp	47A CG	Tyr	32L OH	3.74
		Tyr	32L CE2	3.79
Asp	47A OD2	Tyr	32L CZ	3.88
		Tyr	32L OH	3.34 *
		Tyr	32L CE2	3.54
Asp	72A CB	Ser	30L OG	3.87
Asp	72A CG	Ser	30L OG	3.62
Asp	72A OD2	Ser	30L CB	3.58
		Ser	30L OG	2.93 ***

5

Table 9

KIR2DL1 – 1-7F9 Fab' VH chain interactions. A cut-off of 4.0 Å was used. The contacts were identified by the CONTACT computer program of the CCP4 suite. In the last column "***" indicates a strong possibility for a hydrogen bond at this contact (distance < 3.3 Å) as calculated by CONTACT, " *" indicates a weak possibility (distance > 3.3 Å). Blank indicates that the program considered there to be no possibility of a hydrogen bond.

KIR2DL1 Atoms		1-7F9 Fab' VH Atoms		Distance (Å)
Leu	38A CD1	Ile	54H CD1	3.61
		Phe	31H CB	3.64
Leu	38A CD2	Phe	31H CG	3.70
		Phe	31H CD2	3.74
Asp	47A CG	Ser	103H N	3.73
		Ser	103H OG	3.63
Asp	47A OD1	Gly	102H N	3.99 *
		Gly	102H CA	3.40
		Gly	102H C	3.75
		Ser	103H N	3.09 ***
		Ser	103H OG	3.77 *
Asp	47A OD2	Ser	103H N	3.73 *
		Ser	103H CB	3.36
		Ser	103H OG	2.77 ***
Thr	48A C	Tyr	108H OH	3.74
Thr	48A O	Tyr	108H CZ	3.68
		Tyr	108H OH	2.90 ***
		Tyr	108H CE2	3.65
Leu	49A CA	Tyr	105H O	3.28
Leu	49A CB	Tyr	105H O	3.62
Leu	49A CD2	Tyr	105H O	3.67
		Tyr	105H CB	3.71
		Tyr	105H CD1	3.76
		Ser	103H OG	3.91
Leu	49A C	Tyr	105H O	3.59
Arg	50A N	Tyr	105H C	3.80
		Tyr	105H O	2.94 ***
		Tyr	106H N	3.87 *
		Tyr	108H CE2	3.81
Arg	50A CA	Tyr	105H O	3.97
Arg	50A CB	Tyr	106H O	3.88
		Tyr	108H CE2	3.80
Arg	50A CG	Phe	31H CZ	3.65
		Phe	31H CE2	3.90
		Tyr	106H O	3.99
Arg	50A CD	Phe	31H CZ	3.32
		Phe	31H CE2	3.63
		Tyr	106H O	3.71
Arg	50A NE	Tyr	106H O	2.88 ***
		Asp	107H C	3.94
		Asp	107H O	3.58 *
Arg	50A CZ	Tyr	106H O	3.70
		Asp	107H O	3.74

Arg	50A NH2	Tyr	106H O	3.61 *
		Asp	107H CB	3.56
		Asp	107H O	3.86 *
Arg	50A C	Tyr	106H N	3.86
Arg	50A O	Tyr	106H O	3.56 *
		Tyr	105H C	3.68
		Tyr	105H O	3.72 *
		Tyr	106H N	2.86 ***
		Tyr	106H CA	3.67
		Tyr	106H CB	3.47
Ile	52A CD1	Tyr	106H CD1	3.93
		Tyr	106H CE1	3.68
Phe	64A CD2	Tyr	105H CE1	3.86
Tyr	80A CZ	Phe	55H CE2	3.90
Tyr	80A OH	Phe	55H CE2	3.87
Pro	87A CB	Thr	28H OG1	3.46
Pro	87A CG	Thr	28H OG1	3.66
Pro	87A O	Ser	30H CA	3.87
		Ser	30H C	3.88
Tyr	88A CB	Glu	74H CD	3.39
		Glu	74H OE1	3.38
		Glu	74H OE2	3.40
Tyr	88A CG	Glu	74H CG	3.65
		Glu	74H CD	3.37
		Glu	74H OE1	3.71
		Glu	74H OE2	3.55
Tyr	88A CD1	Glu	74H CB	3.85
		Glu	74H CG	3.72
		Glu	74H CD	3.82
		Glu	74H OE1	3.97
Tyr	88A CE1	Glu	74H CB	3.93
		Glu	74H CG	3.91
		Glu	74H O	3.84
		Ser	77H OG	3.68
		Thr	28H CB	3.97
Tyr	88A CZ	Ser	77H OG	3.51
		Ser	30H N	3.87
		Thr	28H CB	3.71
		Phe	29H N	3.86
Tyr	88A OH	Ser	77H CA	3.73
		Ser	77H CB	3.35
		Ser	77H OG	2.49 ***
		Thr	28H CA	3.88
		Thr	28H CB	3.34
		Thr	28H C	3.96
		Phe	29H N	3.07 ***
		Phe	29H CA	3.86
		Phe	29H CB	3.67
		Phe	29H CD2	3.83
Tyr	88A CE2	Ser	30H N	3.32
		Ser	30H O	3.82
		Phe	29H C	3.92
		Phe	29H CB	3.89
Tyr	88A CD2	Glu	74H CG	3.81
		Glu	74H CD	3.77
		Glu	74H OE2	3.64

		Ser	30H N	3.91
		Ser	30H O	3.58
		Pro	53H CB	3.83
Tyr	88A C	Ile	54H CG2	3.93
Tyr	88A O	Ile	54H CG2	3.75

Example 12: Physical stability of 1-7F9

The biophysical properties and stability of human antibody 1-7F9 were studied. The folding and secondary structure of the protein was studied by circular dichroism (CD) and the oligomerization and aggregation by dynamic light scattering (DLS). In order to mimic storage conditions for two years at 5 °C the protein was subjected to incubation at 37 °C with shaking for 14 days.

Materials and Methods

Sample preparation. 2 mg/ml 1-7F9 was prepared in (a) 50 mM Na-Phosphate, 0.001 % Polysorbate 80 (Sigma, P8074), pH 7.0; (b) 50 mM Na-Phosphate, 0.001 % Polysorbate 80, pH 7.0, 0.5 mM Sucrose; (c) 50 mM Citrate, 0.001 % Polysorbate 80, pH 3.0; and d) 50 mM Tris, 0.001 % Polysorbate 80, pH 8.5.

Circular dichroism (CD). CD measurements were performed at 25 °C with a protein concentration of 2.0 mg/ml on a Chirascan circular dichroism spectrometer (Applied Photophysics) equipped with a peltier element for temperature control. 1-7F9 samples were in cylindrical quartz cells with 0.1 mm path length. Buffer scans were recorded and subtracted for each sample spectra.

Dynamic light scattering (DLS). DLS was performed at 25 °C with a protein concentration of 2.0 mg/ml using a Dynapro 99 temperature controlled DLS instrument (Protein Solutions Inc.). Data analysis was performed using the Dynamics software supplied with the instrument.

Results

Whereas the molecular size did not change for the samples at pH 7.0 after 14 days incubation as evaluated by DLS, both the samples formulated at pH 3.0 and pH 8.5 aggregated heavily during a 14 day period.

The CD measurements showed characteristics of an all beta structure and revealed that the samples formulated at pH 7.0 maintained their secondary structure throughout the accelerated study, although there was a slight drop in the signal for the sample containing only Polysorbate 80 as excipient. This might be due to a weak precipitation of the sample since the overall form of the spectra is unchanged. The sample containing sucrose showed no such decrease over time. The CD measurements of the samples formulated at pH 3.0

and 8.5 showed a strong change in spectral characteristics over time, probably as a result of unfolding or other conformational changes, which could lead to non-functional 1-7F9 protein. The changes were observed immediately and were most significant at pH 3.0.

Overall, 1-7F9 maintained its physical properties and remained stable under stressed conditions (37 °C with shaking) at pH 7.0 with Polysorbate 80 and Sucrose as excipients.

Example 13: Affinity determination (monovalent binding) of 1-7F9 and 1-4F1

The affinities of 1-7F9 and 1-4F1 in monovalent binding to KIR2DL1 and KIR2DL3 antigen, (as opposed to the bivalent binding affinity determinations in Examples 3 and 8), were determined by surface plasmon resonance technology, using a Biacore 3000.

Briefly, an anti-human IgG (Dako RAHIgG, # 0423) was used together with an HBS-EP buffer at a flow rate of 20µL/min. Purified antibodies were diluted to 10µg/mL. For each antibody, KIR2DL1 or KIR2DL3 and buffer was injected. The antigens were tested at concentrations of 2000, 500, 200, 50 og 20nM. Flowrate was maintained at 20µL/min. Regeneration of the surfacec was accomplished by a single 30s injection of Glycine-HCl pH 1.8.

The results are displayed in Table 10. For the binding of 1-4F1 to KIR2DL1, there was a significant shift in baseline. Without being limited to any specific theory, this might represent a buffer effect caused by the matrix, or the binding reaction actually involving 2 steps, *e.g.*, because of a conformational change of the antigen.

Table 10

Monovalent binding affinity of 1-7F9 and 1-4F1 to KIR2DL1 and KIR2DL3. *) indicates fit with RI (response of matrix) fitted globally.

	k_a (1/Ms)	k_d (1/s)	K_D (M)	Chi2
1-4F1/2DL1	8,33E+03	5,09E-15	6,11E-19	1,24
1-4F1/2DL1 *)	2,68E+04	0,0132	4,95E-07	9,1
1-4F1/2DL3	4,16E+05	2,94E-03	7,05E-09	0,398
1-7F9/2DL1	1,51E+05	1,56E-03	1,03E-08	4,02
1-7F9/2DL3	1,51E+05	5,20E-04	3,45E-09	1,55

EXAMPLARY ASPECTS OF THE INVENTION

1. An antibody comprising a light chain variable region comprising an amino acid sequence at least 50% identical to SEQ ID NO:15 or SEQ ID NO:39, and/or a heavy chain variable re-

gion comprising an amino acid sequence at least 50% identical to SEQ ID NO:17 or SEQ ID NO:41.

2. An antibody comprising a light chain variable region comprising an amino acid sequence at least 80% identical to SEQ ID NO:15 or SEQ ID NO:39, and/or a heavy chain variable region comprising an amino acid sequence at least 80% identical to SEQ ID NO:17 or SEQ ID NO:41.

3. An antibody comprising a light chain variable region comprising an amino acid sequence at least 90% identical to SEQ ID NO:15 or SEQ ID NO:39, and/or a heavy chain variable region comprising an amino acid sequence at least 90% identical to SEQ ID NO:17 or SEQ ID NO:41.

4. An antibody comprising a light chain variable region comprising an amino acid sequence at least 95% identical to SEQ ID NO:15 or SEQ ID NO:39, and/or a heavy chain variable region comprising an amino acid sequence at least 95% identical to SEQ ID NO:17 or SEQ ID NO:41.

5. An antibody comprising a light chain variable region comprising an amino acid sequence at least 98% identical to SEQ ID NO:15 or SEQ ID NO:39, and/or a heavy chain variable region comprising an amino acid sequence at least 98% identical to SEQ ID NO:17 or SEQ ID NO:41.

6. The antibody of any of the preceding aspects, wherein the light chain variable region comprises at least one conservative amino acid substitution, each amino acid substitution being conservative as compared to the amino acid at the corresponding position in SEQ ID NO:15 or SEQ ID NO:39.

7. The antibody of any of the preceding aspects, wherein the heavy chain variable region comprises at least one conservative amino acid substitution, each amino acid substitution being conservative as compared to the amino acid at the corresponding position in SEQ ID NO:17 or SEQ ID NO:41.

8. The antibody of any of the preceding aspects, comprising at least one of

(a) a light chain CDR1 amino acid sequence at least 90% identical to residues 24-34 of SEQ ID NO:15;

(b) a light chain CDR2 amino acid sequence at least 90% identical to residues 50-56 of SEQ ID NO:15;

(c) a light chain CDR3 amino acid sequence at least 90% identical to residues 89-97 of SEQ ID NO:15;

(d) a heavy chain CDR1 amino acid sequence at least 90% identical to residues 31-35 of SEQ ID NO:17;

(e) a heavy chain CDR2 amino acid sequence at least 90% identical to residues 50-65 of SEQ ID NO:17; and

(f) a heavy chain CDR3 amino acid sequence at least 90% identical to residues 99-112 of SEQ ID NO:17.

9. The antibody of any of the preceding aspects, comprising at least one of

(a) a light chain CDR1 amino acid sequence corresponding to residues 24-34 of SEQ ID NO:15;

(b) a light chain CDR2 amino acid sequence corresponding to residues 50-56 of SEQ ID NO:15;

(c) a light chain CDR3 amino acid sequence corresponding to residues 89-97 of SEQ ID NO:15.

(d) a heavy chain CDR1 amino acid sequence corresponding to residues 31-35 of SEQ ID NO:17;

(e) a heavy chain CDR2 amino acid sequence corresponding to residues 50-65 of SEQ ID NO:17; and

(f) a heavy chain CDR3 amino acid sequence corresponding to residues 99-112 of SEQ ID NO:17.

10. The antibody of any of the preceding aspects, comprising at least one of

(a) a light chain CDR1 amino acid sequence consisting essentially of residues 24-34 of SEQ ID NO:15;

(b) a light chain CDR2 amino acid sequence consisting essentially of residues 50-56 of SEQ ID NO:15;

(c) a light chain CDR3 amino acid sequence consisting essentially of residues 89-97 of SEQ ID NO:15.

(d) a heavy chain CDR1 amino acid sequence consisting essentially of residues 31-35 of SEQ ID NO:17;

(e) a heavy chain CDR2 amino acid sequence consisting essentially of residues 50-65 of SEQ ID NO:17; and

(f) a heavy chain CDR3 amino acid sequence consisting essentially of residues 99-112 of SEQ ID NO:17.

11. The antibody of any of aspects 8-10, comprising at least 2 of (a) to (f).

12. The antibody of any of aspects 8-10, comprising at least 3 of (a) to (f).

13. The antibody of any of aspects 8-10, comprising at least 4 of (a) to (f).

14. The antibody of any of aspects 8-10, comprising at least 5 of (a) to (f).

15. The antibody of any of aspects 8-10, comprising all of (a) to (f).

16. The antibody of any of the preceding claims, comprising a light chain variable region comprising the amino acid sequence of SEQ ID NO:15, and a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:17.

17. The antibody of any of aspects 1-7, comprising at least one of

(g) a light chain CDR1 amino acid sequence at least 90% identical to residues 24-34 of SEQ ID NO:39;

(h) a light chain CDR2 amino acid sequence at least 90% identical to residues 50-56 of SEQ ID NO:39;

(i) a light chain CDR3 amino acid sequence at least 90% identical to residues 89-97 of SEQ ID NO:39;

(j) a heavy chain CDR1 amino acid sequence at least 90% identical to residues 31-35 of SEQ ID NO:41;

(k) a heavy chain CDR2 amino acid sequence at least 90% identical to residues 50-66 of SEQ ID NO:41; and

(l) a heavy chain CDR3 amino acid sequence at least 90% identical to residues 99-113 of SEQ ID NO:41.

18. The antibody of any aspects 1-7 and 17, comprising at least one of

(g) a light chain CDR1 amino acid sequence corresponding to residues 24-34 of SEQ ID NO:39;

(h) a light chain CDR2 amino acid sequence corresponding to residues 50-56 of SEQ ID NO:39;

(i) a light chain CDR3 amino acid sequence corresponding to residues 89-97 of SEQ ID NO:39;

(j) a heavy chain CDR1 amino acid sequence corresponding to residues 31-35 of SEQ ID NO:41;

(k) a heavy chain CDR2 amino acid sequence corresponding to residues 50-66 of SEQ ID NO:41; and

(l) a heavy chain CDR3 amino acid sequence corresponding to residues 99-113 of SEQ ID NO:41.

19. The antibody of any aspects 1-7 and 17-18, comprising at least one of

(g) a light chain CDR1 amino acid sequence consisting essentially of residues 24-34 of SEQ ID NO:39;

(h) a light chain CDR2 amino acid sequence consisting essentially of residues 50-56 of SEQ ID NO:39;

(i) a light chain CDR3 amino acid sequence consisting essentially of residues 89-97 of SEQ ID NO:39;

(j) a heavy chain CDR1 amino acid sequence consisting essentially of residues 31-35 of SEQ ID NO:41;

(k) a heavy chain CDR2 amino acid sequence consisting essentially of residues 50-66 of SEQ ID NO:41; and

(l) a heavy chain CDR3 amino acid sequence consisting essentially of residues 99-113 of SEQ ID NO:41.

20. The antibody of any of aspects 17-19, comprising at least 2 of (a) to (f).

21. The antibody of any of aspects 17-19, comprising at least 3 of (a) to (f).

22. The antibody of any of aspects 17-19, comprising at least 4 of (a) to (f).

23. The antibody of any of aspects 17-19, comprising at least 5 of (a) to (f).

24. The antibody of any of aspects 17-19, comprising all of (a) to (f).

25. The antibody of any of claims 1-7 and 17-24, comprising a light chain variable region comprising the amino acid sequence of SEQ ID NO:39, and a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:41.

26. The antibody of any of the preceding aspects, which antibody does not bind at least one of KIR2DS4 and KIR2DS3.

27. The antibody of any aspects 1-15 and 26, which antibody blocks KIR2DL1 and KIR2DL2/3 binding to an HLA-C class I molecule.

28. The antibody of any aspects 1-15 and 26-27, which potentiates the lytic activity of an NK cell against a human target cell expressing an HLA-C class I molecule.

29. The antibody of any of aspects 1-15 and 26-28 which antibody is more efficient than DF200 in potentiating the lytic activity of an NK cell against a human target cell expressing an HLA-C Class I molecule.

30. The antibody of any of aspects 1-15 and 26-28, which antibody is more efficient than NKVSF1 (Pan2D) in potentiating the lytic activity of an NK cell against a human target cell expressing an HLA-C Class I molecule.

31. The antibody of any of aspects 1-15 and 26-28, which antibody is more efficient than EB6 in potentiating the lytic activity of an NK cell against a human target cell expressing an HLA-C Class I molecule

32. The antibody of any of the preceding aspects, which is a human or humanized antibody.

33. The antibody of any of the preceding aspects, which is an IgG1, IgG2, IgG3, or IgG4 antibody.

34. The antibody of any of the preceding aspects, which is a human IgG4 antibody.

35. The antibody of any of aspects 1-34, which is a human IgG2 antibody.

36. A human IgG4 antibody comprising a light chain variable region comprising the amino acid sequence of SEQ ID NO:15, and a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:17.

37. A human IgG2 antibody comprising a light chain variable region comprising the amino acid sequence of SEQ ID NO:39, and a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:41.

38. An antibody that competes with 1-7F9 in the binding to KIR2DL1 or KIR2DL2/3, wherein the antibody is not 1-4F1, DF200, gl183, A210, A803(g), or EB6.

39. An antibody that competes with 1-4F1 in the binding to KIR2DL1 or KIR2DL2/3, wherein the antibody is not 1-7F9, DF200, gl183, A210, A803(g), or EB6.

40. The antibody of aspect 38 , wherein the antibody is not 1-4F1.

41. The antibody of aspects 39, wherein the antibody is not 1-7F9

42. The antibody of any of aspects 38-39, wherein the antibody is not DF200.

43. The antibody of any of aspects 38-39, wherein the antibody is not gl183.

44. The antibody of any of aspects 38-39, wherein the antibody is not EB6.

45. The antibody of any of the preceding aspects, which has a K_d to KIR2DL1 of no more than about 20 nM.

46. The antibody of any of the preceding aspects, which has a K_d to KIR2DL1 of no more than 10.9 nM.

47. The antibody of any of the preceding aspects, which has a K_d to KIR2DL1 of no more than 0.45 nM.

48. The antibody of any of the preceding aspects, which has a K_d to KIR2DL3 of no more than about 20 nM.

49. The antibody of any of the preceding aspects, which has a K_d to KIR2DL3 of no more than 2.0 nM.

50. The antibody of any of the preceding aspects, which has a K_d to KIR2DL3 of no more than 0.025 nM.

51. The antibody of any of the preceding aspects, which is human.

52. A human or humanized antibody that competes with 1-7F9 in the binding to KIR2DL1.

53. A human or humanized antibody that competes with 1-4F1 in the binding to KIR2DL1.

54. A human or humanized antibody that competes with 1-7F9 in the binding to KIR2DL2.

55. A human or humanized antibody that competes with 1-4F1 in the binding to KIR2DL2.

56. A human or humanized antibody that competes with 1-7F9 in the binding to KIR2DL3.

57. A human or humanized antibody that competes with 1-4F1 in the binding to KIR2DL3.

58. A human or humanized antibody that competes with 1-7F9 in the binding to KIR2DL1 and KIR2DL2/3.

59. A human or humanized antibody that competes with 1-4F1 in the binding to KIR2DL1 and KIR2DL2/3.

60. The human or humanized antibody of any of aspects 52-59, which does not bind at least one of KIR2DS4 and KIR2DS3.

61. The human or humanized antibody of any of aspects 52-60, which blocks KIR2DL1 or KIR2DL2/3 binding to an HLA-C class I molecule.

62. The human or humanized antibody of any of aspects 52-47, which potentiates the lytic activity of an NK cell against a human target cell expressing an HLA-C class I molecule.

63. The human or humanized antibody of any of aspects 52-62, which antibody comprises a light chain variable region comprising an amino acid sequence at least 90% identical to SEQ ID NO:15, and a heavy chain variable region comprising an amino acid sequence at least 90% identical to SEQ ID NO:17, or a light chain variable region at least 90% identical to SEQ ID NO:39, and a heavy chain variable region comprising an amino acid sequence at least 90% identical to SEQ ID NO:41.

64. The human or humanized antibody of any of aspects 52-63, which is an IgG1, IgG2, IgG3, or IgG4 antibody.

65. The human or humanized antibody of aspect 64, which is an IgG4 antibody.

66. The human antibody of aspect 64, which is an IgG2 antibody.

67. A human or humanized antibody that binds to each one of KIR2DL1, -2, and -3, and which blocks KIR2DL1, -2, or -3 binding to an HLA-C class I molecule.

68. An antibody that interacts with residues M44 and F45 of KIR2DL1.

69. The antibody of aspect 68, which further interacts with one or more of KIR2DL1 residues L38, R41, N46, D47, T48, L49, R50, I52, F64, D72, Y80, P87, and Y88.

70. An antibody that binds to KIR2DL1 exclusively within a region defined by the amino acid residues L38, R41, M44, F45, N46, D47, T48, L49, R50, I52, F64, D72, Y80, P87, and Y88.

71. An antibody that binds to KIR2DL1 and KIR2DL2/3 without interacting with amino acid residues outside the region defined by the residues L38, R41, M44, F45, N46, D47, T48, L49, R50, I52, F64, D72, Y80, P87, and Y88.

72. The antibody of any of aspects 67-71, which is human antibody 1-7F9.

73. An antibody fragment or antibody derivative comprising at least one light chain CDR of the antibody of any of the preceding aspects.

74. An antibody fragment or antibody derivative comprising at least two light chain CDRs of the antibody of any of the preceding aspects.

75. An antibody fragment or antibody derivative comprising at least three light chain CDRs of the antibody of any of the preceding aspects.

76. An antibody fragment or antibody derivative comprising a VL region of the antibody of any of the preceding aspects.

77. An antibody fragment or antibody derivative comprising at least one heavy chain CDR of the antibody of any of the preceding aspects.

78. An antibody fragment or antibody derivative comprising at least two heavy chain CDRs of the antibody of any of the preceding aspects.

79. An antibody fragment or antibody derivative comprising at least three heavy chain CDRs of the antibody of any of the preceding aspects.

80. An antibody fragment or antibody derivative comprising a VH region of the antibody of any of the preceding aspects.

81. An antibody fragment or antibody derivative comprising all CDRs of the antibody of any of the preceding aspects.

82. An antibody fragment or antibody derivative comprising the VH and VL regions of the antibody of any of the preceding aspects.

83. A hybridoma producing the antibody of any of the preceding claims.

84. A nucleic acid encoding the antibody or antibody fragment of any of aspects 1-82.

85. A vector comprising the nucleic acid of aspect 84.

86. A cell comprising the vector of aspect 85.

87. The cell of aspect 86, wherein the cell is selected from a simian COS cell, a CHO cell, and a human myeloma cell.

88. A method of producing an anti-KIR antibody or antibody fragment comprising culturing the cell of aspect 86 under conditions suitable for expression of the anti-KIR antibody, antibody fragment, respectively.

89. A method of producing a derivative of an anti-KIR antibody or anti-KIR antibody fragment comprising providing an an anti-KIR antibody or antibody fragment and conjugating at least one derivative moiety thereto.

90. A method of treating a cancer in a subject, which method comprises administering to a subject suffering from cancer an effective amount of the antibody or antibody fragment or derivative of any of aspects 1-82.

91. The method of aspect 90, further comprising administering to the patient an agent selected from a chemotherapeutic, radiotherapeutic, anti-angiogenic, immunomodulatory, and hormonal agent.

92. A method of treating a disease or disorder caused by a virus in a subject, which method comprises administering to a subject suffering from disease or disorder caused by a virus an effective amount of the antibody or antibody fragment or derivative of any of aspects 1-82.

93. A use of the antibody or antibody fragment or derivative of any of aspects 1-82 in the preparation of a medicament for treating cancer.

94. A use of the antibody or antibody fragment or derivative of any of aspects 1-82 in the preparation of a medicament for treating a disease or disorder caused by a virus.

95. A composition comprising the antibody or antibody fragment or derivative of any of aspects 1-82, and a pharmaceutically acceptable carrier or excipient.

96. The composition of aspect 95, comprising Polysorbate 80.

97. The composition of aspect 95, comprising Sucrose.

98. The composition of aspect 95, comprising Polysorbate 80 and Sucrose.

99. The composition of any of aspects 95-98, further comprising an agent selected from a chemotherapeutic, radiotherapeutic, anti-angiogenic, immunomodulatory, and hormonal agent.

100. The composition of any of aspects 95-99, in which the antibody, antibody fragment, or antibody derivative is covalently conjugated to a tumor-targeting agent.

101. The composition of any of aspects 95-99, in which the antibody, antibody fragment, or antibody derivative is encapsulated in a liposome.

102. The composition of any of aspects 95-99, further comprising an antibody binding to a KIR, wherein the KIR is not KIR2DL1, KIR2DL2, KIR2DL3, KIR2DS1, or KIR2DS2.

103. A method of increasing the lytic activity of a cell selected from a lymphocyte and an NK cell comprising contacting the cell with the antibody, antibody fragment or derivative of any of aspects 1-82.

104. A method of reducing the interaction between a KIR expressed by a cell selected from a lymphocyte, T cell, and an NK cell, and an HLA-C class I molecule expressed by a target cell, the method comprising contacting the cell with the antibody or antibody fragment or derivative of any of aspects 1-82.

105. A method of neutralizing the inhibitory activity of a KIR expressed by an NK cell, the method comprising contacting the NK cell with the antibody or antibody fragment or derivative of any of aspects 1-82.

106. A human antibody that binds to each one of KIR2DL1, KIR2DL2, and KIR2DL3, which antibody blocks the binding of an HLA-Cw4 molecule to KIR2DL1, and the binding of an HLA-Cw3 molecule to at least one of KIR2DL2 or KIR2DL3.

107. The antibody or antibody fragment or derivative according to any of aspects 1-82, wherein the KIR2DL1 comprises the amino acid sequence of SEQ ID NO:23, the KIR2DL2 comprises the amino acid sequence of SEQ ID NO:24, and/or the KIR2DL3 comprises the amino acid sequence of SEQ ID NO:25.

108. A compound that binds to substantially the same KIR2DL1 epitope as the antibody, antibody fragment, or antibody derivative of any of aspects 1-82.

109. A compound that binds to essentially the same KIR2DL1 epitope as the antibody, antibody fragment, or antibody derivative of any of aspects 1-82.

110. An antibody or antibody derivative comprising a light chain comprising the sequence of SEQ ID NO:36.

111. An antibody or antibody derivative comprising a heavy chain comprising the sequence of SEQ ID NO:37.

112. An antibody or antibody derivative comprising the light chain of aspect 109 and the heavy chain of aspect 110.

113. An antibody consisting of a light chain comprising the sequence of SEQ ID NO:36 and a heavy chain consisting of the sequence of SEQ ID NO:37.

* * *

All references, including publications, patent applications and patents, cited herein are hereby incorporated by reference to the same extent as if each reference was individually and specifically indicated to be incorporated by reference and was set forth in its entirety herein.

All headings and sub-headings are used herein for convenience only and should not be construed as limiting the invention in any way,

Any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

The terms "a" and "an" and "the" and similar referents as used in the context of describing the invention are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context.

Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. Unless otherwise stated, all exact values provided herein

are representative of corresponding approximate values (*e.g.*, all exact exemplary values provided with respect to a particular factor or measurement can be considered to also provide a corresponding approximate measurement, modified by "about," where appropriate).

All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context.

The use of any and all examples, or exemplary language (*e.g.*, "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise indicated. No language in the specification should be construed as indicating any element is essential to the practice of the invention unless as much is explicitly stated.

The citation and incorporation of patent documents herein is done for convenience only and does not reflect any view of the validity, patentability and/or enforceability of such patent documents,

The description herein of any aspect or embodiment of the invention using terms such as "comprising", "having", "including" or "containing" with reference to an element or elements is intended to provide support for a similar aspect or embodiment of the invention that "consists of", "consists essentially of", or "substantially comprises" that particular element or elements, unless otherwise stated or clearly contradicted by context (*e.g.*, a composition described herein as comprising a particular element should be understood as also describing a composition consisting of that element, unless otherwise stated or clearly contradicted by context).

This invention includes all modifications and equivalents of the subject matter recited in the aspects or claims presented herein to the maximum extent permitted by applicable law.

CLAIMS

1. An isolated human antibody that binds to each one of KIR2DL1, KIR2DL2, and KIR2DL3, but which does not bind KIR2DS4.

2. The isolated human antibody of claim 1, which further does not bind to KIR2DS3.

3. The isolated human antibody of any of the preceding claims, which antibody blocks the binding of at least one of KIR2DL1, KIR2DL2, and KIR2DL3 to an HLA-C class I molecule.

4. The isolated human antibody of any of the preceding claims, which antibody blocks the binding of an HLA-Cw4 molecule to KIR2DL1, and the binding of an HLA-Cw3 molecule to at least one of KIR2DL2 or KIR2DL3.

5. The isolated human antibody of any of the preceding claims, which antibody potentiates the lytic activity of an NK cell against a human target cell expressing an HLA-C class I molecule.

6. The isolated human antibody of any of the preceding claims, which antibody competes with an antibody comprising a light chain variable region comprising the amino acid sequence of SEQ ID NO:15, and a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:17, in the binding to at least one of KIR2DL1, KIR2DL2, and KIR2DL3.

7. The isolated human antibody of any of the preceding claims, which antibody competes with an antibody comprising a light chain variable region comprising the amino acid sequence of SEQ ID NO:15, and a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:17, in the binding to each of KIR2DL1, KIR2DL2, and KIR2DL3.

8. The isolated human antibody of any of the preceding claims, which antibody comprises a light chain variable region comprising the amino acid sequence of SEQ ID NO:15.

9. The isolated human antibody of any of the preceding claims, which antibody comprises

(a) a heavy chain CDR1 amino acid sequence corresponding to residues 31-35 of SEQ ID NO:17;

(b) a heavy chain CDR2 amino acid sequence corresponding to residues 50-65 of SEQ ID NO:17; and

(c) a heavy chain CDR3 amino acid sequence corresponding to residues 99-112 of SEQ ID NO:17.

10. The isolated human antibody of any of the preceding claims, which antibody comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:17.

11. The isolated human antibody of any of the preceding claims, which antibody has a dissociation constant (K_d) for KIR2DL1 of no more than about 0.45 nM.

12. The isolated human antibody of any of the preceding claims, which antibody has a K_d for KIR2DL3 of no more than about 0.025 nM.

13. The isolated human antibody of any of the preceding claims, which antibody binds to a KIR2DL1 epitope substantially comprising the amino acid residues L38, R41, M44, F45, N46, D47, T48, L49, R50, I52, F64, D72, Y80, P87, and Y88.

14. The isolated human antibody of any of the preceding claims, which antibody blocks the binding of an HLA-Cw4 molecule to residues M44, F45 and D72 of the extracellular portion of KIR2DL1 (SEQ ID NO:23).

15. The isolated human antibody of any of the preceding claims, which antibody is a monoclonal antibody.

16. The isolated human antibody of any of the preceding claims, which antibody is an IgG1, IgG2, IgG3, or IgG4 antibody.

17. The isolated human antibody of any of the preceding claims, which antibody is an IgG4 antibody.

18. A nucleic acid encoding the human antibody of any of the preceding claims.

19. A vector comprising the nucleic acid of claim 18.

20. A cell comprising the vector of claim 19.

21. A method of producing a human anti-KIR antibody, comprising culturing the cell of claim 20 under conditions suitable for expression of the anti-KIR antibody.

22. A pharmaceutical composition comprising the human antibody of any of claims 1-17, or the human antibody produced by the method of claim 21, in an amount effective to detectably potentiate NK cell cytotoxicity in a patient, and a pharmaceutically acceptable carrier or excipient.

23. The pharmaceutical composition of claim 22, comprising Polysorbate 80, sucrose, or both Polysorbate 80 and sucrose.

24. A method of potentiating NK cell activity in a subject in need thereof, which method comprises administering to the subject an effective amount of the composition of any of claims 22 to 23.

25. The method of claim 24, wherein the subject is a patient suffering from cancer.

26. The method of claim 25, wherein the patient is suffering from a cancer selected from acute myeloid leukaemia, chronic myeloid leukaemia, multiple myeloma, and non-Hodgkin's lymphoma.

27. The method of claim 25, wherein the patient is suffering from a cancer selected from colorectal cancer, renal cancer, ovarian cancer, lung cancer, breast cancer, and malignant melanoma.

28. The method of claim 24, wherein the subject is a patient suffering from an infectious disease.

29. The method of any of claims 24 to 28, further comprising administering a therapeutic agent selected from an immunomodulatory agent, a hormonal agent, a chemotherapeutic agent, an anti-angiogenic agent, an apoptotic agent, a second antibody that binds to an inhibitory KIR, an anti-infective agent, a targeting agent, and an adjunct compound.

FIGURE 1

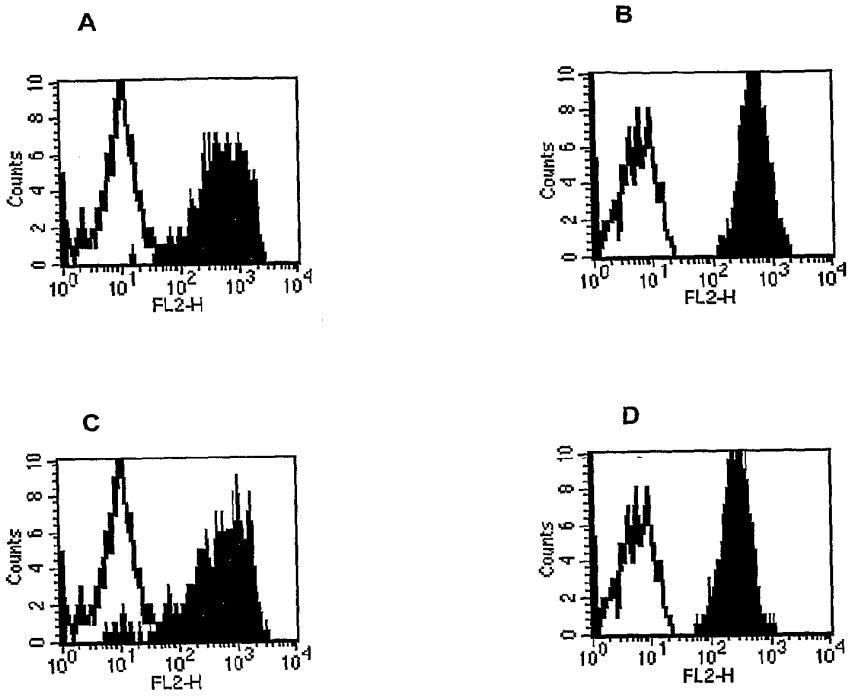


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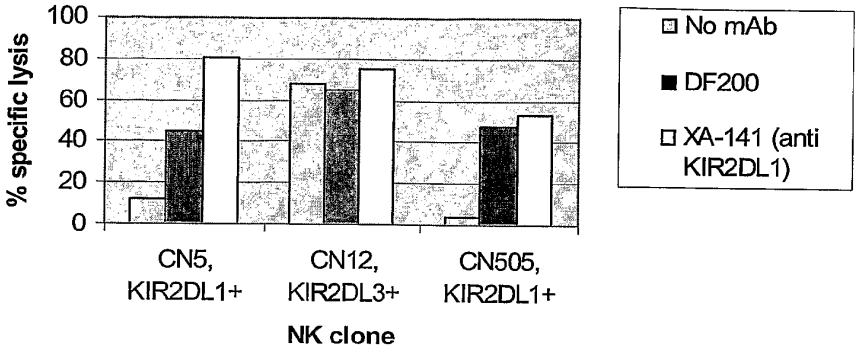

100
80
60
40
20
0
% specific lysis
CN5, KIR2DL1+
CN12, KIR2DL3+
CN505, KIR2DL1+
NK clone
No mAb
DF200
XA-141 (anti KIR2DL1)

FIGURE 3

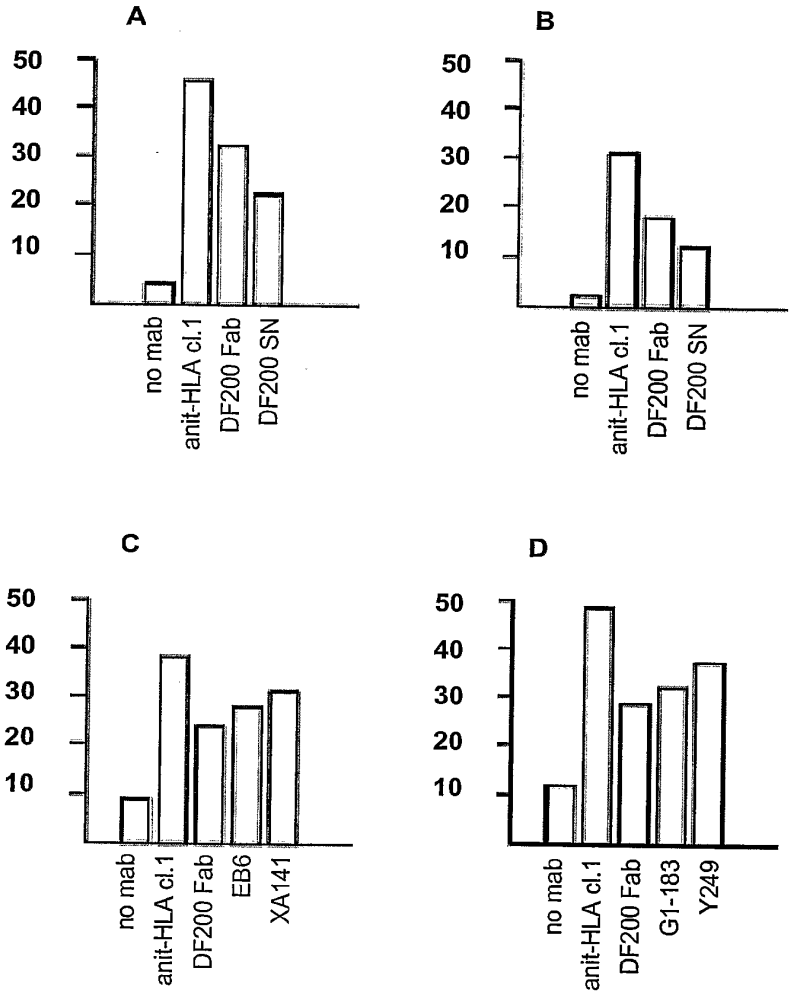
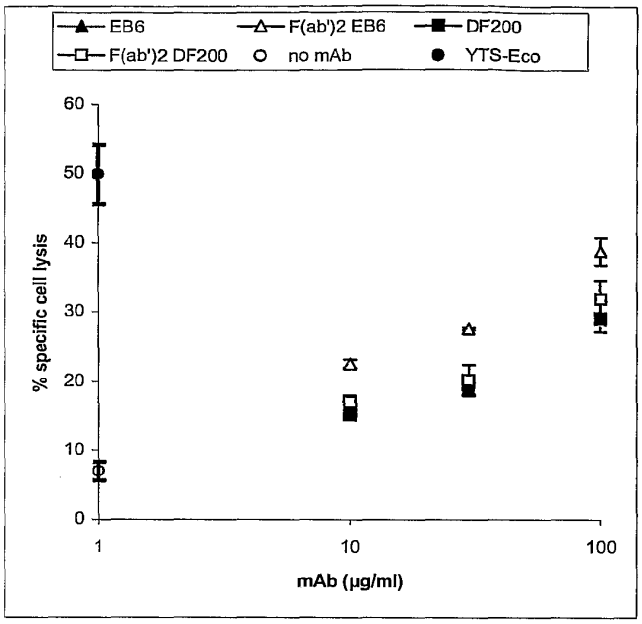

A
50
40
30
20
10
no mab
anit-HLA cl.1
DF200 Fab
DF200 SN
B
50
40
30
20
10
no mab
anit-HLA cl.1
DF200 Fab
DF200 SN
C
50
40
30
20
10
no mab
anit-HLA cl.1
DF200 Fab
EB6
XA141
D
50
40
30
20
10
no mab
anit-HLA cl.1
DF200 Fab
G1-183
Y249

FIGURE 4

A


EB6
F(ab')2 EB6
DF200
F(ab')2 DF200
no mAb
YTS-Eco
% specific cell lysis
60
50
40
30
20
10
0
1
10
100
mAb (µg/ml)

B

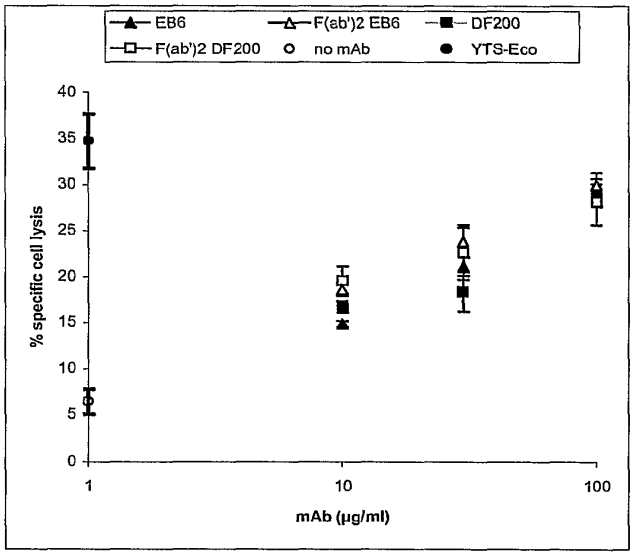
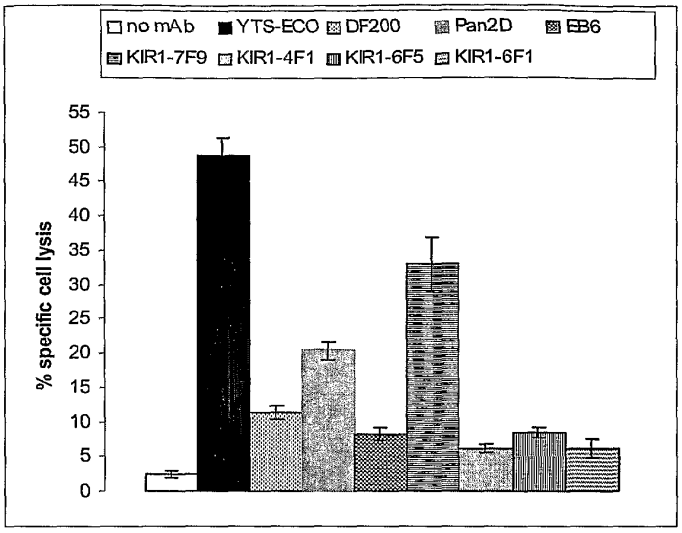

EB6
F(ab')2 EB6
DF200
F(ab')2 DF200
no mAb
YTS-Eco
% specific cell lysis
40
35
30
25
20
15
10
5
0
1
10
100
mAb (µg/ml)

FIGURE 5

A


no mAb
YTS-ECO
DF200
Pan2D
EB6
KIR1-7F9
KIR1-4F1
KIR1-6F5
KIR1-6F1
% specific cell lysis
55
50
45
40
35
30
25
20
15
10
5
0

B

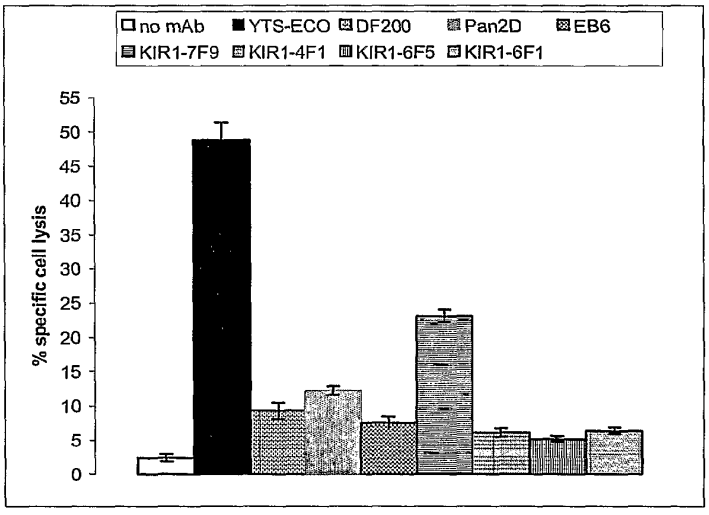
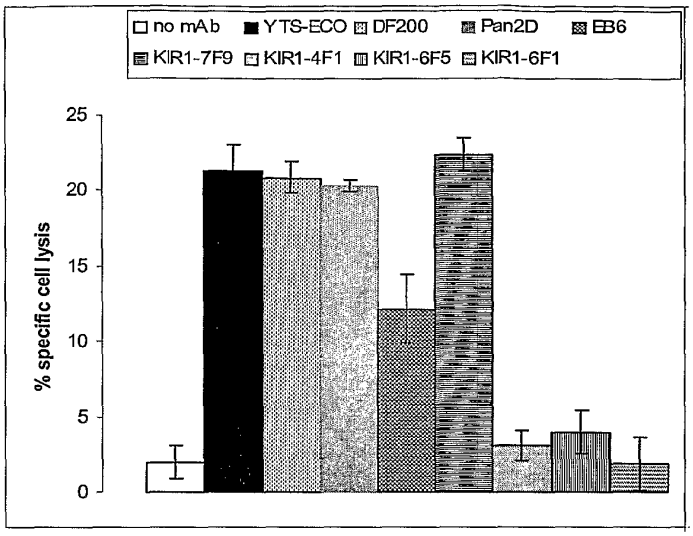

no mAb
YTS-ECO
DF200
Pan2D
EB6
KIR1-7F9
KIR1-4F1
KIR1-6F5
KIR1-6F1
% specific cell lysis
55
50
45
40
35
30
25
20
15
10
5
0

FIGURE 6

A


no mAb
YTS-ECO
DF200
Pan2D
EB6
KIR1-7F9
KIR1-4F1
KIR1-6F5
KIR1-6F1
% specific cell lysis
25
20
15
10
5
0

B

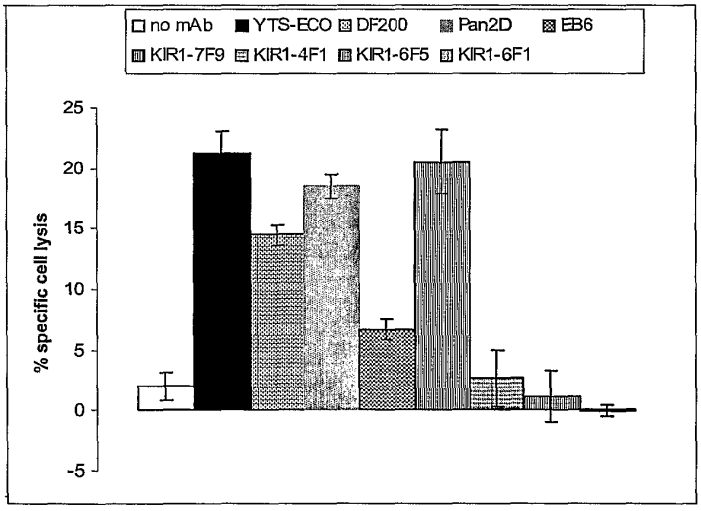

no mAb
YTS-ECO
DF200
Pan2D
EB6
KIR1-7F9
KIR1-4F1
KIR1-6F5
KIR1-6F1
% specific cell lysis
25
20
15
10
5
0
-5

FIGURE 7

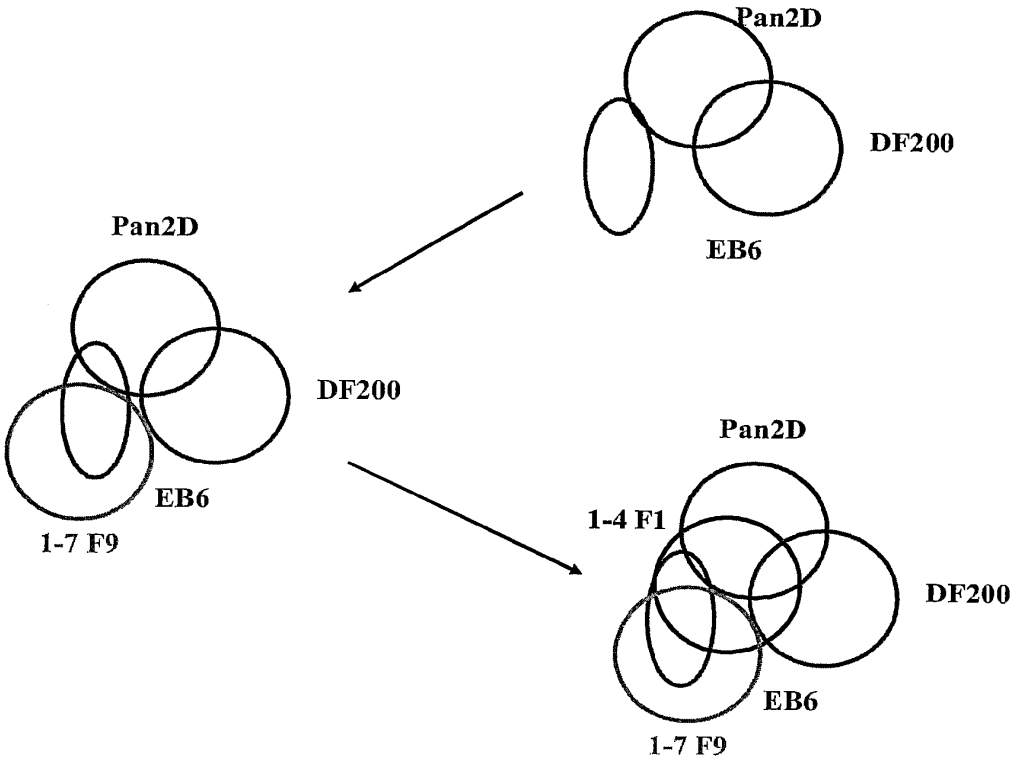

Pan2D
DF200
EB6
Pan2D
DF200
EB6
1-7 F9
Pan2D
1-4 F1
DF200
EB6
1-7 F9

FIGURE 8

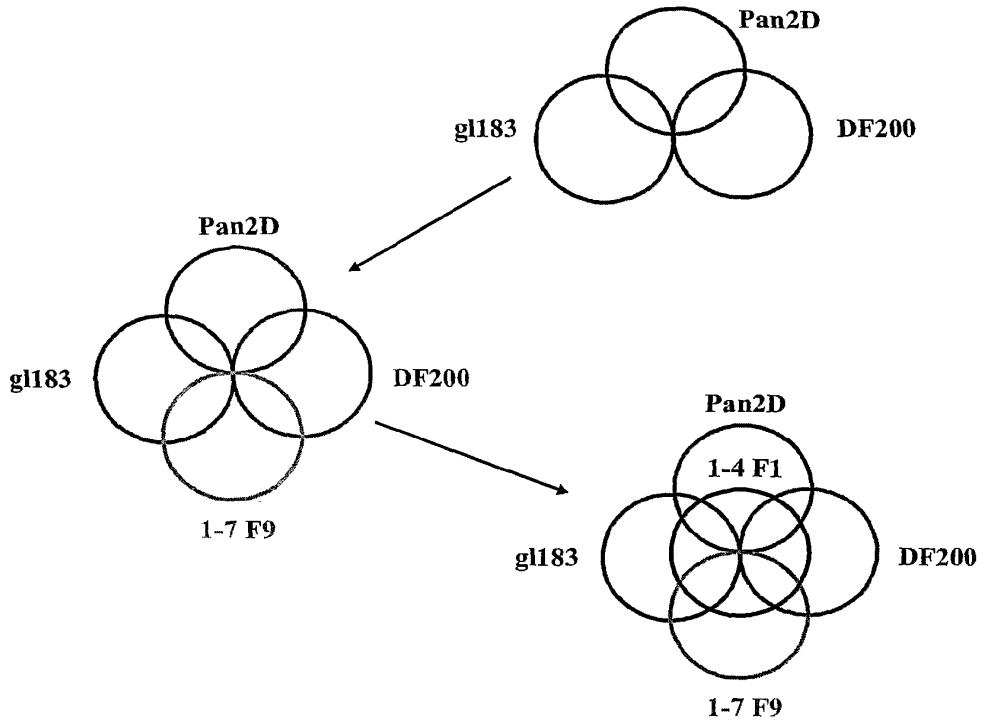

Pan2D
gl183
DF200
Pan2D
gl183
DF200
1-7 F9
Pan2D
1-4 F1
gl183
DF200
1-7 F9

FIGURE 9

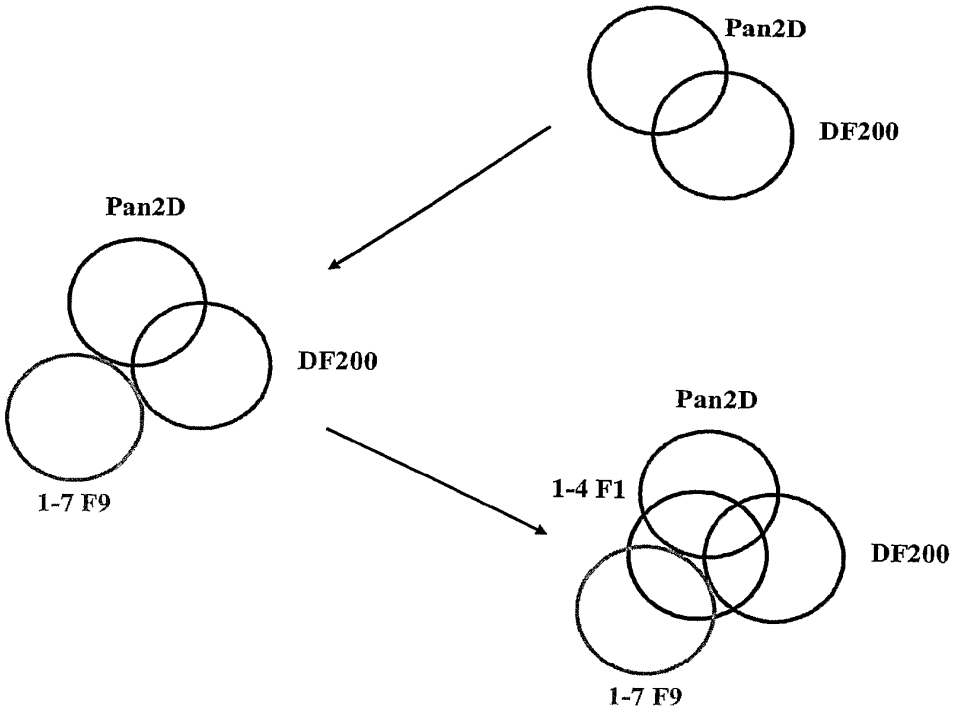


FIGURE 10

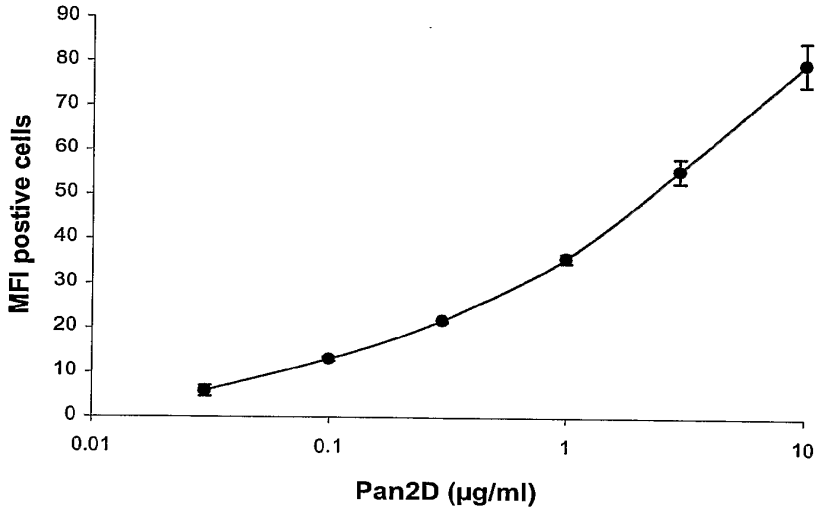
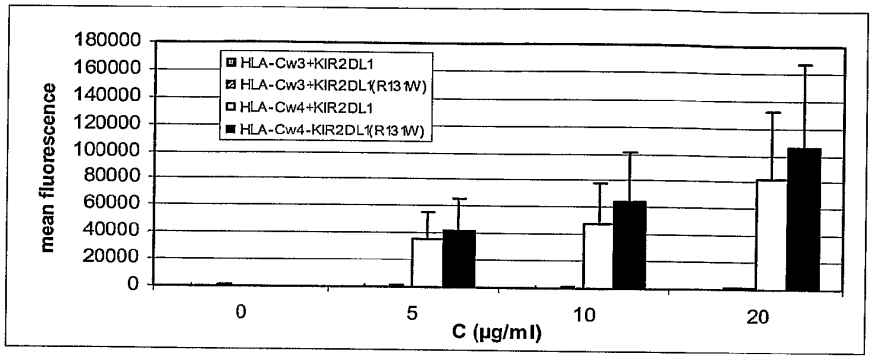

MFI postive cells
90
80
70
60
50
40
30
20
10
0
0.01
0.1
1
10
Pan2D (µg/ml)

FIGURE 11

A


mean fluorescence
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
HLA-Cw3+KIR2DL1
HLA-Cw3+KIR2DL1(R131W)
HLA-Cw4+KIR2DL1
HLA-Cw4-KIR2DL1(R131W)
0
5
10
20
C (µg/ml)

B

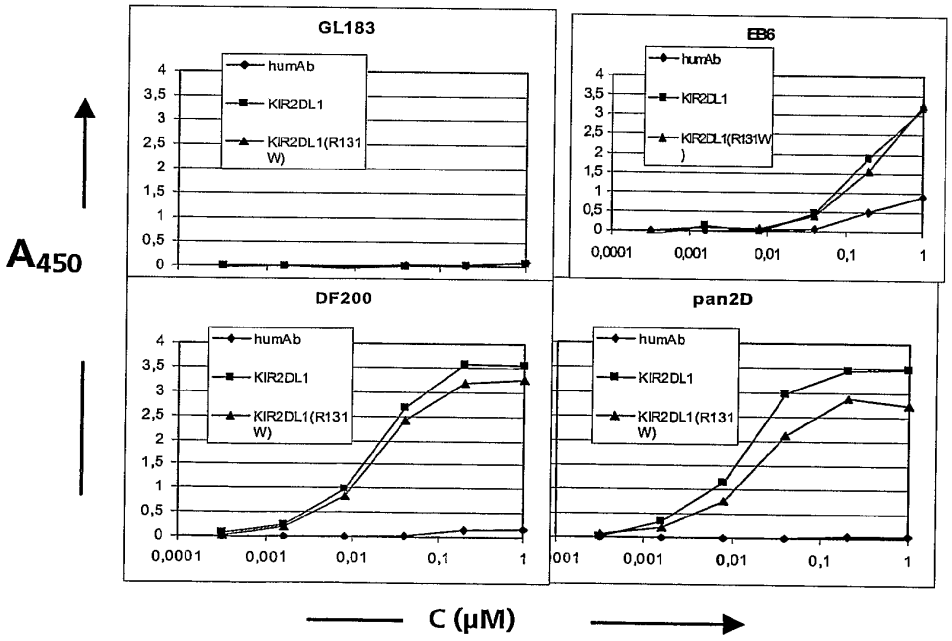

A450
GL183
EB6
DF200
pan2D
humAb
KIR2DL1
KIR2DL1(R131W)
0,0001
0,001
0,01
0,1
1
C (µM)

FIGURE 12

A

```
                          1                                                  50
DF-200 light variable     (1) M--ESQTLVFISILLWIYGADGNIVMTQSPKSMSMSVGERVTLTCKASEN
 PAN2D-Light-variable     (1) MDFQVQIFSFLLISASVIMSRGQIVLTQSPASMSASLGERVTMTCTASSS
           Consensus      (1)      Q   FI I   L  A GNIVLTQSP SMS SLGERVTLTC AS
                          51                                                 100
DF-200 light variable     (49) VVI-YVSWYQQKPEQSPKLLIYGASNRYIGVPDRFIGSGSATDFTLTISS
 PAN2D-Light-variable     (51) VSSSYLYWYQQKPGSSPKLWIYSTSNLASGVPARFSGSGSGTSYSLTISS
           Consensus      (51) V S YL WYQQKP  SPKL IY  SN  SGVP RFSGSGSAT FSLTISS
                            101                                 131
DF-200 light variable     (98) VQAEDLADYHCGQGYSYPYTFGGGTKLEIKR
 PAN2D-Light-variable     (101)MEAEDAATYYCHQYHRSPPTFGGGTKLEIKR
           Consensus      (101) M AED A YHC Q H  P TFGGGTKLEIKR
```

B

```
DF-200 light variable    (44) KASENVVI-YVS (SEQ ID NO.3)
 PAN2D-Light-variable    (46) TASSSVSSSYIY (SEQ ID NO.4)
           Consensus         AS  V S YL
```

C

```
DF-200 light variable    (70) GASNRYT (SEQ ID NO.5)
 PAN2D-Light-variable    (73) STSNLAS (SEQ ID NO.6)
           Consensus         SN  S
```

D

```
DF-200 light variable    (109) GQGYSYPYT (SEQ ID NO.7)
 PAN2D-Light-variable    (112) HQYHRSPPT (SEQ ID NO.8)
           Consensus          Q H  P T
```

FIGURE 13

DF200 VH

A

MAVLGLLFCLVTFPSCVLS

QVQLEQSGPGLVQPSQSLSITCTVSGFSFTPYGVHWVRQSPGKGLEWLGVIWSGGNTDY
NAAFISRLSINKDNSKSQVFFKMNSLQVNDTAIYYCARNPRPGNYPYGMDYWGQGTSVT
VSS (SEQ ID NO:9)

B

GFSFTPYGVH (SEQ ID NO:10)

C

VIWSGGNTDYNAAFIS (SEQ ID NO:11)

D

NPRPGNYPYGMDY (SEQ ID NO:12)

FIGURE 14

1-7F9 VL and VH

A

EIVLTQSPVTLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPARFSGSGSG
TDFTLTISSLEPEDFAVYYCQQRSNWMYTFGQGTKLEIKRT (SEQ ID NO:15)

B

gaaattgtgttgacacagtctccagtcaccctgtctttgtctccaggggaaagagccaccctctcctg
cagggccagtcagagtgttagcagctacttagcctggtaccaacagaaacctggccaggctcccaggc
tcctcatctatgatgcatccaacagggccactggcatcccagccaggttcagtggcagtgggtctggg
acagacttcactctcaccatcagcagcctagagcctgaagattttgcagtttattattgtcagcagcg
tagcaactggatgtacacttttggccaggggaccaagctggagatcaaacgaact (SEQ ID
NO:16)

C

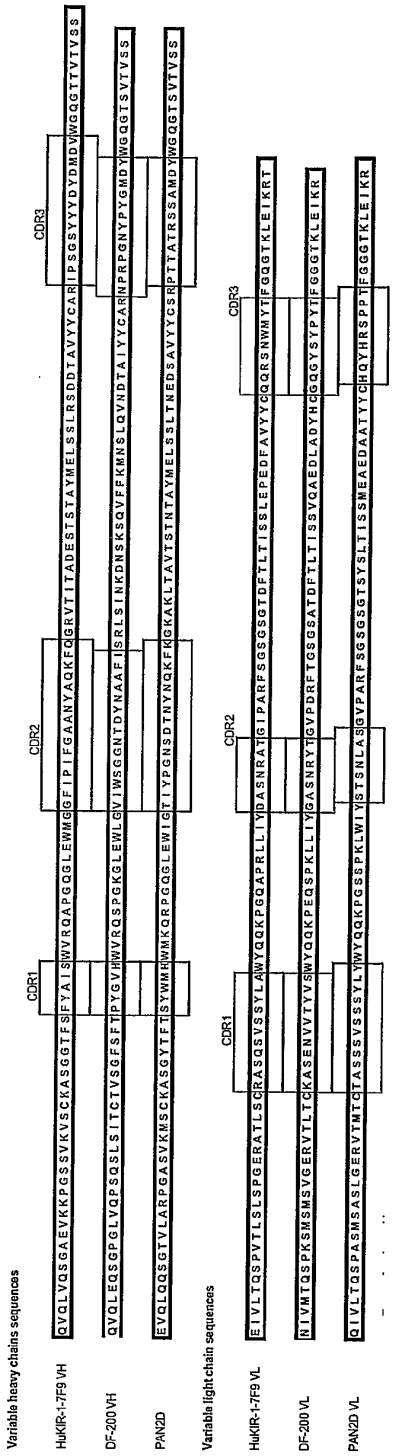
QVQLVQSGAEVKKPGSSVKVSCKASGGTFSFYAISWVRQAPGQGLEWMGGFIPIFGAANYAQKFQGRV
TITADESTSTAYMELSSLRSDDTAVYYCARIPSGSYYYDYDMDVWGQGTTVTVSS (SEQ ID
NO:17)

D

caggtccagctggtgcagtctggggctgaggtgaagaagcctgggtcctcggtgaaggtctcctgcaa
ggcttctggaggcaccttcagtttctatgctatcagctgggtgcgacaggcccctggacaagggcttg
agtggatgggagggttcatccctatctttggtgcagcaaactacgcacagaagttccagggcagagtc
acgattaccgcggacgaatccacgagcacagcctacatggaactgagcagcctgagatctgacgacac
ggccgtgtattactgtgcgagaatccctagtgggagctactactacgactacgatatggacgtctggg
gccaagggaccacggtcaccgtctcctca (SEQ ID NO:18)

15/20

FIGURE 15



16/20

FIGURE 16

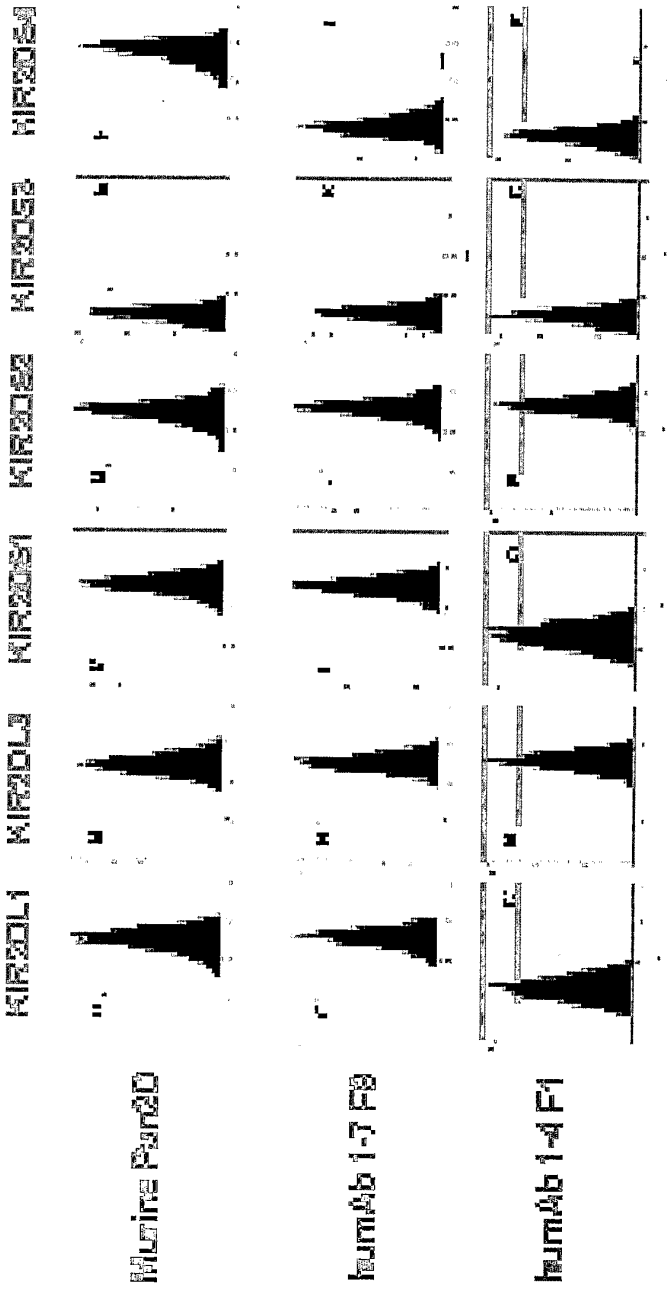
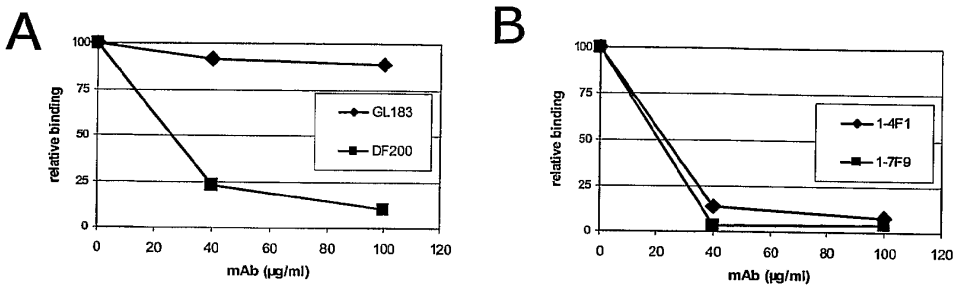


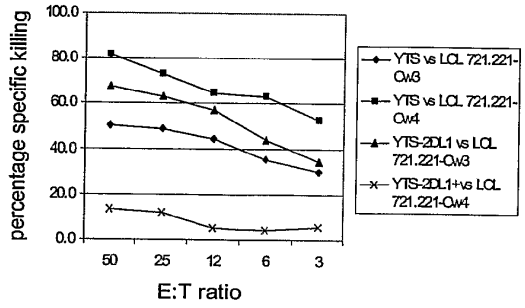
FIGURE 17


A
relative binding
100
75
50
25
0
0
20
40
60
80
100
120
mAb (µg/ml)
GL183
DF200
B
relative binding
100
75
50
25
0
0
20
40
60
80
100
120
mAb (µg/ml)
1-4F1
1-7F9

18/20

FIGURE 18

A



B

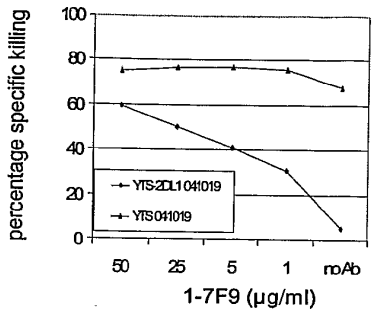


FIGURE 19

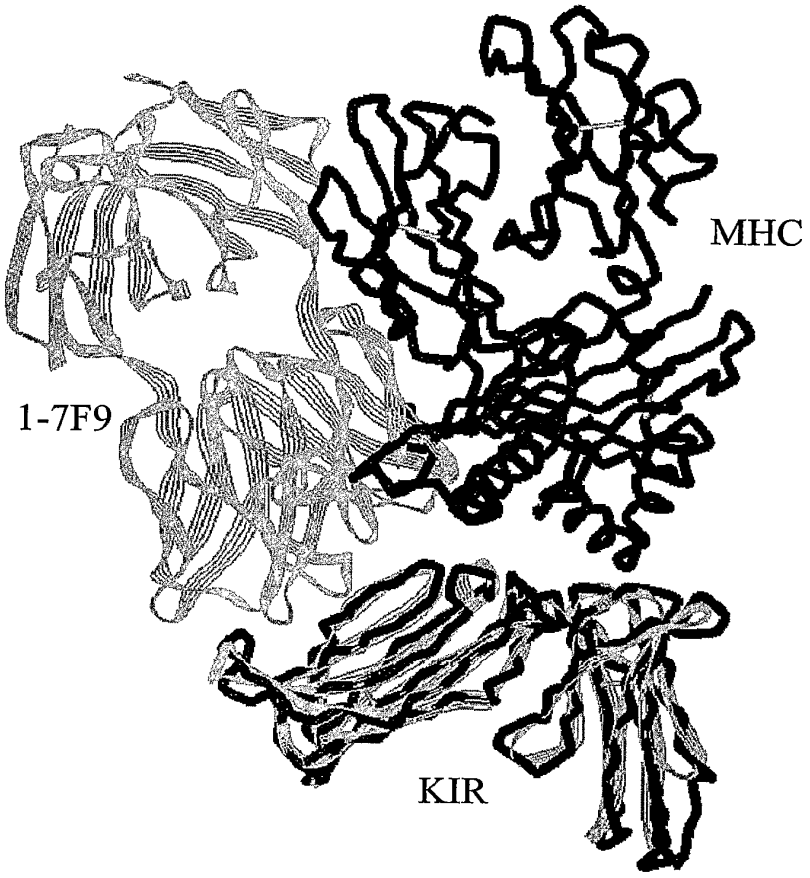


FIGURE 20

```
  1   HEGVHRKPSL LAHPGRLVKS EETVILQCWS DVMFEHFLLH REGMFNDTLR
 51   LRGEHHDGVS KANFSISRMT QDLAGTYRCY GSVTHSPYQV SAPSDPLDIV
101   IIGLYEKPSL SAQLGPTVLA GENVTLSCSS RSSYDMYHLS REGEAHERRL
151   PAGPKVNGTF QADFPLGPAT HGGTYRCFGS FHDSPYEWSK SSDPLLVSVT
201   GNPSNSWPSP TEPSSKTGNP RHLH
```

dwcgi-2004-1120031544-500.txt
SEQUENCE LISTING

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<120> HUMAN ANTI-KIR ANTIBODIES

<130> 7121.504-WO

<150> PCT/DK2004/000470
<151> 2004-07-01

<150> PA 2005 00025
<151> 2005-01-06

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<170> PatentIn version 3.3

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<212> PRT
<213> Mus musculus

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Met Glu Ser Gln Thr Leu Val Phe Ile Ser Ile Leu Leu Trp Leu Tyr
1 5 10 15

Gly Ala Asp Gly Asn Ile Val Met Thr Gln Ser Pro Lys Ser Met Ser
20 25 30

Met Ser Val Gly Glu Arg Val Thr Leu Thr Cys Lys Ala Ser Glu Asn
35 40 45

Val Val Thr Tyr Val Ser Trp Tyr Gln Gln Lys Pro Glu Gln Ser Pro
50 55 60

Lys Leu Leu Ile Tyr Gly Ala Ser Asn Arg Tyr Thr Gly Val Pro Asp
65 70 75 80

Arg Phe Thr Gly Ser Gly Ser Ala Thr Asp Phe Thr Leu Thr Ile Ser
85 90 95

Ser Val Gln Ala Glu Asp Leu Ala Asp Tyr His Cys Gly Gln Gly Tyr
100 105 110

Ser Tyr Pro Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg
115 120 125

<210> 2
<211> 131
<212> PRT
<213> Mus musculus

<400> 2

Met Asp Phe Gln Val Gln Ile Phe Ser Phe Leu Leu Ile Ser Ala Ser
1 5 10 15

Samsung Bioepis Exhibit No. 1002
Page 2388

Val Ile Met Ser Arg Gly Gln Ile Val Leu Thr Gln Ser Pro Ala Ser
20 25 30

Met Ser Ala Ser Leu Gly Glu Arg Val Thr Met Thr Cys Thr Ala Ser
35 40 45

Ser Ser Val Ser Ser Ser Tyr Leu Tyr Trp Tyr Gln Gln Lys Pro Gly
50 55 60

Ser Ser Pro Lys Leu Trp Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly
65 70 75 80

Val Pro Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu
85 90 95

Thr Ile Ser Ser Met Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys His
100 105 110

Gln Tyr His Arg Ser Pro Pro Thr Phe Gly Gly Gly Thr Lys Leu Glu
115 120 125

Ile Lys Arg
130

<210> 3
<211> 11
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<400> 3

Lys Ala Ser Glu Asn Val Val Thr Tyr Val Ser
1 5 10

<210> 4
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<400> 4

Thr Ala Ser Ser Ser Val Ser Ser Ser Tyr Leu Tyr
1 5 10

<210> 5
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<212> PRT
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Gly Ala Ser Asn Arg Tyr Thr
1 5

<210> 6
<211> 7
<212> PRT

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<400> 6

Ser Thr Ser Asn Leu Ala Ser
1 5

<210> 7
<211> 9
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Gly Gln Gly Tyr Ser Tyr Pro Tyr Thr
1 5

<210> 8
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<400> 8

His Gln Tyr His Arg Ser Pro Pro Thr
1 5

<210> 9
<211> 140
<212> PRT
<213> Mus musculus

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Met Ala Val Leu Gly Leu Leu Phe Cys Leu Val Thr Phe Pro Ser Cys
1 5 10 15

Val Leu Ser Gln Val Gln Leu Glu Gln Ser Gly Pro Gly Leu Val Gln
20 25 30

Pro Ser Gln Ser Leu Ser Ile Thr Cys Thr Val Ser Gly Phe Ser Phe
35 40 45

Thr Pro Tyr Gly Val His Trp Val Arg Gln Ser Pro Gly Lys Gly Leu
50 55 60

Glu Trp Leu Gly Val Ile Trp Ser Gly Gly Asn Thr Asp Tyr Asn Ala
65 70 75 80

Ala Phe Ile Ser Arg Leu Ser Ile Asn Lys Asp Asn Ser Lys Ser Gln
85 90 95

Val Phe Phe Lys Met Asn Ser Leu Gln Val Asn Asp Thr Ala Ile Tyr
100 105 110

Tyr Cys Ala Arg Asn Pro Arg Pro Gly Asn Tyr Pro Tyr Gly Met Asp
115 120 125

Tyr Trp Gly Gln Gly Thr Ser Val Thr Val Ser Ser
130 135 140

<210> 10
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Gly Phe Ser Phe Thr Pro Tyr Gly Val His
1 5 10

<210> 11
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<212> PRT
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<400> 11

Val Ile Trp Ser Gly Gly Asn Thr Asp Tyr Asn Ala Ala Phe Ile Ser
1 5 10 15

<210> 12
<211> 13
<212> PRT
<213> Mus musculus

<400> 12

Asn Pro Arg Pro Gly Asn Tyr Pro Tyr Gly Met Asp Tyr
1 5 10

<210> 13
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<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 13
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<211> 26
<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 14
cgggatccca ggtgtctggg gttacc 26

<210> 15
<211> 109
<212> PRT
<213> Homo sapiens

<400> 15

Glu Ile Val Leu Thr Gln Ser Pro Val Thr Leu Ser Leu Ser Pro Gly
1 5 10 15

Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser Ser Tyr
20 25 30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile
35 40 45

Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro
65 70 75 80

Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser Asn Trp Met Tyr
85 90 95

Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys Arg Thr
100 105

<210> 16
<211> 327
<212> DNA
<213> Homo sapiens

<400> 16
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ggccaggctc ccaggctcct catctatgat gcatccaaca gggccactgg catcccagcc 180

aggttcagtg gcagtgggtc tgggacagac ttcactctca ccatcagcag cctagagcct 240

gaagattttg cagtttatta ttgtcagcag cgtagcaact ggatgtacac ttttggccag 300

gggaccaagc tggagatcaa acgaact 327

<210> 17
<211> 123
<212> PRT
<213> Homo sapiens

<400> 17

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser
1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Gly Thr Phe Ser Phe Tyr
20 25 30

Ala Ile Ser Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
35 40 45

Gly Gly Phe Ile Pro Ile Phe Gly Ala Ala Asn Tyr Ala Gln Lys Phe

50 55 60

Gln Gly Arg Val Thr Ile Thr Ala Asp Glu Ser Thr Ser Thr Ala Tyr
65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Ile Pro Ser Gly Ser Tyr Tyr Tyr Asp Tyr Asp Met Asp Val
100 105 110

Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
115 120

<210> 18
<211> 369
<212> DNA
<213> Homo sapiens

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cctggacaag ggcttgagtg gatgggaggg ttcatcccta tctttggtgc agcaaactac 180

gcacagaagt tccagggcag agtcacgatt accgcggacg aatccacgag cacagcctac 240

atggaactga gcagcctgag atctgacgac acggccgtgt attactgtgc gagaatccct 300

agtgggagct actactacga ctacgatatg gacgtctggg gccaagggac cacggtcacc 360

gtctcctca 369

<210> 19
<211> 121
<212> PRT
<213> Mus musculus

<400> 19

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Ser Leu Ser Ile Thr Cys Thr Val Ser Gly Phe Ser Phe Thr Pro Tyr
20 25 30

Gly Val His Trp Val Arg Gln Ser Pro Gly Lys Gly Leu Glu Trp Leu
35 40 45

Gly Val Ile Trp Ser Gly Gly Asn Thr Asp Tyr Asn Ala Ala Phe Ile
50 55 60

Ser Arg Leu Ser Ile Asn Lys Asp Asn Ser Lys Ser Gln Val Phe Phe
65 70 75 80

Lys Met Asn Ser Leu Gln Val Asn Asp Thr Ala Ile Tyr Tyr Cys Ala

85 90 95

Arg Asn Pro Arg Pro Gly Asn Tyr Pro Tyr Gly Met Asp Tyr Trp Gly
100 105 110

Gln Gly Thr Ser Val Thr Val Ser Ser
115 120

<210> 20
<211> 121
<212> PRT
<213> Mus musculus

<400> 20

Glu Val Gln Leu Gln Gln Ser Gly Thr Val Leu Ala Arg Pro Gly Ala
1 5 10 15

Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
20 25 30

Trp Met His Trp Met Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
35 40 45

Gly Thr Ile Tyr Pro Gly Asn Ser Asp Thr Asn Tyr Asn Gln Lys Phe
50 55 60

Lys Gly Lys Ala Lys Leu Thr Ala Val Thr Ser Thr Asn Thr Ala Tyr
65 70 75 80

Met Glu Leu Ser Ser Leu Thr Asn Glu Asp Ser Ala Val Tyr Tyr Cys
85 90 95

Ser Arg Pro Thr Thr Ala Thr Arg Ser Ser Ala Met Asp Tyr Trp Gly
100 105 110

Gln Gly Thr Ser Val Thr Val Ser Ser
115 120

<210> 21
<211> 108
<212> PRT
<213> Mus musculus

<400> 21

Asn Ile Val Met Thr Gln Ser Pro Lys Ser Met Ser Met Ser Val Gly
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Glu Arg Val Thr Leu Thr Cys Lys Ala Ser Glu Asn Val Val Thr Tyr
20 25 30

Val Ser Trp Tyr Gln Gln Lys Pro Glu Gln Ser Pro Lys Leu Leu Ile
35 40 45

Tyr Gly Ala Ser Asn Arg Tyr Thr Gly Val Pro Asp Arg Phe Thr Gly
50 55 60

Ser Gly Ser Ala Thr Asp Phe Thr Leu Thr Ile Ser Ser Val Gln Ala
65 70 75 80

Glu Asp Leu Ala Asp Tyr His Cys Gly Gln Gly Tyr Ser Tyr Pro Tyr
85 90 95

Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg
100 105

<210> 22
<211> 109
<212> PRT
<213> Mus musculus

<400> 22

Gln Ile Val Leu Thr Gln Ser Pro Ala Ser Met Ser Ala Ser Leu Gly
1 5 10 15

Glu Arg Val Thr Met Thr Cys Thr Ala Ser Ser Ser Val Ser Ser Ser
20 25 30

Tyr Leu Tyr Trp Tyr Gln Gln Lys Pro Gly Ser Ser Pro Lys Leu Trp
35 40 45

Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly Val Pro Ala Arg Phe Ser
50 55 60

Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Ser Met Glu
65 70 75 80

Ala Glu Asp Ala Ala Thr Tyr Tyr Cys His Gln Tyr His Arg Ser Pro
85 90 95

Pro Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg
100 105

<210> 23
<211> 224
<212> PRT
<213> Homo sapiens

<220>
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<223> Xaa is Pro or Leu

<220>
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<220>
<221> MISC_FEATURE
<222> (114)..(114)
<223> Xaa is Pro or Leu

<400> 23

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His Glu Gly Val His Arg Lys Pro Ser Leu Leu Ala His Pro Gly Xaa
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Leu Val Lys Ser Glu Glu Thr Val Ile Leu Gln Cys Trp Ser Asp Val
            20                  25                  30

Met Phe Glu His Phe Leu Leu His Arg Glu Gly Met Phe Asn Asp Thr
        35                  40                  45

Leu Arg Leu Ile Gly Glu His His Asp Gly Val Ser Lys Ala Asn Phe
    50                  55                  60

Ser Ile Ser Arg Met Thr Gln Asp Leu Ala Gly Thr Tyr Arg Cys Tyr
65                  70                  75                  80

Gly Ser Val Thr His Ser Pro Tyr Gln Val Ser Ala Pro Ser Asp Pro
                85                  90                  95

Leu Asp Ile Val Ile Ile Gly Leu Tyr Glu Lys Pro Ser Leu Ser Ala
            100                 105                 110

Gln Xaa Gly Pro Thr Val Leu Ala Gly Glu Asn Val Thr Leu Ser Cys
        115                 120                 125

Ser Ser Arg Ser Ser Tyr Asp Met Tyr His Leu Ser Arg Glu Gly Glu
    130                 135                 140

Ala His Glu Arg Arg Leu Pro Ala Gly Pro Lys Val Asn Gly Thr Phe
145                 150                 155                 160

Gln Ala Asp Phe Pro Leu Gly Pro Ala Thr His Gly Gly Thr Tyr Arg
                165                 170                 175

Cys Phe Gly Ser Phe His Asp Ser Pro Tyr Glu Trp Ser Lys Ser Ser
            180                 185                 190

Asp Pro Leu Leu Val Ser Val Thr Gly Asn Pro Ser Asn Ser Trp Pro
        195                 200                 205

Ser Pro Thr Glu Pro Ser Ser Lys Thr Gly Asn Pro Arg His Leu His
    210                 215                 220
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<210> 24
<211> 224
<212> PRT
<213> Homo sapiens

<400> 24

His Glu Gly Val His Arg Lys Pro Ser Leu Leu Ala His Pro Gly Arg
1 5 10 15

Leu Val Lys Ser Glu Glu Thr Val Ile Leu Gln Cys Trp Ser Asp Val
20 25 30

Arg Phe Glu His Phe Leu Leu His Arg Glu Gly Lys Phe Lys Asp Thr
35 40 45

Leu His Leu Ile Gly Glu His His Asp Gly Val Ser Lys Ala Asn Phe
50 55 60

Ser Ile Gly Pro Met Met Gln Asp Leu Ala Gly Thr Tyr Arg Cys Tyr
65 70 75 80

Gly Ser Val Thr His Ser Pro Tyr Gln Leu Ser Ala Pro Ser Asp Pro
85 90 95

Leu Asp Ile Val Ile Thr Gly Leu Tyr Glu Lys Pro Ser Leu Ser Ala
100 105 110

Gln Pro Gly Pro Thr Val Leu Ala Gly Glu Ser Val Thr Leu Ser Cys
115 120 125

Ser Ser Arg Ser Ser Tyr Asp Met Tyr His Leu Ser Arg Glu Gly Glu
130 135 140

Ala His Glu Cys Arg Phe Ser Ala Gly Pro Lys Val Asn Gly Thr Phe
145 150 155 160

Gln Ala Asp Phe Pro Leu Gly Pro Ala Thr His Gly Gly Thr Tyr Arg
165 170 175

Cys Phe Gly Ser Phe Arg Asp Ser Pro Tyr Glu Trp Ser Asn Ser Ser
180 185 190

Asp Pro Leu Leu Val Ser Val Ile Gly Asn Pro Ser Asn Ser Trp Pro
195 200 205

Ser Pro Thr Glu Pro Ser Ser Lys Thr Gly Asn Pro Arg His Leu His
210 215 220

<210> 25
<211> 224
<212> PRT
<213> Homo sapiens

<400> 25

His Glu Gly Val His Arg Lys Pro Ser Leu Leu Ala His Pro Gly Pro
1 5 10 15

Leu Val Lys Ser Glu Glu Thr Val Ile Leu Gln Cys Trp Ser Asp Val
20 25 30

Arg Phe Gln His Phe Leu Leu His Arg Glu Gly Lys Phe Lys Asp Thr
35 40 45

Leu His Leu Ile Gly Glu His His Asp Gly Val Ser Lys Ala Asn Phe
50 55 60

Ser Ile Gly Pro Met Met Gln Asp Leu Ala Gly Thr Tyr Arg Cys Tyr
65 70 75 80

Gly Ser Val Thr His Ser Pro Tyr Gln Leu Ser Ala Pro Ser Asp Pro
85 90 95

Leu Asp Ile Val Ile Thr Gly Leu Tyr Glu Lys Pro Ser Leu Ser Ala
100 105 110

Gln Pro Gly Pro Thr Val Leu Ala Gly Glu Ser Val Thr Leu Ser Cys
115 120 125

Ser Ser Arg Ser Ser Tyr Asp Met Tyr His Leu Ser Arg Glu Gly Glu
130 135 140

Ala His Glu Arg Arg Phe Ser Ala Gly Pro Lys Val Asn Gly Thr Phe
145 150 155 160

Gln Ala Asp Phe Pro Leu Gly Pro Ala Thr His Gly Gly Thr Tyr Arg
165 170 175

Cys Phe Gly Ser Phe Arg Asp Ser Pro Tyr Glu Trp Ser Asn Ser Ser
180 185 190

Asp Pro Leu Leu Val Ser Val Thr Gly Asn Pro Ser Asn Ser Trp Pro
195 200 205

Ser Pro Thr Glu Pro Ser Ser Glu Thr Gly Asn Pro Arg His Leu His
210 215 220

<210> 26
<211> 45
<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 26
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<210> 27
<211> 21
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<213> Artificial

<220>
<223> Primer

<400> 27
ctaatacgac tcactatagg g 21

<210> 28
<211> 32
<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 28
gcaggcacac aacagaggca gttccagatt tc 32

<210> 29
<211> 45
<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 29
ctaatacgac tcactatagg gcaagcagtg gtatcaacgc agagt 45

<210> 30
<211> 21
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<213> Artificial

<220>
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<400> 30
ctaatacgac tcactatagg g 21

<210> 31
<211> 23
<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 31
gtgccagggg gaagaccgat ggg 23

<210> 32
<211> 16
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<213> Artificial

<220>
<223> Primer

<400> 32
gtaaaacgac ggccag 16

<210> 33
<211> 17
<212> DNA
<213> Artificial

<220>
<223> Primer

<400> 33
caggaaacag ctatgac 17

<210> 34
<211> 108
<212> PRT
<213> Homo sapiens

<400> 34

Val Val Met Thr Gln Ser Pro Ser Thr Leu Ser Ala Ser Val Gly Asp
1 5 10 15

Thr Ile Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Glu Thr Trp Leu
20 25 30

Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr
35 40 45

Lys Ala Ser Thr Leu Lys Thr Gly Val Pro Ser Arg Phe Ser Gly Ser
50 55 60

Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Gly Leu Gln Phe Asp
65 70 75 80

Asp Phe Ala Thr Tyr His Cys Gln His Tyr Ala Gly Tyr Ser Ala Thr
85 90 95

Phe Gly Gln Gly Thr Arg Val Glu Ile Lys Arg Thr
100 105

<210> 35
<211> 119
<212> PRT
<213> Homo sapiens

<400> 35

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Lys Ala Gly Gly
1 5 10 15

Ser Leu Ile Leu Ser Cys Gly Val Ser Asn Phe Arg Ile Ser Ala His
20 25 30

Thr Met Asn Trp Val Arg Arg Val Pro Gly Gly Gly Leu Glu Trp Val
35 40 45

Ala Ser Ile Ser Thr Ser Ser Thr Tyr Arg Asp Tyr Ala Asp Ala Val

Samsung Bioepis Exhibit No. 1002
Page 2400

50 55 60

Lys Gly Arg Phe Thr Val Ser Arg Asp Asp Leu Glu Asp Phe Val Tyr
65 70 75 80

Leu Gln Met His Lys Met Arg Val Glu Asp Thr Ala Ile Tyr Tyr Cys
85 90 95

Ala Arg Lys Gly Ser Asp Arg Leu Ser Asp Asn Asp Pro Phe Asp Ala
100 105 110

Trp Gly Pro Gly Thr Val Val
115

<210> 36
<211> 214
<212> PRT
<213> Homo sapiens

<400> 36

Glu Ile Val Leu Thr Gln Ser Pro Val Thr Leu Ser Leu Ser Pro Gly
1 5 10 15

Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser Ser Tyr
20 25 30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile
35 40 45

Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro
65 70 75 80

Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser Asn Trp Met Tyr
85 90 95

Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys Arg Thr Val Ala Ala
100 105 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
115 120 125

Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
130 135 140

Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
145 150 155 160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
165 170 175

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Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
            180                 185                 190

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
        195                 200                 205

Phe Asn Arg Gly Glu Cys
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<212> PRT
<213> Homo sapiens

<400> 37

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Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser
1               5                   10                  15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Gly Thr Phe Ser Phe Tyr
            20                  25                  30

Ala Ile Ser Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
        35                  40                  45

Gly Gly Phe Ile Pro Ile Phe Gly Ala Ala Asn Tyr Ala Gln Lys Phe
    50                  55                  60

Gln Gly Arg Val Thr Ile Thr Ala Asp Glu Ser Thr Ser Thr Ala Tyr
65                  70                  75                  80

Met Glu Leu Ser Ser Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Ile Pro Ser Gly Ser Tyr Tyr Tyr Asp Tyr Asp Met Asp Val
            100                 105                 110

Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Ala Ser Thr Lys Gly
        115                 120                 125

Pro Ser Val Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser
    130                 135                 140

Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val
145                 150                 155                 160

Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe
                165                 170                 175

Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
            180                 185                 190
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Thr Val Pro Ser Ser Ser Leu Gly Thr Lys Thr Tyr Thr Cys Asn Val
        195                 200                 205

Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys Arg Val Glu Ser Lys
    210                 215                 220

Tyr Gly Pro Pro Cys Pro Ser Cys Pro Ala Pro Glu Phe Leu Gly Gly
225                 230                 235                 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
                245                 250                 255

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser Gln Glu
            260                 265                 270

Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu Val His
        275                 280                 285

Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr Tyr Arg
    290                 295                 300

Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys
305                 310                 315                 320

Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro Ser Ser Ile Glu
                325                 330                 335

Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr
            340                 345                 350

Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val Ser Leu
        355                 360                 365

Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp
    370                 375                 380

Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val
385                 390                 395                 400

Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr Val Asp
                405                 410                 415

Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val Met His
            420                 425                 430

Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Leu
        435                 440                 445

Gly Lys
    450
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<210> 38
<211> 100
<212> PRT
<213> Homo sapiens

<400> 38

Gln Glu Gly Val His Arg Lys Pro Ser Phe Leu Ala Leu Pro Gly His
1 5 10 15

Leu Val Lys Ser Glu Glu Thr Val Ile Leu Gln Cys Trp Ser Asp Val
20 25 30

Met Phe Glu His Phe Leu Leu His Arg Glu Gly Lys Phe Asn Asn Thr
35 40 45

Leu His Leu Ile Gly Glu His His Asp Gly Val Ser Lys Ala Asn Phe
50 55 60

Ser Ile Gly Pro Met Met Pro Val Leu Ala Gly Thr Tyr Arg Cys Tyr
65 70 75 80

Gly Ser Val Pro His Ser Pro Tyr Gln Leu Ser Ala Pro Ser Asp Pro
85 90 95

Leu Asp Met Val
100

<210> 39
<211> 109
<212> PRT
<213> Homo sapiens

<220>
<221> MISC_FEATURE
<222> (1)..(109)
<223> Xaa 3 is Q or R. Xaa 4 is L or M. Xaa 9 is S or F. Xaa 24 is R or W. Xaa 32 is A or Y. Xaa 41 is G or A. Xaa 47 is L or F. Xaa 50 is D or Y. Xaa 55 is E or Q. Xaa 71 is F or Y. Xaa 74 is A or T.

<400> 39

Ala Ile Xaa Xaa Thr Gln Ser Pro Xaa Ser Leu Ser Ala Ser Val Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Xaa Xaa Ala Ser Gln Gly Ile Ser Ser Xaa
20 25 30

Leu Ala Trp Tyr Gln Gln Lys Pro Xaa Lys Ala Pro Lys Leu Xaa Ile
35 40 45

Tyr Xaa Ala Ser Ser Leu Xaa Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Xaa Thr Leu Xaa Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Tyr Ser Thr Pro Leu
85 90 95

Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys Arg Thr
100 105

<210> 40
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<212> DNA
<213> Homo sapiens

<220>
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<222> (1)..(153)
<223> n8 is a or g. n10 is t or a. n26 is c or t. n70 is c or t. n75 is a or c. n94 is g or t. n95 is c or a. n114 is g or a. n122 is g or c. n123 is g or a. n129 is t or c. n139 is c or t. n141 is g or c. n148 is g or t. n153 is c or a.

<220>
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<222> (154)..(327)
<223> n162 is g or a. n163 is g or c. n207 is a or g. n212 is t or a. n220 is g or a.

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atcacttgcn gggcnagtca gggcattagc agtnntttag cctggtatca gcanaaacca 120

gnnaaagcnc ctaagctcnt natctatnat gcntccagtt tnnaaagtgg ggtcccatca 180

aggttcagcg gcagtggatc tgggacngat tncactctcn ccatcagcag cctgcagcct 240

gaagattttg caacttatta ctgtcaacag tattatagta ccccgctcac tttcggcgga 300

gggaccaagg tggagatcaa acgaact 327

<210> 41
<211> 124
<212> PRT
<213> Homo sapiens

<400> 41

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser
1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Gly Thr Ser Asn Ser Tyr
20 25 30

Ser Ile Asn Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
35 40 45

Gly Gly Ile Ile Pro Ile Phe Gly Thr Ala Asn Tyr Ala Gln Lys Phe
50 55 60

Gln Gly Arg Val Thr Ile Ser Ala Asp Glu Ser Thr Arg Thr Val Tyr
65 70 75 80

Met Glu Leu Asn Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Gly Tyr Tyr Asp Ile Phe Lys Asp Tyr Tyr Tyr Gly Met Asp
100 105 110

Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
115 120

<210> 42
<211> 372
<212> DNA
<213> Homo sapiens

<400> 42
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tcctgcaagg cttctggagg cacctccaac agctattcta ttaactgggt gcgacaggcc 120

cctggacaag ggcttgagtg gatgggaggg atcatcccta tatttggtac agcaaactac 180

gcacagaagt tccagggcag agtcacgatt tccgcggacg aatccacgcg cacagtctac 240

atggagctga acagtctgag atctgaggat acggccgtgt attactgtgc gagaggatat 300

tacgatattt tcaaggacta ctattacggt atggacgtct ggggccaagg gaccacggtc 360

accgtctcct ca 372

Immunoglobulin Light Chain Variable Region Gene Sequences for Human Antibodies to *Haemophilus influenzae* Type b Capsular Polysaccharide Are Dominated by a Limited Number of V_κ and V_λ Segments and VJ Combinations

Elisabeth E. Adderson,* Penelope G. Shackelford,* Richard A. Insel,§ Anthony Quinn,* Patricia M. Wilson,‡ and William L. Carroll‡

*Division of Infectious Diseases and ‡Hematology Oncology, Mallinckrodt Department of Pediatrics, Washington University School of Medicine, St. Louis Children's Hospital, St. Louis, Missouri 63110; and §Department of Pediatrics, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

Abstract

The immune repertoire to *Haemophilus influenzae* type b capsular polysaccharide (Hib PS) appears to be dominated by certain light chain variable region genes (IgV_L). In order to examine the molecular basis underlying light chain bias, IgV_L genes have been cloned from a panel of heterohybridomas secreting human anti-Hib PS (antibody) (anti-Hib PS Ab). One hybridoma, representative of the predominant serum clonotype of anti-Hib PS Ab in older children and adults following immunization or Hib infection, uses a V_κII segment identical to the germline gene A2, and a JK3 segment. A second kappa hybridoma uses a member of the V_κI family and a JK4 segment. Four lambda antibodies, all cross-reactive with the structurally related antigen *Escherichia coli* K100 PS, use V_λVII segments which are 96–98% homologous to one another, and may originate from a single germline gene. Two additional lambda antibodies, not K100-cross-reactive, are encoded by members of the V_λII family. All lambda antibodies use highly homologous J_λ2 or J_λ3 segments. The VJ joints of all lambda antibodies and the V_κII-encoded antibody are notable for the presence of an arginine codon, suggesting an important role in antigen binding. Although more complex than heavy chain variable region gene usage, a significant portion of serum anti-Hib PS Ab is likely to be encoded by a limited number of V_κ and V_λ segments and VJ combinations, which may be selectively expressed during development, or following antigen exposure. (*J. Clin. Invest.* 1992: 89:729–738.) Key words: B-cell repertoire • somatic mutation • gene rearrangement • carbohydrate antigen

Introduction

The development of the antibody repertoire involves the recombination of individual members of two or three groups of germline segments, nucleotide insertion or deletion at these junctions, and somatic mutation of rearranged genes (1–4). The individual contributions of these molecular mechanisms to the generation of the immune repertoire against a specific antigen has been extensively studied in murine systems, but it is uncertain whether these findings can be extrapolated to humans. The chromosomal organization of the human heavy and light chain variable region segments within the immunoglobulin loci differ from those of mice (5–7). Furthermore, the response of highly inbred laboratory animal strains, and the use of simple haptens in most studies, may not be comparable with the human response to clinically relevant antigens, such as those presented by microbial pathogens.

The immune response to *Haemophilus influenzae* type b capsular polysaccharide (Hib PS)[1] is an excellent model to examine the immune repertoire to a functionally significant antigen. Hib is a major bacterial pathogen in young children and antibody directed against the capsular polysaccharide, a repetitive polymer of polyribosylribitol phosphate, protects against invasive infection (8). Similar to the response to other polysaccharide antigens, the immune response to Hib PS is age-dependent. Children under 5 yr have lower antibody responses and are at greatest risk of infection. Additionally, there appear to be variations in individual and ethnic susceptibilities to invasive Hib disease (9–11). Analysis of the immune response to Hib PS will contribute to our understanding of the development of the human antibody repertoire, and provide insight into age-related and possible genetic predisposition to infection caused by Hib.

Previous studies employing partial amino acid sequencing of anti-Hib PS serum antibody purified to clonality have shown that light chains of anti-Hib PS antibody are encoded by V_λ, and at least three V_κ gene families (12). Although highly informative, these studies have inherent technologic limitations. To obtain sufficient antibody for analysis, only individuals with antibody concentrations much higher than those elicited in young infants could be studied (12). Amino acid sequences were unobtainable on V_λ-encoded antibodies, and certain regions of V_κ-encoded antibodies (12, 13). Obtaining nucleic acid sequences of variable region genes encoding anti-Hib PS Abs would permit precise comparison of variable region genes obtained from different individuals, allowing classification of these genes into germline families and subfamilies, analysis of D- and J-segment usage, and assessment of the role of somatic mutation in the clonal evolution of this immune response. Additionally, this information could be applied to examination of the germline and expressed immune repertoire

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Address correspondence and reprint requests to Dr. William L. Carroll, Program in Human Molecular Biology and Genetics, University of Utah, Bldg. 533, Salt Lake City, UT 84112.

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1. *Abbreviations used in this paper:* Ars, anti-p-azophenylarsonate; Hib, Haemophilus influenzae type b; Hib PS, Hib capsular polysaccharide; IgV_L, light chain variable region gene; PCR, polymerase chain reaction; SSC, standard saline citrate.

Samsung Bioepis Exhibit No. 1002
Page 2407

of individuals of various ages, and populations at high risk for invasive Hib infection.

Our previous work, and that of others, indicated that the heavy chains of anti-Hib PS Abs are encoded by a very restricted number of V_HIII gene segments, in combination with a variety of D and J_H segments (12, 14). We have extended our studies to examine light chain variable region gene (IgV_L) usage in the human anti-Hib PS response. A major component of serum anti-Hib PS Ab expresses a cross-reactive idiotype associated with the use of a V_κII gene (15, 16). Antibodies expressing this light chain account for the majority of anti-Hib PS antibody in older infants and adults following invasive Hib disease or immunization. Most lambda-encoded anti-Hib antibodies and some kappa-encoded (V_κI and V_κIII) anti-Hib PS Abs cross-react with the capsular polysaccharide of *E. coli* K100 (12, 17). Naturally acquired antibody of the majority of children expresses K100 PS cross-reactivity, and thus, colonization with this organism may provide protective immunity before exposure to Hib (18). Our results indicate that two important anti-Hib PS light chains, one associated with V_κII expression and the other with lambda antibodies cross-reactive with K100 PS, are encoded by two germline elements that may be expressed with little or no somatic mutation. Moreover, a nongermline-encoded arginine at the VJ joint is seen in the majority of anti-Hib PS Ab light chains, a result suggesting an essential role in antigen recognition.

Methods

Hybridomas. Heterohybridomas secreting human monoclonal anti-Hib PS antibody were obtained, as previously described (14). Volunteers were immunized with either plain Hib-PS vaccine (Praxis Biologicals, Rochester, NY) or Hib-PS diphtheria toxoid conjugate vaccine (Hib-PS-D) (Connaught Laboratories, Swiftwater, PA). 7 d following immunization, peripheral blood lymphocytes were harvested and fused to the nonsecreting mouse myeloma SP2/O-Ag14. Anti-Hib PS Ab was detected by binding to Hib PS in an ELISA, as previously described (14). Specificity was documented by antibody binding to ^{125}I-labeled antigen (19), and by inhibition of binding by 1.25 μg/ml of soluble Hib PS in an ELISA (20). Cross-reactivity with *E. coli* K100 PS was assayed by inhibition of binding to Hib PS-poly-L-lysine with 100 μg/ml of soluble K100 PS in an ELISA. An antibody concentration was selected that gave half-maximum absorbance in the ELISA. Greater than 15% inhibition was considered positive. The *E. coli* K100 PS was prepared from *E. coli* strain Easter, by the same method used to prepare Hib PS (19). The generation of hybridoma line 16M3C8 was described previously (21).

Cloning and sequencing of IgV_L genes. Total RNA was prepared from 10^6 hybridoma cells using the guanidine isothiocyanate method (22). 2 to 8 μg of total RNA served as template for first-strand cDNA synthesis, as previously described (14). 10 pmol of a consensus antisense primer corresponding to conserved sequences within the human kappa or lambda constant region (23) (C_κ codons 181-172:5′CAGCGTCAAGCTTCTGCTGTGGCTGTAGGT 3′, C_λ codons 171-181:5′CAGGCTCAGGAAGCTTCTGGCCGCGTACTTGTT 3′) was used to initiate cDNA synthesis by avian myeloblastosis virus (AMV) reverse transcriptase (RT; Life Sciences, St. Petersburg, FL). One half (10 μl) of the cDNA reaction was directly diluted into polymerase chain reactions (PCR), (24).

PCR conditions were as previously described, except that 70 pmol of an internal constant region consensus primer (C_κ codons 125-117:5′CAACGGATCCTCTGATGGCGGGAAGAT 3′, C_λ codons 125-117:5′TGGGGATCCAGCTCCTCAGAGGAGGG 3′) and 70 pmol of a degenerate 3′ kappa or lambda leader sequence primer (kappa:5′GGGAATTCATGGACATG(GA)(GA)(GA)(GAT)(TC)CC-(ACT)(GAC)G(TC)(GT)CA(GC)CTT 3′, lambda 5′GGGAATTCATG(GA)CCTG(GC)(AT)C(TC)CCTCTC(TC)T(TC)CT(GC)(AT)-(TC)C 3′) were used to amplify rearranged variable region genes (14, 25).

PCR products were isolated from low melting-point agarose gels (FMC Bioproducts, Rockland, ME). Artificial restriction enzymes sites in each primer allowed directional cloning into M13mp18 and M13mp19 phage vectors (Boehringer Mannheim Corp., Indianapolis, IN). Sequencing of V_L genes was performed by the dideoxy technique (26). Two to five positive clones were obtained and sequenced for each hybridoma gene.

The 16M3C8 light chain was generated by amplification of first-strand cDNA, with primers corresponding to amino acid positions −7 of the leader, to position 6 of the lambda light chain (5′CTGCACAGGGTCCTGGGCCGAGCTCGTGGTGACTCA 3′), and the C_λ constant region (antisense, 5′GCATTCTAGACTATTATGAACATTCTGTAGGGGC3′). Blunt-ended PCR products were cloned into SmaI cut M13 mp18 and M13mp19 and sequencing was also performed by the dideoxy method.

Southern blot analysis of genomic DNA. Peripheral blood was obtained from 10 unrelated healthy adult volunteers, from L.S. (the donor of hybridoma LSF2), and from a parent of L.S. Lymphocytes were separated on a Ficoll-Hypaque gradient, and genomic DNA was prepared by Proteinase K digestion (27). 10 μg of each subject's genomic DNA was digested with BamHI (4 U/μg DNA) (Boehringer Mannheim Corp.) at 37°C for 16 h, according to manufacturer's specifications. Digested DNA was separated by electrophoresis in a 0.8% agarose gel using TAE buffer (0.04 M Tris-acetate/0.001 M EDTA), and was transferred to Duralon UV membranes (Stratagene Inc., La Jolla, CA), using a positive pressure blotting apparatus (Posiblotter; Stratagene, Inc.). To examine germline representation of anti-Hib PS Ig genes, genomic DNA was hybridized with a 320-bp EcoRI/SmaI V_λ segment from hybridoma LSF2 that lacked the associated J_λ and C_λ regions. Probe was radiolabeled to high specific activity using T7 DNA polymerase (28). Prehybridization and hybridizations were carried out in 5× standard saline citrate (SSC), 5× Denhardt's solution, and 200 μg/ml sheared and denatured salmon sperm DNA (Boehringer Mannheim Corp.) at 42°C overnight. Filters were washed with 2× SSC, 0.2% SDS at room temperature twice, then with 0.2× SSC, 0.2% SDS at 55 to 60°C twice, and exposed to X-OMAT AR film (Eastman Kodak Co., Rochester, NY) for 2–7 d.

In order to further localize germline candidates within V region families, an oligonucleotide probe corresponding to conserved sequences within the CDR-1 region (sense oligonucleotide: 5′GGCTCCAGCACTGGAGATGTCACCAGT 3′, antisense oligonucleotide: 5′GTAGGGATAATGACCACTGGTGACATCTCC 3′) was hybridized to genomic DNA using the method described by Hellman (29). 15 μg of BamHI-digested human genomic DNA was separated by electrophoresis in 0.8% agarose gel using TBE buffer (0.09 M Tris-borate/0.002 M EDTA). Agarose gels were dried under vacuum and hybridized in 5× saline/sodium phosphate/EDTA (SSPE), 0.1% SDS, and 10^6 μg/ml of sheared and denatured salmon sperm DNA with 2×10^6 cpm of probe/ml of hybridization solution at 60°C for 16 h (30, 31). Hybridization conditions were previously adjusted to minimize nonspecific binding. Gels were washed with 6× SSC at room temperature for 20 min twice, followed by a 4-h room temperature wash. The gel was finally washed in 6× SSC for 3 min at 64°C, and exposed to X-OMAT AR film for 4 d.

Results

Hybridomas

Eight hybridoma cell lines from seven individuals were initially selected from 20 cell lines. All individuals demonstrated substantial increases in serum anti-Hib PS Ab 1 mo after vaccination (Table I). Hybridomas were selected to exemplify serologic characteristics (kappa or lambda expression, and K100 PS

Samsung Bioepis Exhibit No. 1002
Page 2408

*Table I. Summary of Hybridoma Cell Lines**

Hybridoma	Isotype	Age	Vaccine Form	K100 Inh of mAb	Serum Anti-Hib PS Ab: Total Ab Pre	Total Ab Post	λK100 Inh Pre	λK100 Inh Post
RC3	IgA/k	Adult	PS-D	−	1.1	300	11	11
ED6.1	IgG_2/k	4 yr	PS-D	−	0.8	22	0	0
SB5/D6	IgA_1/λ	Adult	PS-D	+	2.0	688	30	27
RAY4	IgA_2/λ	Adult	PS-D	+	1.9	28	0	42
LSF2	IgA_1/λ	Adult	PS	+	ND	138	ND	29
JB32	IgA_1/λ	11 yr	PS	+	0.3	125	19	33
JB21	IgG_2/λ	11 yr	PS	−	0.3	125	19	33
16M3C8	IgG_2/λ	Adult‡	PS	−	ND	ND	ND	ND

* Columns show name of hybridoma cell line, isotype of anti-Hib PS Ab secreted, age of subject at immunization, type of vaccine (PS is plain polysaccharide, PS-D is Hib PS covalently linked to diphtheria toxoid), presence (+) or absence (−) of inhibition of binding of anti-Hib PS Ab by *E. coli* K100 PS, concentration of total serum anti-Hib PS AB pre- and 1 mo post-immunization, and percent inhibition of binding of serum lambda antibodies to Hib PS by *E. coli* K100 PS in pre- and 1 mo post-immunization sera. ‡ Pool of 3 adult donors. ND, not done.

cross-reactivity) and to represent the response to "T-independent" plain polysaccharide, or "T-dependent" polysaccharide-protein conjugate forms of antigen. (Table I).

Four lambda-expressing anti-Hib PS Ab, obtained from four different subjects, cross-reacted with K100 PS. One of these individuals, RAY4, had no detectable serum cross-reactivity before immunization. The Abs JB32 and JB21 were obtained from the same individual, and differed in heavy chain isotype and K100 cross-reactivity.

IgV_L genes. IgV_L nucleic acid sequences were obtained from two kappa and six lambda hybridomas. Candidate germline IgV_L sequences were sought by comparing hybridoma sequences to those registered with EMBL and GenBank (32).

Four lambda-expressing hybridomas, derived from four unrelated subjects, use V_λ segments which are 96–98% homologous to one another, and are members of the germline V_λVII family of which a single member, 4A, has been previously described (33) (Fig. 1). Notably, each of these four V_λVII-encoded hybridomas cross-reacts with K100 PS. The high degree of homology between these segments suggests that these genes may be encoded by a single, or group of, closely related germline elements. The four hybridoma genes share a number of nucleic acid differences (maximum nucleic acid sequence homology 89%) from the only previously sequenced V_λVII germline gene 4A, implying that these genes may be derived from another, as yet undescribed, V_λVII germline element.

The homology of the translated amino acid sequences of these 4 V_λVII sequences parallels the nucleic acid homology. The hybridoma V_λVII genes are 90 to 97% homologous at the amino acid level (Fig. 2). Framework regions are 94 to 100% homologous, whereas CDR regions are 72 to 96% homologous. Whether these minor differences reflect genetic polymorphisms, or somatic mutation, cannot be resolved from these data.

JB21 and 16M3C8, the lambda-expressing hybridomas which do not cross-react with K100 PS, are encoded by a V_λII gene family member (34), (Fig. 3). JB21 is 91% homologous to the germline V_λ2.1 gene (99% homologous in framework regions and 74% homologous in CDRs). 16M3C8 is 89% homologous to V_λ2.1 (95% in framework regions and 77% in CDRs). JB21 shares 87% amino acid homology with the translated V_λ2.1 germline sequence (99% in framework regions and 60% in CDR regions), and 16M3C8 shares 82% amino acid homology (92% in framework regions and 60% in CDRs) (Fig. 4).

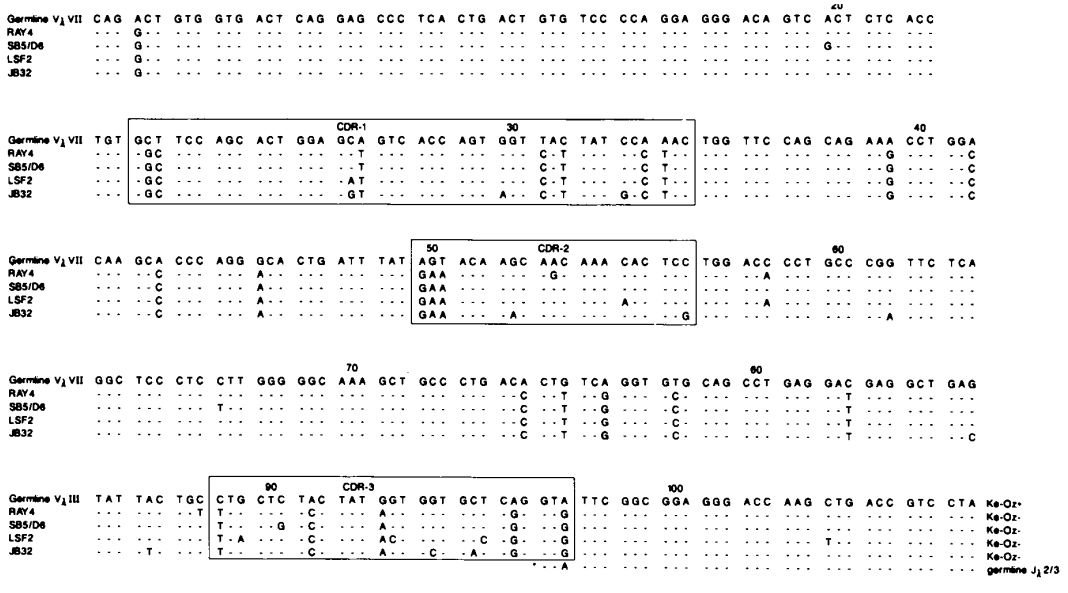


Figure 1. Nucleotide sequences of the V_λ and J_λ genes from four hybridoma cell lines (RAY4, SB5/D6, LSF2, JB32), and from the V_λVII germline gene, 4A (33). Numbering is according to Kabat (23). These sequence data are available from EMBL/GenBank Data Libraries under accession numbers M80917 (RAY4), M80918 (SB5/D6), M80915 (LSF2), and M80919 (JB32).

Samsung Bioepis Exhibit No. 1002
Page 2409

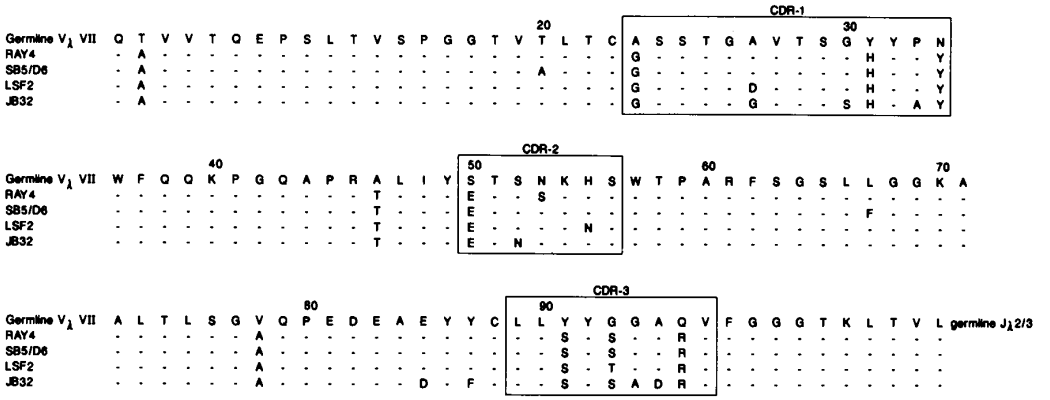


Figure 2. Translated amino acid sequences of V_λ and J_λ from four hybridoma cell lines (RAY4, SB5/D6, LSF2, JB32), and from the V_λVII germline gene, 4A (33). (See Fig. 1.)

These two V_λII-encoded gene segments are 91% homologous to one another, and have in common a number of nucleic acid differences from the V_λ2.1 germline gene (especially in CDRs). Whether they derive from another germline gene, or whether these differences reflect mutation, is unknown.

All four of the V_λVII-encoded antibodies are also encoded by V_HIII segments closely homologous to the germline gene 9.1 (5). Similar V_H segments also encode Ab utilizing other V_λ and V_κ gene segments (Table II) (manuscript in preparation). It is uncertain whether pairing of 9.1-like V_H and V_λVII-encoded heavy and light chains contributes to binding specificity or avidity. JB21 and RC3, also encoded by 9.1-like V_H gene segments, but paired with V_λII- and V_κII-encoded light chains, respectively, do not cross-react with K100 PS.

All six of the lambda light chains are encoded by a common J_λ segment closely homologous to both J_λ2 and J_λ3 germline genes (Fig. 1) (23). J_λ2 is associated with the use of the (Kern$^-$Oz$^-$)C_λ segment 2, and J_λ3 with the (Kern$^-$Oz$^+$)Cλ segment 3 (37). Each of these J_λ segments substitutes the codon GTG for the reported germline sequence GTA at amino acid position 97. Interestingly, this change does not result in an amino acid replacement, and is therefore not of importance in antigen binding. This minor sequence difference may reflect a shared genetic polymorphism, rather than somatic mutation.

The hybridoma RC3 uses a V_κII segment identical to the germline V_κII gene A2, and a J_κ3 segment closely homologous to the germline sequence (Fig. 5) (13). The second kappa hybridoma, ED6.1, is encoded by a member of the V_κI family

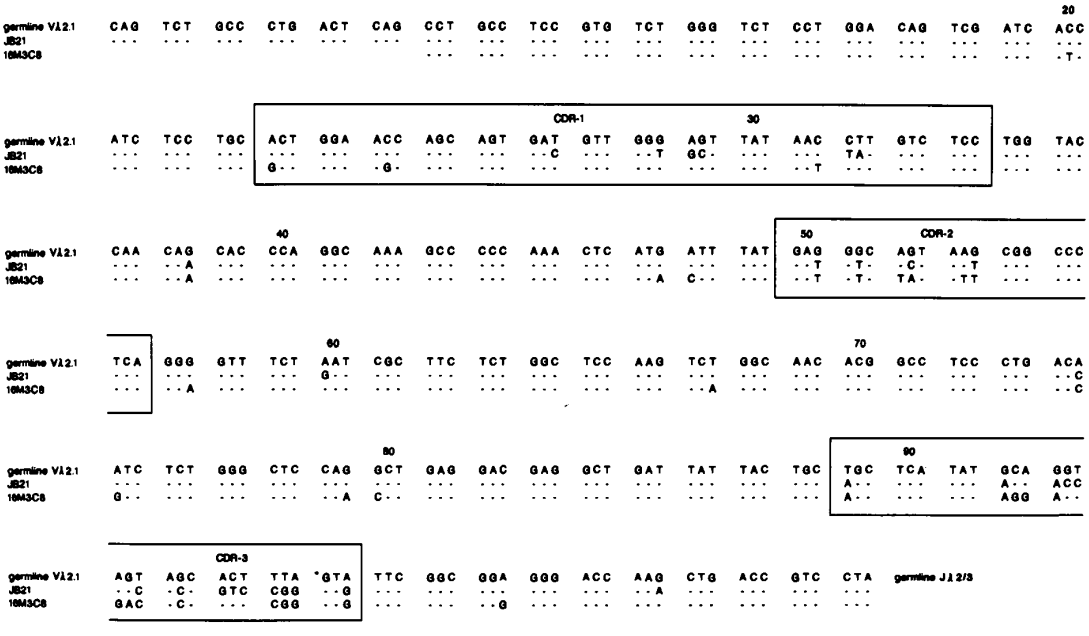


Figure 3. Nucleotide sequences of V_λ and J_λ genes from two hybridoma cell lines (JB21 and 16M3C8). Also shown is a germline gene V2.1 (34), and germline J_λ2/3 gene (23). An asterisk denotes the beginning of the J_λ segment. Numbering is according to Kabat (23). These sequence data are available from EMBL/GenBank Data Libraries under accession numbers M80916 (JB21) and M80921 (16M3C8).

Samsung Bioepis Exhibit No. 1002
Page 2410

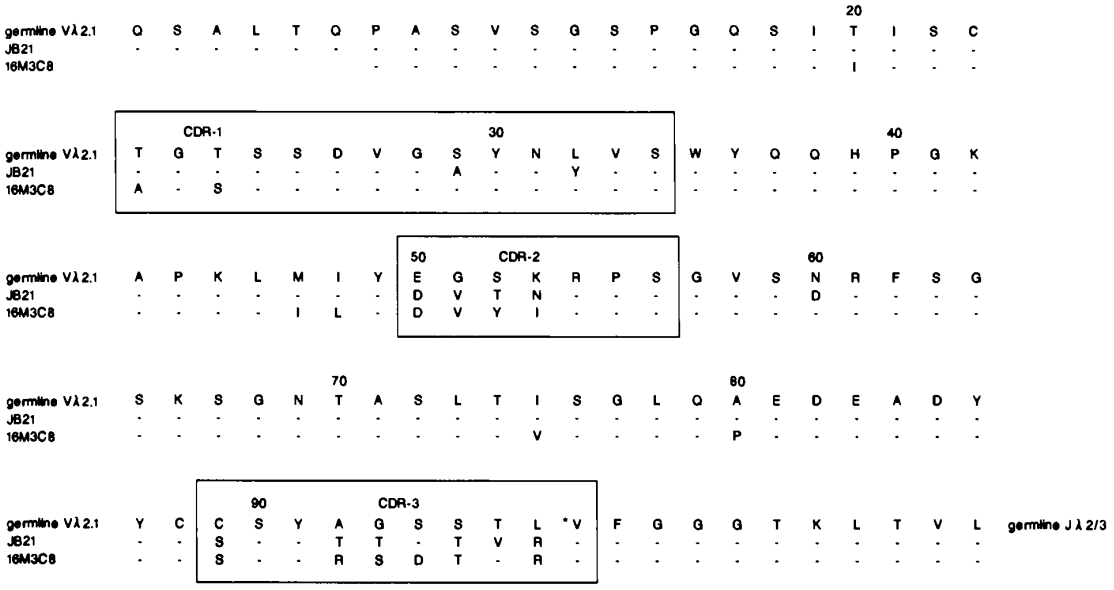


Figure 4. Translated amino acid sequences of V_λ and J_λ genes from two hybridoma cell lines (JB21 and 16M3C8). Shown for comparison is the translated sequence of the V_λ germline gene $V_\lambda 2.1$, and $J_\lambda 2/3$ germline gene. An asterisk denotes the beginning of the J_λ segment. (See Fig. 3.)

(36), and uses a $J_\kappa 4$ segment differing from the germline sequence by a single base (Fig. 6). The ED6.1 sequence is identical to the partial amino acid sequence reported previously for 2 kappa antibodies purified from serum (12).

VJ joints of seven of these eight hybridoma V_L genes are notable for the presence of an arginine codon (Fig. 7). Comparison of germline sequences indicates that in the RC3 V_L gene, the codon CGA could only have arisen by the addition of a non-germline-encoded G at the $V_\kappa J_\kappa$ joint. In the V_λVII-encoded antibodies, the relevant germline V_λ segment is unknown. The arginine codon may be germline-encoded, or result from either mutation or N sequence addition. The VJ splice junction in all lambda antibodies is identical, implying this V_λ/J_λ combination is a necessary prerequisite for generation of the arginine. Since the CDR-3 region encodes the third hypervariable region, the frequent finding of an arginine residue at this location, and particularly its presence in both kappa and lambda anti-Hib PS Ab clonotypes, implies this residue is of considerable importance in antigen binding.

*Table II. Summary of V_H and V_L Gene Segments**

Hybridoma	V_H Segment	V_L Segment
SB5/D6	V_HIII 9.1 (5)	V_λVII 4A (33)
RAY4	V_HIII 9.1	V_λVII 4A
JB32	V_HIII 9.1	V_λVII 4A
JB21	V_HIII 9.1	V_λII 2.1 (34)
RC3	V_HIII 9.1	V_κII A2 (13)
LSF2	V_HIII 9.1	V_λVII 4A
16M3C8	V_HIII VH26 (35)	V_λII 2.1
ED6.1	N.K.	V_κI clone KC-1 (36)

* Columns show name of hybridoma cell line and most closely homologous known germline V_H and V_L segments. Reference for germline shown in parentheses. NK, Not known.

Southern blots/analysis of germline V_λVII genes

The available repertoire of V_λVII family members was examined by hybridization of human genomic DNA with the V_λVII probe generated from LSF2. 10 prominent bands, as well as an additional 5 fainter bands, were demonstrated (Fig. 8). Notably, polymorphism such as the presence or absence of a 5.0-kb fragment, and varying intensity of the 3.5-kb fragment, was demonstrated even within this relatively small sample. The human lambda variable region locus is believed to contain about 60 members, of which 10 are members of the V_λVII family (33, 38). The five less prominent bands may represent members of other V_λ families sharing significant homology with the V_λVII family. To determine if the V_λVII-encoded antibodies derive from a single germline gene, an oligonucleotide probe corresponding to conserved V_λVII CDR-1 sequence was used to identify a single 3.7-kb genomic DNA fragment. Although more than one V gene may be present in a single restriction fragment, both the repeated isolation of highly homologous V_λVII genes, and the single prominant band detected with the oligonucleotide probe, suggests that a single V_λ gene may be responsible for the generation of lambda anti-Hib PS Ab that cross-reacts with K100 PS (Fig. 9). Less prominant higher molecular weight bands are also present, but hybridize weakly to the oligomer after subtraction of nonspecific background binding by high molecular weight DNA.

Discussion

To examine the molecular basis of the immune response to Hib PS, a panel of hybridomas secreting human monoclonal anti-Hib PS Ab has been developed. Our previous studies showed exclusive use of members of the V_HIII gene family in this im-

Samsung Bioepis Exhibit No. 1002
Page 2411

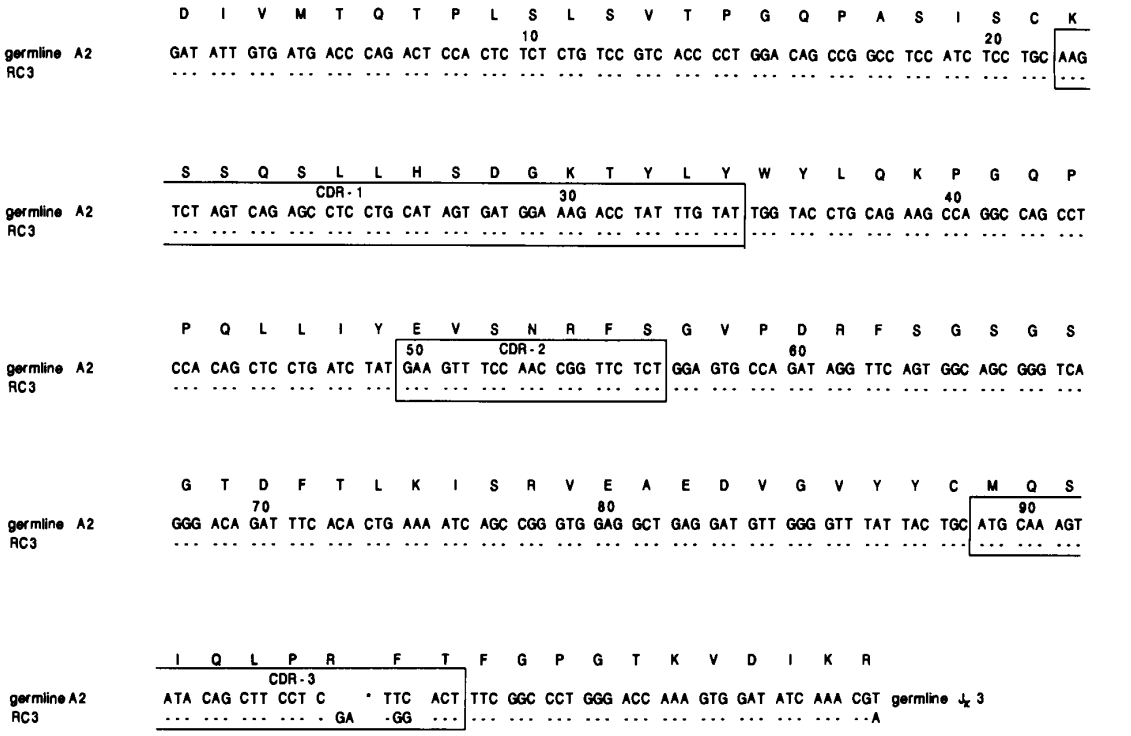


Figure 5. Nucleotide sequences of V_κ and J_κ genes from the hybridoma cell line RC3. Also shown is the translated amino acid sequence of the RC3 V_κ gene. Shown for comparison is the nucleotide sequence of a germline V_κ gene, A2 (13), and germline $J_\kappa 3$ gene. An asterisk denotes the beginning of the J_κ segment. Numbering is according to Kabat (23). This sequence data is available from EMBL/GenBank Data Libraries under accession number M80914 (RC3).

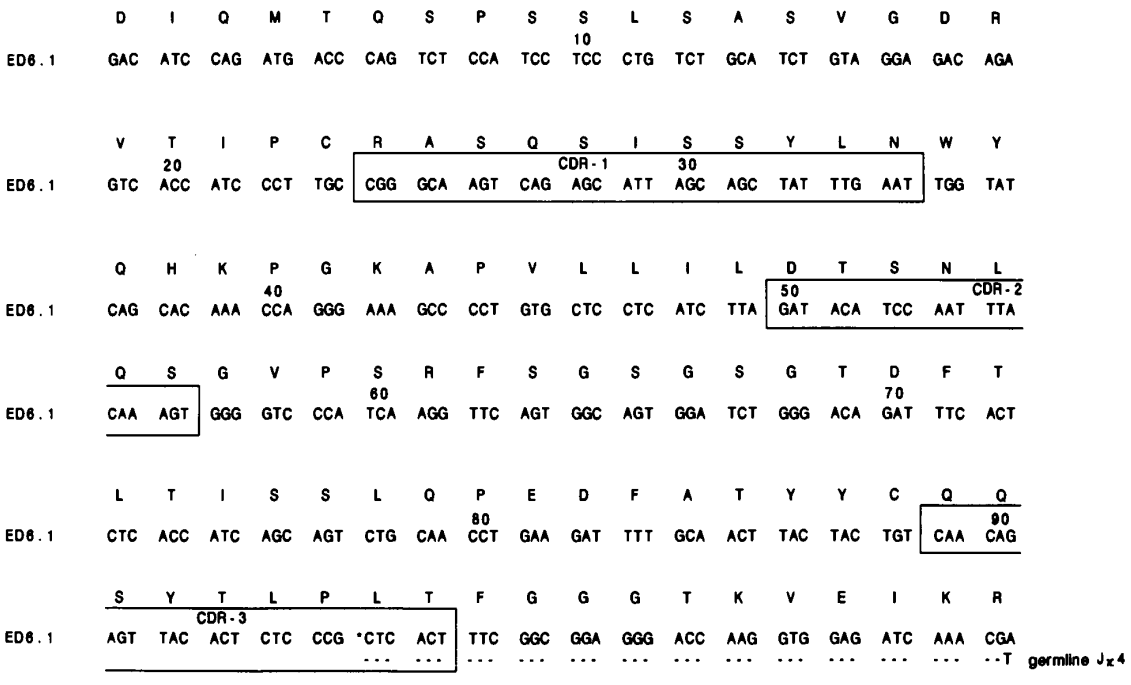


Figure 6. Nucleotide sequence of V_κ and J_κ genes from hybridoma cell line ED6.1. Also shown is the translated amino acid sequence of the ED6.1 V_κ gene. An asterisk denotes the beginning of the J_κ segment. Numbering is according to Kabat (23). This sequence data is available from EMBL/GenBank Data Libraries under accession number M80920 (ED6.1).

Samsung Bioepis Exhibit No. 1002
Page 2412

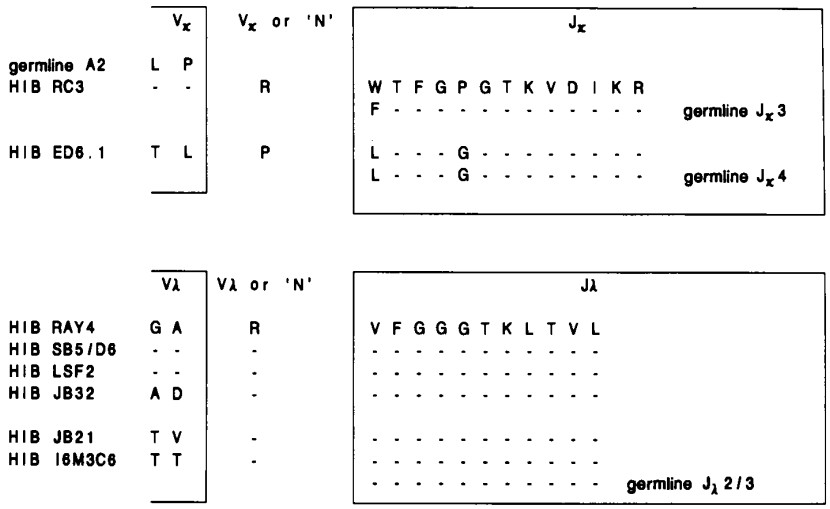


Figure 7. Translated amino acid sequences of VJ joints from seven hybridoma cell lines (RC3, ED6.1, RAY4, SB5/D6, LSF2, JB21, and 16M3C8). Also shown are translated amino acid sequences of the germline V_κ gene A2, and germline $J_\kappa 3$, $J_\kappa 4$, and $J_\lambda 2/3$ genes. (See Figs. 2, 4, and 6.)

mune response, and the apparent restriction to two or three germline V_H genes. In contrast, V_L gene usage is more complex, with antibody encoded by a number of V_λ and V_κ families (12). Analysis of light chain variable region genes obtained from cell lines selected to represent various characteristics of serum antibodies showed that despite diversity in the variety of light chain variable region gene segments utilized, most serum anti-Hib PS Abs may be encoded by a limited number of V_κ and V_λ germline genes.

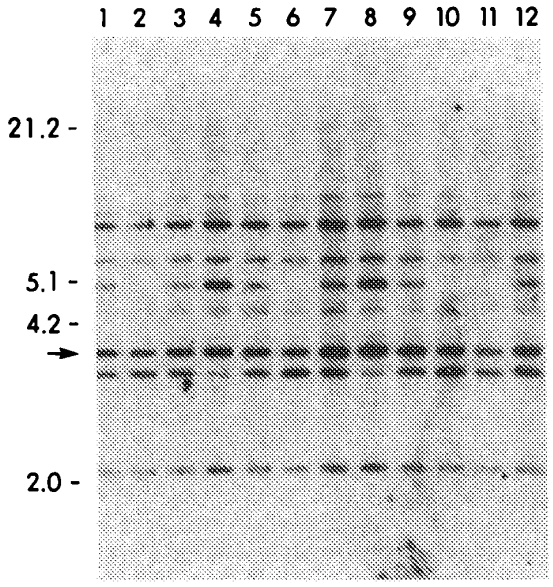


Figure 8. Southern blot analysis for V_λVII family members in BamHI-digested human genomic DNA. Lane *12* contains genomic DNA from the subject from whom hybridoma LSF2 was obtained. Lane *9* contains genomic DNA from a parent of LS. The remaining 10 donors are unrelated. An arrow indicates the 3.7-kb fragment which is identified by the oligonucleotide probe illustrated in Fig. 9.

The light chain distribution of anti-Hib PS Ab is characterized by a predominance of kappa light chains, although some individuals predominantly or exclusively express lambda light chains (39, 40). Scott et al. have shown that V_κII light chains are the most commonly expressed light chains among clonally purified serum anti-Hib PS antibodies from adults with high responses to immunization. Limited amino acid sequences of a number of these serum antibodies showed that greater than 60% of subjects immunized with plain Hib PS produce antibody encoded by a V_κII family member, and further, that a number of these antibodies show identical amino acid homology with the translated amino acid sequence of the germline V_κII gene A2 (12, 13). Lucas et al. have described an anti-idiotypic monoclonal antibody (Hibid-1) that identifies anti-Hib PS antibodies expressing this light chain (16). On average, 60%

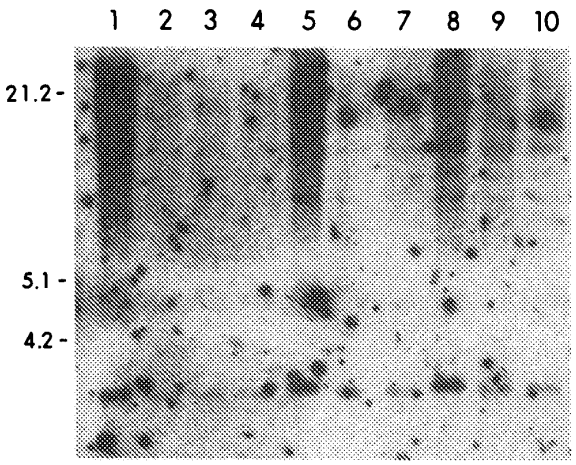


Figure 9. Dried gel hybridization of BamHI-digested human genomic DNA with an oligonucleotide probe corresponding to conserved V_λVII hybridoma CDR-1 sequences. Genomic DNA in lanes *1*, *4*, *5*, *6*, *7*, *9* and *10* of Fig. 8 corresponds to that in lanes *2*, *7*, *4*, *8*, *10*, *5* and *9* of Fig. 9, respectively.

Samsung Bioepis Exhibit No. 1002
Page 2413

of the total anti–Hib PS antibody of most children and adults immunized with either Hib PS or Hib PS-conjugate vacine expresses this idiotype (15, 16). Taken together, this suggests the most common clonotype of anti–Hib PS Ab is encoded by a single V_κII gene. The V_κ locus contains approximately 50 functional genes, of which 40% are members of the V_κII family (41). The V_κII A2 germline gene is known to be present in a single copy (13). Our findings definitively demonstrate that at least a portion of V_κII-encoded antibodies are encoded by unmutated germline elements. Our subject R.C. was immunized with PS-protein conjugate vaccine, whereas Scott's subjects were immunized with the plain PS vaccine. Thus it is possible that the unmutated V_κII A2 segment is utilized irrespective of antigen presentation in a T-independent or T-dependent form.

Lambda variable region gene usage appears similarly restricted. The majority of lambda-expressing anti–Hib PS Ab in adults with high antibody responses to vaccine cross-reacts with *E. coli* K100 PS (17). The capsular polysaccharides of Hib and *E. coli* K100 are structurally similar and gastrointestinal colonization with *E. coli* K100 induces antibody to Hib PS in humans (42). "Naturally occurring" anti–Hib PS Ab in children commonly cross-reacts with K100 PS, whereas Ab of individuals immunized with Hib PS or responding to Hib infection is less frequently cross-reactive (18). The kappa/lambda ratio of natural anti–Hib PS Ab is lower in infants than in older children, and in the vaccine-induced antibody of adults (39). Further, Hibid-1 (the CRI associated with expression of the V_κII light chain) is expressed less frequently and comprises a smaller proportion of total anti–Hib PS Ab in preimmune compared with postimmunization sera. Expression of Hibid-1 following immunization is similar in adults and children. These results imply that cross-reactive antigens may induce the natural antibody of children. K100 PS-cross-reactive antibody may provide protective immunity in young children upon first exposure to Hib. These cross-reactive antibodies appear to be subsequently replaced or dominated by antibody specific for Hib PS when individuals are exposed to this antigen by immunization, colonization, or infection. We demonstrate that cross reactivity of lambda antibodies may be mediated through a single light chain germline gene. The mechanism for the clonal dominance of the V_κII antibodies is unclear; the avidity for Hib PS of these K100 PS–cross-reactive antibodies from adults is similar to that of Hib specific PS antibodies (P. Shackelford, unpublished observation).

Lambda antibodies expressing the V_λII gene did not cross-react with *E. coli* K100 PS. Whether all noncross-reactive antibodies are encoded by closely related members of the V_λII gene family will require further characterization of additional antibodies.

By the age of about 5 yr most individuals have acquired protective anti–Hib PS Ab. All of the hybridomas in this study were derived from older children or adults, who had detectable preimmunization antibody. The B cells immortalized by fusion, therefore, most likely represent a secondary immune response. Despite this, V_H gene segments obtained from these hybridomas appear to be relatively unmutated (14), and it appears that some V_L genes are similarly conserved. The RC3 V_κ gene segment is identical to the A2 germline gene, and although the pertinent V_λVII germline gene is not known, the paucity of differences in V_λVII segments from four different subjects suggests these gene segments are relatively unmutated. J_κ and J_λ segments are also highly homologous to germline elements. Thus, at least a portion of the anti–Hib PS response represents the use of unmutated germline elements.

VJ joints of the majority of rearranged V_L genes also appear to be an additional source of restriction in the anti–Hib PS immune response. Although there is variability in the use of J_κ segments, V_κII-encoded V_L genes are characterized by the presence of an arginine at amino acid position 95a (13). In the RC3 V_L gene and at least one of the antibodies (B-G2a) described by Scott, this appears to have arisen by N sequence addition. In four other kappa antibodies described by Scott, alternative joining may have generated this invariant arginine (13). All of the lambda V_L genes in the present study are similarly characterized by an invariant arginine-96. The germline V_λVII segment is not known; therefore the mechanism used to generate this codon is uncertain. The invariant presence of this residue in many anti–Hib PS Abs supports the previous suggestion that it may be of great importance in antigen binding (13). This also is supported by studies of the murine anti-*p*-azophenylarsonate (Ars) response. Anti-Ars light chains also contain an invariant arginine at position 96, encoded by intracodonic splicing of a single V_κ (V_κ10-Ars) and a single J_κ (J_κ1) segment (43). This arginine is essential for Ars binding, and the substitution of tyrosine for arginine has been shown to abolish binding (44). The presence of arginine residues clustered within the CDR's of the heavy chains of murine anti–DNA antibodies correlates with increased affinity, and the arginine requirement in the anti–Hib PS response may indicate its participation in the recognition of the similar phosphate–ester linkage of the repeating carbohydrate moiety (45). Thus, although diversity exists in the use of a variety of V and J segments, certain VJ combinations, which produce arginine codons by junctional flexibility or N segment addition, may be strongly selected.

Does the restricted nature of V_H and V_L segment usage in the human anti–Hib PS response explain the age-related acquisition of immunity to this organism? In both mice and humans, preferential use of certain V_H gene segments is observed in early development. In mice, biased usage of J_H-proximal V_H gene segments occurs in fetal and neonatal animals (46, 47). Developmental regulation of V_L gene usage is less extensively studied than V_H genes. Teale and Morris noted preferential expression of certain V_κ families in LPS-stimulated murine fetal B cells compared to adult B cells, with no evidence for position-dependent bias (48). Kaushik et al. also described nonstochastic use of V_κ families in adult mice, suggesting molecular constraints may persist beyond the neonatal period (49). Two or three V_H gene segments, and a single V_κ and a single V_λ gene segments, appear to encode a large proportion of anti–Hib PS Ab in immune subjects. The use of highly conserved V gene segments in human anti–Hib PS response may place considerable restraints on the immune response. The inability to utilize these genes, whether due to chromosomal position or other molecular constraints, may be responsible for the age-related susceptibility to Hib infection. The lack of somatic hypermutation in a portion of these antibodies would also place greater dependence on the germline repertoire, in that it may be impossible to improve antibody affinity by antigen-driven selection of favorable mutations. The absence or polymorphism of these genes also may contribute to individual and ethnic susceptibility to invasive Hib disease. In addition, the repertoire differences detected in some individuals (such as the absence of kappa antibody, or the absence of the V_κII-associated CRI) may be explained by the use of other minor light chains. An individ-

Samsung Bioepis Exhibit No. 1002
Page 2414

ual missing the A2 germline gene has been described, although the functional implications of such a deletion are not known (13).

Expression of certain V_H gene family members in mice is correlated with B cell subpopulations (50). An alternative explanation for the poor response to plain Hib PS may be a maturational delay in recruitment of a critical B cell population that selectively expresses important variable region gene segments. We and others note that an arginine is expressed at the VJ joint in the great majority of anti-Hib PS light chains. In some circumstances N segment addition is required to generate this codon. The addition of these extra nucleotides is infrequent in light chain recombination, suggesting that these B cell precursors may be relatively infrequent (51). Moreover, N segment addition also appears to be developmentally regulated. Neonatal murine B cells lack N sequences, and their presence in heavy chain recombination increases with time (52). In addition, N sequence addition may also correlate with certain B cell subsets. N segments appear to be rare in Ly1-B cell-derived populations (53).

In contrast to plain polysaccharide vaccines, most young infants respond well to the newer Hib-PS protein conjugate vaccines (54). It is presently unknown if this antigen formulation recruits an alternative antibody repertoire, or if, by an uncertain mechanism, developmental restriction of a "dormant" B cell subset is overcome. To address this question, V gene expression in infants must be examined.

Acknowledgments

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Samsung Bioepis Exhibit No. 1002
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Samsung Bioepis Exhibit No. 1002
Page 2416

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

AMGEN INC.
Petitioner,

v.

ALEXION PHARMACEUTICALS
Patent Owner.

Case IPR2019-00740
U.S. Patent No. 9,718,880

PETITION FOR *INTER PARTES* REVIEW OF U.S. PATENT NO. 9,718,880

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TABLE OF CONTENTS

I. Statement of Precise Relief Requested and Reasons Therefor (37 C.F.R. §42.22(a)) 1

II. Introduction 1

III. Summary 2

IV. The '880 patent and its prosecution history 7

V. State of the art before March 15, 2007 11

- A. Humanized monoclonal antibodies were well-known 11
- B. The prior art taught that eculizumab is a humanized anti-C5 monoclonal antibody (h5G1.1) containing a hybrid IgG2/IgG4 constant region 13
- C. The art taught eculizumab's amino acid sequence 14
- D. Eculizumab was a known, effective treatment for PNH 18

VI. Person of ordinary skill in art 21

VII. Claim construction 22

VIII. Identification of the challenge (37 C.F.R. §42.104(b)) 22

IX. The same or substantially the same prior art or arguments were not previously presented to the Office 24

X. Ground 1: Hillmen anticipates claim 2 25

- A. Hillmen explicitly disclosed every element of claim 2, except for eculizumab's amino acid sequence 25
- B. Alexion admitted that Hillmen's eculizumab necessarily has the claimed sequence 26
- C. Hillmen's disclosure is enabling 28

XI. Ground 2: Hill '05 anticipates claim 2 31

- A. Hill '05 explicitly disclosed every element of claim 2, except for eculizumab's amino acid sequence 32
- B. Hill '05's eculizumab necessarily has the claimed sequence 32
- C. Hill '05's disclosure is enabling 34

XII. Ground 3: claims 1 and 3 would have been obvious over Hillmen, Bell, and Wang 36

XIII. Ground 4: claims 1 and 3 would have been obvious over Hill '05, Bell, and Wang. 44

XIV. Ground 5: claims 1-3 would have been obvious over Bell, Wang, Bowdish, and Evans. 46

A. Bell and Wang taught all the limitations of claims 1-3 except eculizumab's amino acid sequence. 46
B. Bowdish and Evans taught the claimed amino acid sequences. 50
C. A POSA would have had a reason to combine the references with a reasonable expectation of success. 55

XV. Ground 6: claims 1-3 would have been obvious over Bell, Wang, Evans, and Meuller. 58

A. Bell and Wang taught all the limitations of claims 1-3 except eculizumab's amino acid sequence. 58
B. Evans and Mueller taught eculizumab's amino acid sequences. 59
C. A POSA would have had a reason to combine the references with a reasonable expectation of success. 66

XVI. Objective indicia do not support patentability 68

XVII. Certification that the Patent May Be Contested via *Inter Partes* Review by the Petitioner and Standing (37 C.F.R. §42.104(a)). 70

XVIII. Mandatory Notices (37 C.F.R. §42.8(a)(1). 70

XIX. Conclusion. 72

EXHIBIT LIST

Exhibit #	*Description*
1001	Bell, L., *et al.*, "Treatment of Paroxysmal Nocturnal Hemoglobinuria Patients by an Inhibitor of Complement," U.S. Patent No. 9,718,880 B2 (filed May 6, 2016; issued August 1, 2017)
1002	Declaration of Joseph P. Balthasar, Ph.D.
1003	Curriculum Vitae of Joseph P. Balthasar, Ph.D.
1004	Hillmen, P., *et al.*, "Effect of Eculizumab on Hemolysis and Transfusion Requirements in Patients with Paroxysmal Nocturnal Hemoglobinuria," *N. Engl. J. Med.* 350:552-559 (2004)
1005	Bell, L. and Rother, R., "Method of Treating Hemolytic Disease," U.S. Patent Application Publication No. 2005/0191298 A1 (filed February 3,2005; published September 1, 2005)
1006	Bowdish, K., *et al.*, "Rationally Designed Antibodies," U.S. Patent Application Publication No. 2003/0232972 A1 (filed December 2, 2002; published December 18, 2003)
1007	Evans, M., *et al.*, "C5-Specific Antibodies for The Treatment of Inflammatory Diseases," U.S. Patent No. 6,355,245 B1 (filed June 7, 1995; issued March 12, 2002)
1008	Mueller, J., *et al.*, "Porcine Cell Interaction Proteins," International Patent Application Publication No. WO 97/11971 A1 (filed September 27 1996; published April 3, 1997)
1009	Patent Term Extension Application for U.S. Patent No. 6,355,245 B1
1010	Patent Term Extension Certificate for U.S. Patent No. 6,355,245 B1
1011	Hill, A. "Sustained Control of Hemolysis and Symptoms and Reduced Transfusion Requirements over a Period of 2 Years in Paroxysmal Nocturnal Hemoglobinuria (PNH) with Eculizumab Therapy," *Blood* 104: Abstract 2823 (2004)
1012	Hillmen, P., *et al.*, "The Complement Inhibitor Eculizumab in Paroxysmal Nocturnal Hemoglobinuria," *N. Engl. J. Med.* 355:1233-1243 (2006)
1013	Young, N., *et al.*, "Safety and Efficacy of the Terminal Complement Inhibitor Eculizumab in Patients with Paroxysmal Nocturnal Hemoglobinuria: Interim Shepherd Phase III Clinical Study," *Blood* 108: Abstract 971 (2006)
1014	File History for U.S. Patent No. 9,725,504 B2

Exhibit #	***Description***
1015	File History for U.S. Patent No. 9,732,149 B2
1016	File History for U.S. Patent No. 9,718,880 B2
1017	Opposition File History for European Patent No. 1720571
1018	Harlow and Lane, Chapter 2:Antibody Molecules, in *Antibodies, A Laboratory Manual* (1988)
1019	Brekke, O. and Sandlie, I., "Therapeutic antibodies for human diseases at the dawn of the twenty-first century," *Nature Reviews Drug Discovery* 2: 52-62 (2003)
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1024	FDA Medical Review for Application Number 125166 (eculizumab), available at https://www.accessdata.fda.gov/drugsatfda_docs/nda/2007/125166s000TOC.cfm
1025	Australian Government Department of Public Health, Pharmaceutical Benefits Advisory Committee, "Public Summary Document" for Eculizumab, available at http://www.pbs.gov.au/info/industry/listing/elements/pbac-meetings/psd/2009-03/pbac-psd-eculizumab-march09
1026	File History of U.S. Application No. 13/426,973
1027	Opposition File History for European Patent No. 2359834
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1064	Centocor Ortho Biotech, LP, Orthoclone OKT3 Package Insert, available at https://dailymed.nlm.nih.gov/dailymed/archives/fdaDrugInfo.cfm?archiveid=17321
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1066	Genentech, Inc., Raptiva 2009 Package Insert, available at https://www.fda.gov/downloads/Drugs/DrugSafety/PostmarketDrugSafetyInformationforPatientsandProviders/UCM143346.pdf
1067	Centocor, Inc., Remicade 1998 Package Insert, available at https://www.accessdata.fda.gov/drugsatfda_docs/label/1998/inflcen082498lb.pdf
1068	Eli Lilly & Co., ReoPro 1997 Package Insert, available at https://www.accessdata.fda.gov/drugsatfda_docs/label/1997/abcicen110597-lab.pdf
1069	Genentech, Inc., Rituxan 1997 Package Insert, available at https://www.accessdata.fda.gov/drugsatfda_docs/label/1997/ritugen112697-lab.pdf
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I. Statement of Precise Relief Requested and Reasons Therefor (37 C.F.R. §42.22(a)).

Amgen Inc. petitions for *Inter Partes* Review, seeking cancellation of claims 1-3 of U.S. Patent No 9,718,880 ("'880 patent"; AMG1001), assigned to Alexion Pharmaceuticals, Inc. The challenged claims are unpatentable under 35 U.S.C. §§ 102 and/or 103. This Petition is supported by the declaration of Dr. Joseph Balthasar (AMG1002), an expert in the development and evaluation of therapeutic monoclonal antibodies. AMG1002, ¶¶1-10. Because the petition demonstrates a reasonable likelihood that claims 1-3 are unpatentable, institution is warranted.

II. Introduction.

The challenged claims encompass methods of treating paroxysmal nocturnal hemoglobinuria ("PNH") using a humanized anti-C5 antibody having a specified sequence. During prosecution of the '880 patent, Alexion asserted—incorrectly, yet repeatedly—that the claimed sequence "was not disclosed in the prior art; nor was it available to the public." AMG1016, 179-180; *see also* 720("[n]either eculizumab nor its complete sequence, including the sequence of its unique, non-naturally occurring, protein engineered heavy chain, was in the public domain prior to the March 15, 2007 effective filing date of the present application."). Indeed, Alexion's argument that the claimed sequence was not publicly known was the only stated basis for withdrawing the rejections and issuing a notice of allowance:

"none of the applied references in the rejections recite using an antibody [with the claimed sequence]." *Id.*, 762. But, as shown herein, long before the '880 patent's alleged priority date Alexion repeatedly published using pharmaceutical compositions for intravenous infusion of the monoclonal anti-C5 antibody eculizumab to treat a patient suffering from PNH. AMG1002, ¶¶29-43.

Alexion admitted that it performed at least 17 clinical trials treating PNH with a humanized anti-C5 antibody ("eculizumab"), and that the antibody used in those trials had the claimed amino acid sequence. *Id.* 735-737. Thus, by Alexion's admission, prior art publications of those trials necessarily disclose the claimed sequence, providing an inherent disclosure of the claimed sequence. In addition, Alexion's own prior art publications of the sequence and structure of a humanized anti-C5 antibody, were such that the skilled artisan could and would have made and used an antibody formulation as claimed, with a reasonable expectation of success. The challenged claims, therefore, offer nothing novel or inventive.

III. Summary.

Eculizumab (Soliris®) is a monoclonal antibody that binds complement protein C5 and inhibits C5 cleavage. Alexion obtained U.S. Patent No. 6,355,245 ("Evans"), which is prior art to the '880 patent, on March 12, 2002. Alexion contends that Evans "claims the approved product" (Soliris®; eculizumab), and

provides both written description and enablement support for claims directed to eculizumab. AMG1009, 4; AMG1010, 2; AMG1049, 838-839.

The FDA approved Soliris® for treatment of patients with PNH on March 16, 2007. AMG1009, 2. Exactly *one day* before receiving FDA approval, Alexion filed PCT Application No. PCT/US2007/006606[1] ("the '606 application") and began prosecuting a chain of new patents directed to eculizumab. This was no coincidence.

To secure new eculizumab patents from the '606 application, Alexion repeatedly told the USPTO that eculizumab's amino acid sequence—specifically its IgG2/IgG4 heavy chain constant region—was not available in the prior art. But Alexion did not inform the UPSTO that it had been repeatedly publishing on eculizumab, its amino acid sequence—including its engineered IgG2/IgG4 heavy chain—years, and its use in intravenous formulations for treating PNH before March 15, 2007. Indeed, Alexion proudly boasted about widespread knowledge of the IgG2/IgG4 constant region in the art when it suited Alexion's interests, but remained silent about it to the USPTO. AMG1049, 838-839. Alexion's carefully timed pursuit of the '606 application one day before product approval improperly

[1] The '880 patent claims priority to the '606 application.

seeks to ensnare and monopolize that which is already in the public domain, through Alexion's own publications nonetheless.

Well before March 15, 2007, artisans were aware that eculizumab was safe and effective for treating PNH. Alexion itself admitted, prior to that date, that eculizumab was administered via intravenous infusion to patients in at least 17 different clinical trials, many of which were published (summarized below in Table 1). AMG1014, 735-737. Alexion also admitted that the eculizumab used in these trials has the claimed amino acid sequence. *Id* Thus, by Alexion's admission, the humanized anti-C5 antibody administered in these published trials (eculizumab) necessarily has the claimed sequence.

In addition, and contrary to Alexion's misrepresentation, the amino acid sequence and structure of eculizumab *were* known in the art, and a skilled artisan would have had ample reasons, guidance, and direction to make and use eculizumab as claimed, rendering the challenged claims obvious. Indeed, it was Alexion who placed the claimed amino acid sequence into the public domain, yet failed to inform the examiner of this.

For example, Alexion's patent application publication US 2003/0232972 A1 ("Bowdish"), *not* raised by the examiner during prosecution, published in 2003, used eculizumab as the starter "scaffold" antibody for creating a recombinant thrombopoietin (TPO) peptide-antibody, and provided the full eculizumab amino

acid sequence except for the heavy chain CDR3 ("HCDR3") sequence that it had replaced with the TPO peptide. AMG1006, ¶¶[0191]-[0193], Figs. 13A-13B, and SEQ ID NOs. 67 and 69. But the missing HCDR3 sequence was taught in Evans.

Bowdish explicitly incorporated by reference Evans[2] (another Alexion patent) for making eculizumab: "[c]onstruction of 5G1.1 [i.e., eculizumab] is described in U.S. Application Ser. No. 08/487,283, incorporated herein by reference."[3] *Id.*, ¶[0191]. As Dr. Balthasar explains, the skilled artisan would have readily identified the heavy chain CDR3 sequences in Evans, thereby obtaining the complete amino acid sequence of the claimed antibody. *See* AMG1002, ¶¶55-56.

The claimed amino acid sequence was also taught in the art through the combination of Evans and another Alexion publication, WO 97/11971 ("Mueller"; AMG1008). In addition to disclosing its heavy chain CDR3 sequence, Evans taught the amino acid sequences of eculizumab's light and heavy chain variable regions. AMG1007, 44:4-13; AMG1002, ¶¶56, 146. With the heavy and light chain variable region sequences in hand, the artisan would have needed only to

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[2] Alexion patent application publication US 2005/0191298 A1 ("Bell") also explicitly incorporates by reference Evans for preparing eculizumab. AMG1005, ¶[0052].

[3] Evans issued from U.S. Application No. 08/487,283. AMG1007, face.

identify the sequences for the light and heavy chain constant regions—information found in Evans (light chain) and Mueller (both the light and heavy chains).

Notably, Alexion *never* provided Mueller to the examiner. Published in 1997, Mueller taught methods of creating recombinant antibodies with chimeric IgG2/IgG4 constant regions that were known not to activate the complement system. AMG1008, 7:28-31, 8:23-26, 12:27-32. Mueller further described using eculizumab—a humanized anti-C5 antibody with the same IgG2/IgG4 constant region as the experimental antibody—as a control antibody, and provided amino acid sequences of its IgG2/IgG4 heavy chain constant region and light chain constant region. *Id.*, 12:35-37, Fig. 15, 52-53, 58-61; AMG1002, ¶¶57-59, 166. Dr. Balthasar explains that a skilled artisan also would have readily obtained the complete amino acid sequence of the humanized anti-C5 antibody that results from the combination of Evans and Mueller; a sequence that Alexion now claims to be novel. *See* AMG1002, ¶¶170-175.

Alexion admittedly placed methods of treating PNH using a pharmaceutical composition comprising a humanized anti-C5 antibody, as claimed, squarely in the prior art, and it's numerous prior art publications of eculizumab clinical trials results would have given the artisan ample reason to formulate eculizumab to treat PNH. The prior art also supplied sufficient information about the sequence and structure of eculizumab such that the artisan could and would have made and used

a humanized anti-C5 antibody, as claimed, with a reasonable expectation of success. The challenged claims, therefore, offer nothing novel or inventive over what was well known in the art to a POSA before March 15, 2007.

The examiner was not aware that eculizumab's amino acid sequence was already in the prior art. The Board here has the benefit of a more complete record, and should take the opportunity to correct the examiner's error by determining that claims 1-3 of the '880 patent are unpatentable as anticipated and obvious.

IV. The '880 patent and its prosecution history.

The '880 patent issued on August 1, 2017, from U.S. Appl. No. 15/148,839, filed on May 6, 2016, and claims a priority date of March 15, 2007.[4] Its claims recite:

> 1. A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein the composition is a sterile, preservative free, 300 mg single-use dosage form comprising 30 ml of a 10 mg/ml antibody solution, wherein the antibody comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4.

[4] Petitioner does not concede that the '880 patent is entitled to any of its claimed priority dates.

> 2. A pharmaceutical composition comprising an anti-C5 antibody, wherein the anti-C5 antibody comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4.
>
> 3. The pharmaceutical composition of claim 2, wherein the pharmaceutical composition is a sterile, preservative free 300 mg single-use dosage form comprising 30 ml of a 10 mg/ml anti-C5 antibody solution.

EX1001, 39:1-16.

During prosecution, the examiner initially rejected Alexion's claims as obvious over Hillmen, which disclosed methods of using infusions of eculizumab for treating PNH, in view of Evans, which teaches "effective" doses of "antibody 5G1.1, which is the same as eculizumab," which "may be formulated in a pharmaceutically effective carrier such [as] saline, which is considered to be a "preservative free solution," and Wang, which "teaches that eculizumab is highly soluble, including at up to 30 mg per ml," and that eculizumab can be formulated in a sterile, preservative-free solution, AMG1016, 120-121.

In response, Alexion asserted—incorrectly—that "the complete structure of eculizumab was not disclosed in the prior art; nor was it available to the public;" that Hillmen "fails to teach any part of the sequence;" and Evans "fails to teach or

in any way suggest the unique, non-naturally occurring, protein-engineered full heavy chain of eculizumab." *Id.*, 179-180.

In a final rejection, the examiner relied upon Thomas, for a teaching of the claimed sequence. *Id.* 598-599. Again, Alexion's response was that "[n]either eculizumab nor its complete sequence, including the sequence of its unique, non-naturally occurring, protein engineered heavy chain, was in the public domain prior to the March 15, 2007 effective filing date of the present application. *Id.*, 720. Indeed, Alexion's argument that eculizumab's amino acid sequence was not publicly known was the only stated basis for withdrawing the rejections and issuing a notice of allowance: "none of the applied references in the rejection recite using an antibody [with the claimed sequence]." *Id.*, 762.

As shown herein, the examiner was misled. And Alexion has repeatedly made these same misleading arguments to obtain additional eculizumab patents related to the '880 patent[5] that claim subject matter already in the public domain. For example, when prosecuting U.S. 9,732,149 (claiming eculizumab), Alexion

[5] Prosecution of parent applications is considered part of the file history of the child application. *Omega Engg, Inc., v. Raytek Corp.*, 334 F.3d 1314, 1333 (Fed. Cir. 2003); *see also*, *Microsoft Corp. v. Multi-Tech Sys., Inc.*, 357 F.3d 1340, 1349 (Fed. Cir. 2004).

argued that "[n]either eculizumab nor its complete sequence … was in the public domain prior to the March 15, 2007 effective filing date." AMG1015, 709-712. And when prosecuting U.S. 9,725,504 (claiming methods of using eculizumab formulations), Alexion argued that "the complete structure of eculizumab was not disclosed in the prior art; nor was it available to the public." AMG1014, 586-588.

Meanwhile, Alexion was saying the exact opposite in a European opposition proceeding over an eculizumab-related EP patent being challenged for sufficiency of disclosure. There—contrary to what it was telling the USPTO—Alexion stated that "the sequence for eculizumab was publicly available prior to the [February 3, 2004] priority and filing date" and "a sequence for eculizumab was submitted to Chemical Abstract Services (CAS) and entered into their STN database on 14 February 1999…."[6] AMG1017, 277, 291(¶5.1.2). And during prosecution of related U.S. Application No. 11/127,438, Alexion argued that its provisional applications provided written description for claims to "eculizumab" and an

[6] Alexion later tried to take this statement back during prosecution of a different European application, arguing that the eculizumab sequence information submitted in February 1999 had unintentional errors in it and therefore was not a public disclosure of the true eculizumab amino acid sequence, notwithstanding Alexion's intent to disclose it to the public. AMG1054, 247-254, 292-293.

antibody containing a "mutated Fc portion" because the provisional applications incorporated by reference the Evans prior art:

> Applicant respectfully disagrees and asserts that the priority applications provide ample written support for the claimed descriptions. For example, the priority documents each describe that "Particularly useful anti-C5 antibodies are h5G1.1, h5G1.1-scFv and functional fragments of h5G1.1 are *described in U.S. Patent No. 6,355,245*, the disclosures of which are incorporated herein in their entirely [*sic*] by this reference … Applicant submits that *h5G1.1* … [was] *well-known to one of ordinary skill in the art as eculizumab* … at the time of filing of priority applications.

AMG1049, 838-839 (emphasis added).[7] Alexion cannot have it both ways. Indeed, the European Opposition Division has already revoked at least two of Alexion's European eculizumab-related patents. AMG1017, 368-378; AMG1027, 2667-2685.

V. State of the art before March 15, 2007.

A. Humanized monoclonal antibodies were well-known.

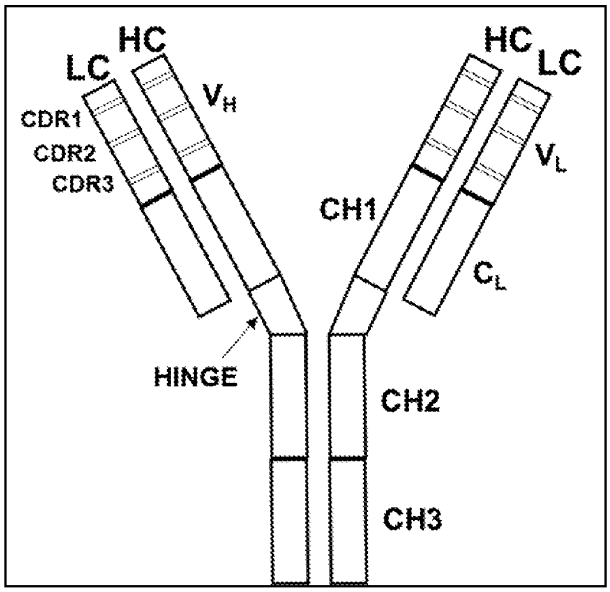
Before March 15, 2007, the structure of humanized monoclonal antibodies was well understood in the art. AMG1002, ¶¶22-28. Antibodies in general were known to be Y-shaped proteins made up of two identical "heavy chain"

[7] Unless otherwise stated, emphasis has been added throughout this Petition.

polypeptides and two identical "light chain" polypeptides. AMG1018, 7. The art taught that these heavy and light chains comprise a **variable region**—denoted as V_L (for the light chain) and V_H (for the heavy chain)—and a **constant region**—denoted as C_L (for the light chain) and C_H (for the heavy chain). AMG1018, 11-12.

The V_L and V_H regions each contain three "complementarity-determining regions" ("CDRs") which provide the antibody with its antigen-binding specificity. *Id.* The term "humanized" refers to an antibody having a human framework into which CDR regions from a non-human monoclonal antibody (*e.g.*, mouse) are inserted. AMG1007, 5:57-67.

This diagram depicts the basic antibody structure:



See AMG1002, ¶26.

B. The prior art taught that eculizumab is a humanized anti-C5 monoclonal antibody (h5G1.1) containing a hybrid IgG2/IgG4 constant region.

Before March 15, 2007, artisans knew that "eculizumab" was more than just a name; it was a known humanized anti- C5 monoclonal antibody derived from the mouse monoclonal antibody "5G1.1," and was frequently referred to as "h5G1.1" or "h5G1.1-mAb." *See* AMG1005, ¶[0052] ("[t]he antibody *h5G1.1-mAb* is currently undergoing clinical trials under the trade name *eculizumab*."); AMG1034, 1279 ("*h5G1.1-mAb*" is synonymous with "*5G1.1, eculizumab*, Alexion Pharmaceuticals."); and AMG1002, ¶¶44-46.[8] Moreover, Alexion has admitted that "*h5G1.1* … [was] well-known to one of ordinary skill in the art *as eculizumab*…." AMG1049, 838.

The prior art also taught structural aspects of eculizumab, including that eculizumab contains a hybrid IgG2/IgG4 constant region. AMG1034, 1279; AMG1049, 838-839; AMG1002, ¶¶47-50. For example, Tacken explicitly described using "h5G1.1-mAb (5G1.1, *eculizumab*; Alexion Pharmaceuticals)"

[8] *See also*, AMG1019, 56 ("*Eculizumab (5G1.1*; Alexion Pharmaceuticals) is a humanized monoclonal antibody."); AMG1020, 2123 ("*Eculizumab (5G1.1)*, the humanized anti-C5 mAb."); AMG1021, 1017 ("*Synonyms 5G1.1, h5G1.1*, C5 complement inhibitor (Alexion), h5G 1.1 scFv").

containing an "*IgG2/IgG4 constant region*." AMG1034, 1279. Likewise, when prosecuting a related application, Alexion told the USPTO that "it was well-known to one of ordinary skill in the art at the time of filing of priority applications [in 2002] that eculizumab has a G2/G4 Fc portion, *i.e.*, a mutated Fc portion."[9] AMG1049, 838-839. It was also well known before March 15, 2007, that antibodies with a hybrid IgG2/IgG4 constant region carried certain benefits, such as a reduced ability to elicit unwanted inflammatory events and lessened propensity to activate the complement system. AMG1032, 11, 19, 28; AMG1031 ("Mueller II"), 451; AMG1002, ¶¶48, 56.

C. The art taught eculizumab's amino acid sequence.

Before March 15, 2007, the art taught eculizumab's amino acid sequence. AMG1002, ¶¶51-60. In 2003, Bowdish described a peptide-antibody recombinant protein using eculizumab as the starter antibody and TPO-mimetic as the agonistic

[9] A POSA would have readily distinguished another "humanized 5G1.1" published in Thomas et al. from eculizumab because Thomas' antibody, was an "IgG4 isotype" called "h5G1.1 HuG4," and not an IgG2/IgG4 isotype. The artisan would have been able to readily distinguish eculizumab from them based on whether a hybrid IgG2/IgG4 constant region was present. AMG1023, 1389, 1396; AMG1002, ¶49.

peptide inserted in place of eculizumab's heavy chain CDR3 sequence. AMG1006, ¶¶[0191]-[0193]. Bowdish provided the full amino acid sequence for the TPO-mimetic-eculizumab antibody, and thus taught eculizumab's amino acid sequence with the exception of its heavy chain CDR3 sequence (HCDR3).[10] *Id*, Figures 13A-13B (SEQ ID NOs: 67 and 69); AMG1002, ¶¶51-53. But that HCDR3 sequence was also known in the art from Evans. Indeed, Bowdish cites and incorporates by reference Evans for making eculizumab. AMG1006, ¶[0191] ("[c]onstruction of 5G1.1 is described in [Evans], incorporated herein by reference.")[11].

Evans disclosed all six CDR regions of the original mouse 5G1.1 antibody, which are underlined in the sequences in Evans' Figures 18 and 19. AMG1007, 9:65-10:20, Figures 18-19. As Dr. Balthasar confirms, a POSA would have had a reason to replace the TPO mimetic from Bowdish's TPO-eculizumab antibody with Evans' HCDR3 to generate eculizumab for use in treating PNH. AMG1002, ¶¶149-152. When combined, the "scaffold" sequences in Bowdish and eculizumab

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[10] As Dr. Balthasar explains, a skilled artisan would have understood that the italicized portions of the sequences in Bowdish's Figures 13A-13B are "leader sequences" that are cleaved off during antibody maturation. AMG1002, ¶53; AMG1006, Figs. 13A-13B; AMG1045, 582.

[11] *See* note 5.

HCDR3 in Evans together form a humanized anti-C5 antibody having the claimed sequence. AMG1002, ¶¶140-148.

Another Alexion publication, Mueller, also provided complementary pieces of the eculizumab amino acid sequence, along with direction and guidance for making and using the antibody. AMG1002, ¶¶57-60. Mueller published in 1997 and disclosed the amino acid sequence of eculizumab's hybrid IgG2/IgG4 constant region and eculizumab's light chain constant region. AMG1008, 52-53, 58-61; AMG1002, ¶¶57-60. Mueller taught methods of developing recombinant antibodies to reduce immune-mediated organ transplant rejection, including antibodies comprising a hybrid IgG2/IgG4 constant region. *Id.*, 8:23-26, 12:27-30. Mueller described using eculizumab (referred to as "h5G1.1 CO12 HuG2/G4 mAb") as a control antibody that shares that same hybrid IgG2/IgG4 constant region as the experimental antibodies. *Id.*, 12:35-37, Figure 15. In these disclosures, Mueller provided the amino acid sequence of the hybrid IgG2/IgG4 constant region used in its antibodies—*i.e.*, the amino acid sequence of eculizumab's hybrid IgG2/IgG4 constant region. *Id.*, 58-61; AMG1002, ¶¶57-60.

Dr. Balthasar's Figure 10 below schematically shows which portions of eculizumab were disclosed in the prior art.[12]

[12] Green represents eculizumab sequences disclosed in the reference; blue

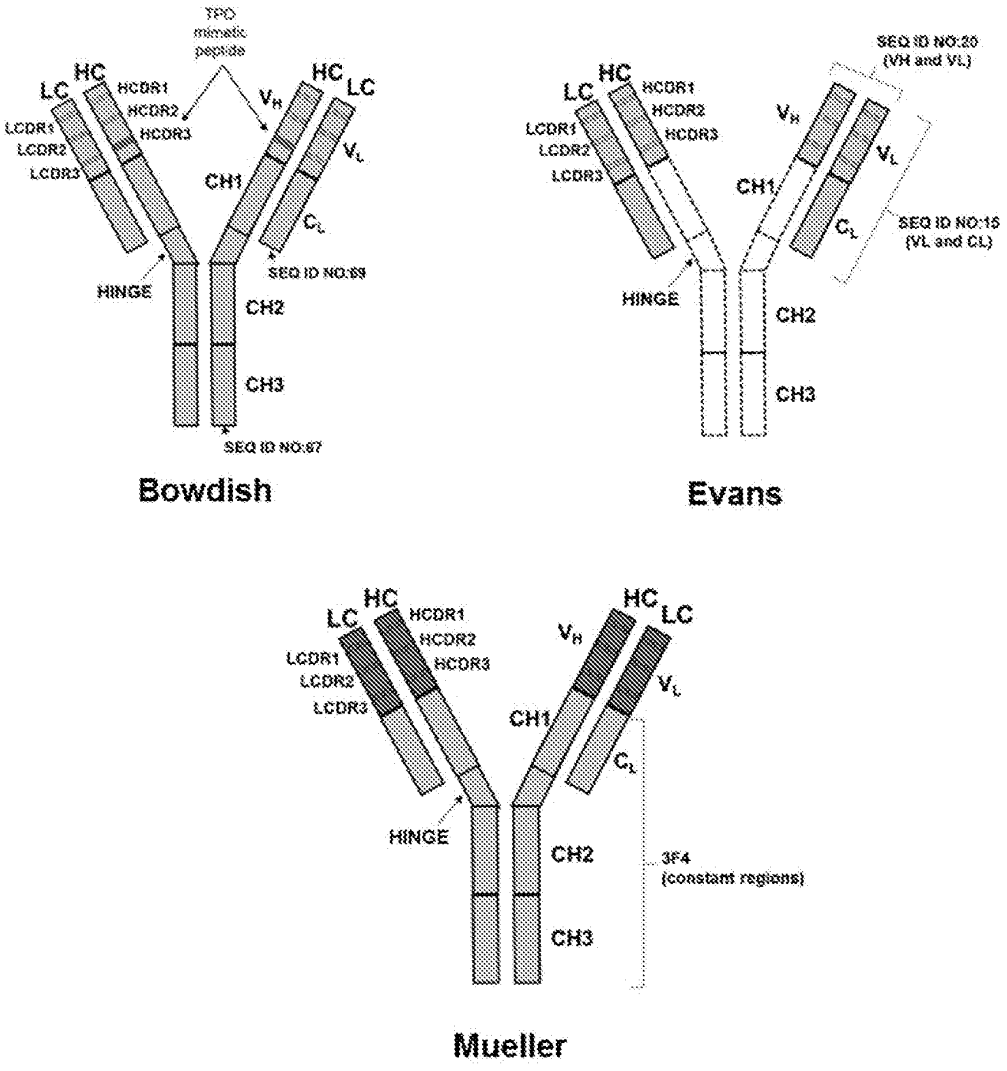


Figure 10.

See AMG1002, ¶¶60.

Though different portions of eculizumab's amino acid sequence were taught

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represents non-eculizumab sequences in each reference. AMG1002, ¶60.

in different references, the POSA is presumed to be knowledgeable of all the pertinent art—*i.e.*, all the portions of eculizumab and its general structure. *Standard Oil Co. v. American Cyanamid Co.*, 774 F.2d 448, 454 (Fed. Cir. 1985).

D. Eculizumab was a known, effective treatment for PNH.

Eculizumab was already a well-known antibody used to treat PNH; a pathological condition that results from complement system dysregulation. AMG1023, 1389. Alexion itself acknowledged that there were at least "17 different clinical studies using eculizumab that occurred prior to March 16, 2007." AMG1016, 734(¶3). Indeed, numerous Alexion publications described clinical studies using eculizumab for successfully treating PNH. AMG1004, Abstract, 554; AMG1042, Abstract; AMG1047, Abstract, 2560; AMG1011, Abstract; AMG1005, ¶¶[0081]-[0096]; AMG1012, Abstract, 1235; AMG1013, Abstract. Each of the publications described intravenous infusion of eculizumab in weekly 600 mg doses for four weeks followed by a 900 mg dose one week later, then biweekly 900 mg maintenance doses (summarized below).

Thus, the art taught not only that eculizumab was safe and effective for treating PNH, but also taught an effective dosing regimen and pharmaceutical composition.

Table 1 Clinical Trials Using Eculizumab to Treat PNH

Study	Alexion Study Number [13]	Dosing Regimen	Outcome
Hillmen (AMG1004) Phase 2 Pilot Study[14] 11 patients	C02-001	"infusions of 600 mg of eculizumab weekly for four weeks, followed one week later by a 900-mg dose and then by a dose of 900 mg every other week through week 12" AMG1004, 554.	"safe and well tolerated in patients with PNH" "improvement in the quality of life in patients with PNH" AMG1004, Abstract.
Hill '05 (AMG1047) Phase 2 Pilot Study Extension #1 11 patients	E02-001	"maintenance dose of 900 mg intravenously every 14 days" for 52 weeks (64 weeks total) AMG1047, 2560.	"sustained reductions in hemolysis and blood transfusions and continued improvement in quality of life." AMG1047, 2565.
Hill '04 (AMG1011)	X03-001	"10 of the 11 patients from the initial 3 month study have continued to	"eculizumab is well tolerated and has persisting efficacy … as

[13] Study numbers as identified in Alexion's statements made to the USPTO during prosecution. *See, e.g.*, AMG1014, 757, 765.

[14] Alexion also disclosed results from what appears to be the same pilot study in an Abstract published by Hillmen et al. in 2003. AMG1042 ("Hillmen '03").

Table 1 □Clinical Trials Using Eculizumab to Treat PNH

Study	Alexion Study Number [13]	Dosing Regimen	Outcome
Phase 2 Pilot Study Extension #2 10 patients		receive 900 mg eculizumab every other week for 2 years." AMG1011, Abstract.	well as improvement in the symptoms of PNH, for over 2 years of therapy." AMG1011, Abstract.
Bell (AMG1005) Summary of Pilot Study and Extensions 11 patients	N/A	"weekly 600 mg intravenous infusion of [eculizumab] … Patients received 900 mg of eculizumab 1 week later then 900 mg on a bi-weekly basis." AMG1005, ¶[0082].	"reduction in adverse symptoms associated with PNH" AMG1005, ¶[0096].
Hillmen '06 (AMG1012) Phase 3 "TRIUMPH" study 87 patients	C04-001	"infusions of 600 mg of eculizumab or placebo every week (±2 days) for 4 weeks, followed 1 week (±2 days) later by 900 mg of eculizumab or placebo, and then by a maintenance dose of 900 mg of eculizumab or placebo every 2 weeks (±2 days) through week 26." AMG1012, 1235.	"Eculizumab is an effective therapy for PNH." AMG1012, 1233.
Young (AMG1013)	C04-002	"Eculizumab was dosed as follows: 600 mg IV every 7 days x 4; 900	"eculizumab treatment markedly reduces intravascular hemolysis,

Table 1 Clinical Trials Using Eculizumab to Treat PNH

Study	Alexion Study Number [13]	Dosing Regimen	Outcome
Phase 3 "SHEPHERD" study 97 patients		mg 7 days later; and then 900 mg every 14±2 days." AMG1013, Abstract.	thereby providing clinical benefit to treated [PNH] patients." AMG1013, Abstract.

VI. Person of ordinary skill in art.

A person of ordinary skill in the art (POSA) is a hypothetical person, presumed to be aware of all pertinent art, who thinks along conventional wisdom in the art, and is a person of ordinary creativity. *KSR Int'l Co. v. Teleflex Inc.*, 550 US 398, 421 (2007); *Standard Oil*, 774 F.2d at 454. A POSA in the field of the '880 patent had knowledge of the scientific literature and have skills relating to the design and generation of antibodies, the complement system, and the application of antibodies as therapeutics before March 15, 2007. AMG1002, ¶21. A POSA also had knowledge of laboratory techniques and strategies used in immunology research, including practical applications of the same. *Id.* Typically, a POSA would have had an M.D. and/or a Ph.D. in immunology, biochemistry, cell biology, molecular biology, pharmaceutics, or a related discipline, with at least two years of experience in the field. *Id.* Also, a POSA may have worked as part of a

multidisciplinary team and drawn upon not only his or her own skills, but also taken advantage of certain specialized skills of others on the team, *e.g.*, to solve a given problem; . for example, a clinician and a formulation chemist may have been part of a team. *Id.*

VII. Claim construction.

Claims must be given their ordinary and customary meaning in light of the specification—"the meaning that the term would have to a person of ordinary skill in the art in question at the time of the invention." *Phillips v. AWH Corp.*, 415 F.3d 1303, 1312-1313 (Fed. Cir. 2015) (en banc); *see also*, 37 C.F.R. §42.100(b); 83 Fed. Reg. 51340, 51358 (Oct. 11, 2018).

The meaning of all claim terms in the '880 patent are plain on their face and require no further construction. AMG1002, ¶64. Amgen reserves the right to rebut any claim construction arguments Alexion might raise.

VIII. Identification of the challenge (37 C.F.R. §42.104(b)).

Amgen requests IPR based on the grounds summarized below.

Ground	35 U.S.C. Section (pre-AIA)	Claims	References
1	§102(b)	2	Hillmen
2	§102(b)	2	Hill '05
3	§103(a)	1, 3	Hillmen, Bell, and Wang

4	§103(a)	1, 3	Hill '05, Bell, and Wang
5	§103(a)	1-3	Bell, Bowdish, Evans, and Wang
6	§103(a)	1-3	Bell, Evans, Mueller, and Wang

- Hillmen et al., *N. Engl. J. Med.* 350(6):552-559 (2004) ("**Hillmen")** published February 5, 2004. AMG1004, 552.

- Hill et al., *Blood* 106(7):2559-2565 (2005) ("**Hill '05**") published October 1, 2005. AMG1047, 2559.

- US 2005/0191298 A1 ("**Bell**"), published September 1, 2005. AMG1005, face.

- US 2005/0271660 A1 ("**Wang**") published December 8, 2005. AMG1028, face.

- US 2003/0232972 A1 ("**Bowdish**"), published December 18, 2003. AMG1006, face .

- U.S. Patent No. 6,355,245 ("**Evans**'), issued March 12, 2002. AMG1007, face.

- WO 97/11971 ("**Mueller**"), published April 3, 1997. AMG1008, face.

These references are prior art under 35 U.S.C. §102(b) because each

published more than one year before March 15, 2007, the '880 patent's earliest claimed priority date.

IX. The same or substantially the same prior art or arguments were not previously presented to the Office.

The arguments and evidence presented herein were not before the examiner during prosecution and, therefore, do not constitute "the same or substantially the same prior art or arguments" under 35 U.S.C. §325(d).

As discussed in §III.B, the examiner rejected Alexion's claims as obvious over combinations of Hillmen, Evans, Wang and Thomas during prosecution. Thomas was the only reference relied upon for eculizumab sequence information. AMG1016, 596-602. And, the examiner allowed the '880 patent claims mistakenly believing—because of Alexion's mischaracterization of the art—that the sequence and structure of eculizumab were not already known. *Id.*, 762-763.

Though Hillmen, Evans, and Wang were referenced by the examiner during prosecution, this Petition presents them in a different light, along with new references—Bell, Bowdish, and Mueller—which teach the IgG2/IgG4 constant domain missing from the art combination raised during prosecution.

Bell and a parent application to Bowdish (US 2003/0049683 A1) was cited but not relied upon during prosecution, and Mueller was not cited at all. Thus, the art combinations here, which were not raised by the examiner during prosecution,

provide the complete sequence of eculizumab, thereby teaching the very thing the examiner mistakenly concluded was missing from the prior art. Consequently, this Petition is not the same/substantially the same as or cumulative of any previous arguments and § 325(d) does not preclude instituting this Petition.

X. Ground 1: Hillmen anticipates claim 2.

Hillmen anticipates claim 2. A reference anticipates when it discloses each and every claim limitation "either expressly or inherently." *In re Crish*, 393 F.3d 1253, 1256 (Fed. Cir. 2004). "Under the principles of inherency, if the prior art necessarily … includes[] the claimed limitations, it anticipates." *MEHL/Biophile Int'l Corp. v. Milgraum*, 192 F.3d 1362, 1363 (Fed. Cir. 1999). Dr. Balthasar confirms that Hillmen discloses all the limitations of claim 2, either expressly or inherently, and is enabling. AMG1002, ¶¶73-81.

A. Hillmen explicitly disclosed every element of claim 2, except for eculizumab's amino acid sequence.

Dr. Balthasar confirms that Hillmen disclosed all the limitations of claim 2, either expressly or inherently. AMG1002, ¶¶74-76. Hillmen expressly disclosed a "pharmaceutical composition comprising an antibody that binds C5" as claimed because Hillmen disclosed administering infusion of *eculizumab* formulations to patients, and eculizumab was a known antibody that binds C5. AMG1004, Abstract. For example, Hillmen disclosed that "patients with PNH received

infusions of *eculizumab*…." *Id.* Hillmen further described eculizumab as an "*antibody against terminal complement protein C5*." *Id.*

B. Alexion admitted that Hillmen's eculizumab necessarily has the claimed sequence.

Hillmen's antibody necessarily "comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4" because Alexion admitted that Hillmen's eculizumab possesses those very amino acid sequences.

During prosecution, Alexion submitted a list of eculizumab clinical studies—including Hillmen's Phase 2 Pilot Study (Study "C02-001")—and stated that "the antibody (eculizumab) used in each of the studies … *contained the heavy and light chain sequences of SEQ ID NOs: 2 and 4*." AMG1014, 767(¶6); *see also, id.*, 765 (study number "C02-001"). This admission is binding on Alexion. *See, e.g., Vitronics Corp. v. Conceptronic, Inc.*, 90 F.3d 1576, 1583 (Fed. Cir. 1996); *Tyler Refrigeration v. Kysar Indus. Corp.*, 777 F.2d 687, 690 (Fed. Cir. 1985).

The FDA Medical Review in Alexion's approval package for Soliris® confirmed that "Study C02-001" was published in "Hillmen, P et al. … NEJM. 2004; 350:552-558" (i.e., Hillmen). AMG1024, 109. Similarly, Australia's Pharmaceutical Benefits Advisory Committee ("PBAC") produced a public summary document for Soliris® showing that clinical trial "C02-001 (Pilot Study)" was published in "N Engl J Med 2004, 350:552-559." AMG1025, 2.

Because Alexion's admission confirms that the "eculizumab" disclosed in Hillmen necessarily comprised a heavy chain consisting of SEQ ID NO:2 and a light chain consisting of SEQ ID NO:4, Hillmen inherently discloses the claimed sequences.

Despite admitting to the Office that the eculizumab disclosed in Hillmen necessarily comprised a heavy chain consisting of SEQ ID NO:2 and a light chain consisting of SEQ ID NO:4, Alexion misleadingly argued during prosecution that its contribution over the art was the specific amino acid sequence of eculizumab. AMG1014, 586-587, 738-744. However, as *Crish* makes clear, "just as the discovery of properties of a known material does not make it novel, *the identification and characterization of a prior art material also does not make it novel*." *Crish*, 393 F.3d at 1258. There is "[a] long line of cases confirm[ing] that one cannot establish novelty by claiming a known material by its properties." *Id.*

In *Crish*, the applicant claimed an hINV promoter region based on its nucleotide sequence. The court stated that the pertinent inquiry for its anticipation analysis is "whether the claimed [invention] was new," and determined that:

> The promoter region of hINV *was not new* … hINV was *known and used years before* … The only arguable contribution to the art that Crish's application makes is the identification of the nucleotide sequence of the promoter region of hINV. However, just as the discovery

> of properties of a known material does not make it novel, *the identification and characterization of a prior art material also does not make it novel*.

Id.; *see also, Atlas Powder Co. v. IRECO Inc.,* 190 F.3d 1342, 1347 (Fed. Cir. 1999 ("the discovery of a previously unappreciated property of a prior art composition … does not render the old composition patentably new to the discoverer."); *Abbott Labs. v. Baxter Pharms.*, 471 F. 3d 1363, 1368 (Fed. Cir. 2006) (lack of knowledge of a compound's property is "wholly irrelevant to the question of whether the [a patent] claims something 'new' over the disclosure of the [prior art].").

This precedent is squarely applicable here and compels finding anticipation. The '880 patent's mere claim to a composition comprising an amino acid sequence that Alexion admits was a property of a prior art compound (eculizumab) contributes nothing over the prior art..

C. Hillmen's disclosure is enabling.

Hillmen also provided an enabling disclosure of the claimed pharmaceutical composition. An anticipatory publication "must be capable, *when taken in conjunction with the knowledge of those skilled in the art* to which it pertains, of placing that invention in the possession of the public." *In re Donohue*, 632 F.2d 123, 125 (CCPA 1980) ("*Donohue I*"); *see also*, *In re Donohue*, 766 F.2d 531, 533

(Fed. Cir. 1985) ("*Donohue II*") (public possession "is effected if one of ordinary skill in the art could have *combined the publication's description* of the invention *with his own knowledge* to make the claimed invention."); and *Elan Pharms. v. Mayo Found.*, 346 F.3d 1051, 1054 (Fed. Cir. 2003).

To determine whether experimentation would be undue, one must examine "(1) the quantity of experimentation; (2) the amount of direction or guidance present; (3) the presence or absence of working examples; (4) the nature of the invention; (5) the state of the prior art; (6) the relative skill of those in the art; (7) the predictability or unpredictability of the art; and (8) the breadth of the claims." *Impax Labs. v. Aventis Pharms.*, 545 F.3d 1312, 1314-1315 (Fed. Cir. 2008) (citing *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988)). Applying these factors, Dr. Balthasar explains that Hillmen, coupled with the general knowledge in the art, would have enabled a POSA to possess a pharmaceutical composition comprising eculizumab having the claimed sequences. AMG1002, ¶¶77-80.

The alleged invention claim 2 are directed to a pharmaceutical composition comprising an antibody (eculizumab). This antibody was expressly disclosed in Hillmen; and Alexion admitted that Hillmen's eculizumab had the claimed sequences. AMG1004, Abstract, 553-554; AMG1016, 737(¶6), 736; AMG1024, 109; AMG1025, 2. And the general knowledge in the prior art was replete with disclosures, direction, and guidance about eculizumab's structure and its amino

acid sequence. AMG1002, ¶78.

Dr. Balthasar's Figure 10 above illustrates the art disclosing eculizumab amino acid sequences. *See* §IV, *supra*; AMG1002, ¶60. Dr. Balthasar explains two independent ways in which a POSA would have obtained eculizumab's amino acid sequence:

(1) A POSA would have known that **Bowdish** disclosed the entire amino acid sequence of eculizumab with the exception of the heavy chain CDR3 region, and that **Evans** disclosed the eculizumab heavy chain CDR3 region (the missing piece from Bowdish). AMG1006, ¶¶[0191]-[0193], Figure 13A-13B (SEQ ID NOs:67 and 69); AMG1007, 44:4-13 (SEQ ID NO:20); AMG1002, ¶78.

(2) A POSA also would have known that **Evans** disclosed the amino acid sequences of eculizumab's heavy and light chain variable domains, and **Mueller** disclosed the hybrid IgG2/IgG4 heavy chain and light chain constant domains of eculizumab. AMG1007, 44:4-13 (SEQ ID NO:20); AMG1008, 52-53, 58-61; AMG1002, ¶78.

And given the high level of skill in the relevant field, the POSA would have readily obtained eculizumab's sequences as claimed from the art using only routine experimentation.

Armed with the general knowledge in the relevant field, a POSA reading

Hillmen would not have "needed to experiment unduly to gain possession of the invention." *Impax*, 545 F.3d at 1315-1316; AMG1002, ¶79. Here, just as in *In re Donohue*, "the primary reference named a composition falling within the scope of the claims and indicated that it had previously been made and tested; additional references showed that a method of making this composition would have been within the knowledge of one of ordinary skill in the art." *Donohue I* at 126.

The law compels finding anticipation here. The claimed antibody was not new—Hillmen taught administering a pharmaceutical composition comprising eculizumab. Under *Crish*, simply adding the amino acid sequence of an already known antibody to the claims does not confer novelty. Moreover, Alexion's admission that Hillmen's eculizumab inherently possesses the claimed sequences is binding under *Vitronics.* And under *Donohue I* and *Donohue II*, Hillmen is enabling in view of the multiple publications of eculizumab's sequence in the art and pharmaceutical compositions that were standard and well-known in the art, particularly for intravenous therapeutic antibody compositions. AMG1002, ¶80. Accordingly, Hillmen anticipates claim 2.

XI. Ground 2: Hill '05 anticipates claim 2.

Dr. Balthasar confirms that Hill '05 disclosed a pharmaceutical composition comprising eculizumab—a known antibody that necessarily possesses the claimed amino acid sequences— and is enabling. AMG1002, ¶¶82-92. Thus, Hill '05

anticipates claim 2. *In re Crish*, 393 F.3d 1253, 1256 (Fed. Cir. 2004).

A. Hill '05 explicitly disclosed every element of claim 2, except for eculizumab's amino acid sequence.

Hill '05 disclosed administering eculizumab formulations to patients over a one-year extension period, concluding that "[r]esults of this 1-year extension study showed that eculizumab therapy continues to be safe and well tolerated in PNH patients." AMG1047, 2565.

Hill '05 disclosed "an antibody that binds C5" as claimed because Hill '05 disclosed administering *eculizumab* formulations to patients, and eculizumab was a known antibody that binds C5. *Id*, Abstract, 2560. For example, Hill '05 disclosed that patients in the extension study received a "maintenance dose of *eculizumab*," and "this maintenance dose of 900 mg intravenously every 14 days was continued throughout the extension study period." *Id*. Hill '05 further described eculizumab as an "a *humanized monoclonal antibody* that specifically targets the *complement protein C5*." *Id*, 2559.

B. Hill '05's eculizumab necessarily has the claimed sequence.

Hill '05 also disclosed the claimed antibody that "comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4" because as already discussed above, Alexion admitted that the eculizumab used in Hill '05's trial—which was already in the public domain—necessarily possesses the

claimed amino acid sequence. AMG1002, ¶¶83-87.

Hill '05 is an Alexion publication describing results from a one-year extension study involving the same 11 patients enrolled in the Hillmen Phase 2 Pilot Study. *Id*, 2559-2560. In the list of eculizumab clinical studies Alexion submitted to the USPTO during prosecution, Alexion also included Hill '05's extension study (Study number "E02-001") when affirmatively stating that "the antibody (eculizumab) used in each of the studies … *contained the heavy and light chain sequences of SEQ ID NOs: 2 and 4*." AMG1016, 737(¶6); *see also, id.*, 735 (study number "E02-001"). Again, Alexion's admission is binding. *Vitronics* 90 F.3d at 1583; *Tyler Refrigeration*, 777 F.2d at 690.

Hill '05 explicitly confirms that it is an extension study of Hillmen's Phase 2 Pilot Study, stating "[w]e previously reported the outcome of an open-label study of eculizumab in patients with PNH [*citing Hillmen*] … Here we report the results of a 1-year follow-up study designed to assess the long-term efficacy and safety of eculizumab in patients with PNH." AMG1047, 2559. Hill '05 also disclosed that "[t]he acute-phase study was an initial 12-week, open-label trial of eculizumab in 11 patients with PNH and has been previously described in detail [*citing Hillmen*] … The current study was an open-label *extension of that acute-phase study*." *Id*, 2560. This is consistent with Alexion's admission to the USPTO that study number E02-001 (Hill '05) is entitled "Extension Study in Patients … who Previously

Participated in Study C02-001 [Hillmen]." AMG1016, 735.

Because Alexion's admission confirms that the "eculizumab" disclosed in Hill '05 necessarily comprised a heavy chain consisting of SEQ ID NO:2 and a light chain consisting of SEQ ID NO:4, as claimed, Hill '05 inherently discloses an antibody comprising the claimed sequences. AMG1002, ¶¶83-87. And, as discussed above, merely claiming the amino acid sequence of a known antibody—as the '880 patent claims—does not confer novelty. *Crish*, 393 F.3d at 1258; *Atlas Powder*, 190 F.3d at 1347; *Abbott Labs.*, 471 F.3d at 1368.

C. Hill '05's disclosure is enabling.

Hill '05 is enabling for the same reasons discussed above in Ground 1. AMG1002, ¶¶88-92. The alleged invention and claim 2 are directed to a pharmaceutical composition comprising an antibody (eculizumab) that was expressly disclosed in Hill '05. AMG1047, Abstract, 2559-2560. And as already discussed, the general knowledge in the prior art had ample disclosures, direction, and guidance on eculizumab and its amino acid sequence. AMG1002, ¶89. *See* §IV, *supra*.

Again, Dr. Balthasar explains two independent ways in which a POSA would have obtained eculizumab's amino acid sequence: (1) from the "5G1.1" scaffold sequences in **Bowdish** and the HCDR3 sequence in **Evans** (AMG1006, ¶¶[0191]-[0193], Figure 13A-13B (SEQ ID NOs:67 and 69); AMG1007, 44:4-13

(SEQ ID NO:20)); or (2) from the heavy and light chain variable region sequences in **Evans** and the heavy and light chain constant domain sequences in **Mueller** (AMG1007, 44:4-13 (SEQ ID NO:20); AMG1008, 52-53, 58-61). AMG1002, ¶89. Given the high level of skill in the relevant field, the POSA would have readily obtained eculizumab's sequences as claimed from the art using only routine experimentation. *Impax*, 545 F.3d at 1315-1316; AMG1002, ¶90. And formulation preparation techniques were standard and well-known in the art, particularly for intravenous therapeutic antibody formulations. AMG1002, ¶91; AMG1056-1076.

As in Ground 1, the law also compels finding anticipation in Ground 2. The claimed antibody was not new because Hill '05 taught administering a pharmaceutical composition comprising eculizumab. Later claiming the amino acid sequence of a known antibody does not make what was already publicly known now novel. *Crish*, 393 F.3d at 1258; *Atlas Powder,* 190 F.3d at 1347; *Abbott Labs.*, 471 F. 3d at 1368. Moreover, Alexion is bound by its admission that Hill '05's eculizumab inherently possesses the claimed sequences. *Vitronics*, 90 F.3d at 1583; *Tyler Refrigeration*, 777 F.2d at 690. And a POSA taking Hill '05's disclosure in conjunction with the knowledge of skill in the art would have been able to possess the claimed pharmaceutical composition without undue experimentation. *Donohue I*, 632 F.2d at 125; *Donohue II*, 766 F.2d at 533. Under *Donohue I* and *Donohue II*, Hill '05 is enabling in view of the multiple publications of eculizumab's sequence

in the art and pharmaceutical compositions that were standard and well-known in the art, particularly for intravenous therapeutic antibody compositions. AMG1002, ¶¶89-91.

XII. Ground 3: claims 1 and 3 would have been obvious over Hillmen, Bell, and Wang.

As already discussed, Hillmen disclosed a pharmaceutical composition comprising eculizumab (an anti-C5 antibody) having the claimed sequences either expressly or inherently. *See* § IX.

Claims 1 and 3 further require that the pharmaceutical composition is "a sterile, preservative free, 300 mg single-use dosage form comprising 30 ml of a 10 mg/ml antibody [or anti-C5 antibody] solution." AMG1001, 39:2-8 and 13-16. Those additional limitations were taught in Bell and Wang. And, a POSA would have had reason to combine Bell and Wang with Hillmen to arrive at claims 1 and 3, with a reasonable expectation of success. AMG1002, ¶¶93-109.

Like Hillmen, Bell taught methods of treating a patient suffering from PNH using a pharmaceutical composition comprising eculizumab. AMG1005, ¶¶[0012], [0052], [0081]-[0096]. Indeed, Bell disclosed a Phase 2 Pilot Study involving 11 PNH patients treated with eculizumab over a period of 12 weeks—identical to that disclosed in Hillmen. *Compare* AMG1005, ¶¶[0081]-[0082] *with* AMG1004, Abstract and 553-554. As Dr. Balthasar explains, Bell and Hillmen shared the

following identical features:

- antibody (eculizumab);
- patient pool (6 male, 5 female);
- administration route (IV);
- dosing regimens;[15] and
- resulting data.

AMG1002, ¶¶40, 111; AMG1005, ¶¶[0081]-[0096].[16] Given the substantial overlap, a POSA would have had ample reason to combine Hillmen and Bell, with

[15] 600 mg eculizumab weekly for four weeks, followed by a 900 mg dose, then 900 mg eculizumab every other week through week 12.

[16] Because the clinical study taught in Bell is the same C02-001 study disclosed in Hillmen, which discloses the eculizumab amino acid sequences of SEQ ID NOs: 2 and 4, Bell, too, therefore anticipates claim 2 for the same reasons discussed above for Hillmen. AMG1016, 737(¶6); *see also, id.*, 735 (study number "C02-001"); AMG1005, ¶[0082]; AMG1002, ¶94, n6. Hill '05, Hill '04, Hillmen '06, and Young '06 also anticipate claim 2 for the same reasons. AMG1016, 737(¶6), 735 (study numbers E02-001, X03-001, C04-001, and C04-002, respectively); AMG1047, 2559-2560; AMG1011, Abstract; AMG1012, 1235; AMG1013, Abstract.

a reasonable expectation of success at achieving the claimed subject matter.

Wang taught therapeutic antibody formulations, and explicitly taught formulating "*eculizumab* as the antibody therapeutic." AMG1028, ¶[0004]. With all three references explicitly teaching aspects of eculizumab, a POSA would have had ample reason to combine Hillmen, Bell, and Wang with a reasonable expectation of success at achieving the claimed subject matter. No objective indicia of nonobviousness support patentability. *See* §XV.

Bell taught "sterile, preservative-free": Bell explains that its eculizumab formulations "*must be sterile* and non-pyrogenic," and that they "*may* contain … preservatives…" (i.e., preservatives are optional). AMG1005, ¶[0062]; AMG1002, ¶98. Dr. Balthasar further explains that a POSA would also have generally known that therapeutic antibodies are routinely prepared in sterile, preservative-free solutions. AMG1002, ¶98. In fact, of 22 monoclonal antibodies approved by the FDA before March 15, 2007—and of which the POSA would have been aware—100% of them were formulated in *sterile, preservative-free* solutions. AMG1002, ¶¶98; AMG1056-AMG1076.

Bell and Hillmen taught a "300mg single-use dosage form": As Dr. Balthasar explains, a POSA would have understood that a "unit dosage form" of an antibody as taught in Bell is formulated for use in a single patient and is therefore a "single-use" dosage form as claimed. AMG1002, ¶99; AMG1046, 2989;

AMG1051, 1. For example, a POSA would have generally known that antibody dosage forms should not be shared among patients or returned to storage after opening, to prevent contamination and unnecessary health risks. AMG1002, ¶100; AMG1046, 2989. Alexion agrees, telling the USPTO during prosecution[17] that skilled artisans "would have been well aware … that single-dose vials are intended for a single procedure or injection and should always be discarded at the end of the procedure/injection. They should never be stored for later use or subsequent procedures, even if for the same patient." AMG1026, 208-209 (internal quotation omitted).

Thus, Bell would have motivated a POSA to prepare eculizumab formulations as single-use dosage forms as claimed in claims 1 and 3 using routine formulation preparation techniques that were standard and well-known in the art, particularly for intravenous therapeutic antibody formulations. AMG1002, ¶¶99-100. Indeed, the '880 patent mentions no "newly discovered" advantages of the single-use unit dosage form claim limitation.

Moreover, a POSA would have arrived at the 300 mg single-use dosage form through simple convenience and a logical application of knowledge in the art.

[17] U.S. Appl. No. 13/426,973 is the abandoned parent application of the '880 patent. AMG1001, face.

AMG1002, ¶¶101-104. Both Hillmen and Bell explicitly taught administering eculizumab in doses of 600 mg and 900 mg (AMG1004, 554; AMG1005, ¶[0082]), thus prompting a POSA, as Dr. Balthasar explains, to administer those same doses because they were each shown to successfully treat PNH. AMG1004, 558-559; AMG1005, ¶¶[0089]-[0096]; AMG1002, ¶101.

To accommodate dosages of 600 mg and 900 mg dosages, a 300 mg single-use dosage amount would have been the most obvious and convenient (and thus predictable) means to a POSA. It would have been easier to achieve 600 mg and 900 mg doses with two and three 300 mg dosages, respectively, than to separately manufacture those two strengths. AMG1002, ¶¶102-103. *See KSR Int'l Co. v. Teleflex Inc.*, 550 US 398, 416 (2007) ("When a work is available in one field, design incentives and other market forces can prompt variations of it … If a person of ordinary skill in the art can implement a predictable variation, and would see the benefit of doing so, § 103 likely bars its patentability.") Tellingly, Alexion admitted during a European opposition proceeding for a related EP patent[18] that there is nothing critical about the claimed single-usedosage form beyond convenience: "*simple dilution* of product required by the label *conveniently* provides the required 600 or 900 mg administration dosages." AMG1027,

[18] EP Patent No. 2359834 is a European counterpart to the '880 patent.

1104(¶5.3.9).

According to Dr. Balthasar, a POSA at the time of invention would have had a reasonable expectation of successfully preparing a 300 mg single-use dosage form because doing so would have been simple, more efficient, and more convenient than developing, validating, and storing separate dosage forms for each of the 600 mg and 900 mg doses taught in Hillmen and Bell—*i.e.*, a "one size fits all" approach. AMG1002, ¶103-104. Moreover, Bell taught methods for preparing unit dosage forms, and both Hillmen and Bell described successfully preparing and administering 600 mg and 900 mg doses. AMG1004, 553-554; AMG1005, ¶¶[0058]-[0060], [0062], [0082]; AMG1002, ¶104.

Wang taught a "30 ml of a 10 mg/ml" solution: Wang taught eculizumab formulations of between *1 and 30 mg/ml*, completely encompassing the claimed 10 mg/ml concentration.[19] AMG1028, Fig. 10, ¶¶[0170]-[0172]; AMG1002, ¶¶105-

[19] Wang's nebulization formulation is equally relevant to intravenous infusion. A POSA would take Wang as confirmation that eculizumab, formulated at a concentration of 1 to 30 mg/mL, would be sufficiently stable and active to be used as a drug. AMG1002, ¶105. And, Wang also shows by SDS-PAGE and HPLC that eculizumab maintains its integrity following formulation and subsequent nebulization. AMG1028, ¶[0173]; AMG1002, ¶105.

108. "[A] prior art reference that discloses a range encompassing a somewhat narrower claimed range is sufficient to establish a *prima facie* case of obviousness." *In re Peterson*, 315 F.3d 1325, 1330 (Fed. Cir. 2003); *In re Wertheim*, 541 F.2d 257 (CCPA 1976) (finding when claimed values "overlap or lie inside ranges disclosed by the prior art," a *prima facie* case of obviousness exists.).

Wang's eculizumab concentrations are also consistent with the general knowledge in the art that 10 mg/ml was a common concentration used for formulating therapeutic antibodies. AMG1002, ¶106; AMG1029, Table 1; AMG1030, Table 1. The law is settled: "[w]here the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experimentation." *In re Aller*, 220 F.2d 454, 456 (CCPA 1955). Moreover, there is no evidence that the claimed 10 mg/ml concentration is in any way critical to the claimed method.[20] AMG1002, ¶107.

Moreover, given the state of the art as discussed above, a POSA would have prepared the eculizumab formulation in 30 ml based on simple arithmetic. As discussed, 300 mg dosage units would be the easiest way to have one dosage form

[20] Alexion argued in Europe that 10 mg/ml improved stability (AMG1027, 1104(¶5.3.8), but as discussed at Section XV, Alexion's arguments are meritless.

achieve 600 mg and 900 mg dosages. Likewise, 10 mg/mL was the prevailing standard concentration for this class of drug. Therefore, a dose of 300 mg concentrated at 10 mg/ml must be in 30 ml of solution (10 mg/ml x 30 ml = 300 mg). AMG1002, ¶108.

Notably, the '880 patent specification mentions no newly discovered advantage of a 30 ml dosage volume to suggest inventiveness in any way. Alexion even stated that the "low-volume liquid formulation … is *easy for the clinician to store and use*" and that the 30 ml size "*minimises the volume which must be stored* on site at each clinic." AMG1027, 1638(¶3.5), 1104(¶5.3.9). Easier storage for lower volumes compared to larger is common knowledge, not inventive. AMG1002, ¶108.

A POSA would have had a reasonable expectation of success here because formulating the doses into 300 mg single-use formulations of 30 ml of a 10 mg/ml sterile, preservative-free solution required mere conventional wisdom and routine skill. AMG1002, ¶104.

* * *

Accordingly, a POSA would have had motivation to arrive at a "sterile, preservative free, 300 mg single-use dosage form comprising 30 ml of a 10 mg/ml [anti-C5] antibody solution," as claimed, with a reasonable expectation of success based on the teachings in Hillmen, Bell and Wang, rendering claims 1 and 3

obvious.

XIII. Ground 4: claims 1 and 3 would have been obvious over Hill '05, Bell, and Wang.

As already discussed, Hill '05 disclosed a pharmaceutical composition comprising eculizumab (an anti-C5 antibody) having the claimed sequences either expressly or inherently. *See* §X. AMG1002, ¶¶83-87, 110. Claims 1 and 3 further require that the pharmaceutical composition is "a sterile, preservative free, 300 mg single-use dosage form comprising 30 ml of a 10 mg/ml antibody [or anti-C5 antibody] solution." AMG1001, 39:2-8 and 13-16. Those additional limitations were taught in Bell and Wang, and, a POSA would have had reason to combine Bell and Wang with Hill '05 to arrive at claims 1 and 3, with a reasonable expectation of success for the same reasons as already discussed for Ground 3. AMG1002, ¶¶110-120.

For example, Hill '05 and Bell taught methods of treating a patient suffering from PNH using a pharmaceutical composition comprising eculizumab. AMG1005, ¶¶[0012], [0052], [0081]-[0096]. Wang taught therapeutic antibody formulations, and explicitly taught formulating "*eculizumab* as the antibody therapeutic." AMG1028, ¶[0004]. With all three references explicitly teaching pharmaceutical compositions of eculizumab, a POSA would have had ample reason to combine Hill '05, Bell, and Wang with a reasonable expectation of

success because:

(i) Bell taught that the humanized antibody unit dose formulation "*must be sterile* and non-pyrogenic" and "*may* contain … preservatives…" (i.e., preservatives are not required). AMG1005, ¶[0062]; AMG1002, ¶116. And a POSA would have known that therapeutic antibodies are routinely prepared in sterile, preservative-free solutions. AMG1002, ¶116; AMG1056-AMG1076.

(ii) Wang taught eculizumab formulations of between 1 to 30 mg/ml, encompassing the claimed 10 mg/ml concentration. AMG1028, Fig. 10, ¶[0067]. Wang's disclosure was also consistent with the general knowledge in the art. *See, e.g.,* AMG1029, Table 1; AMG1030, Table 1; AMG1002, ¶118.

(iii) A POSA would have prepared the eculizumab formulation in 30 ml single-use dosage forms using simple mathematics based on a 300 mg dosage unit concentrated at 10 mg/ml. AMG1002, ¶119. Again, there is nothing critical about a 30 ml volume. AMG1027, 1638(¶3.5), 1104(¶5.3.9). Accordingly, a POSA routinely arriving at a 300 mg single-use dose concentrated at 10 mg/ml would also have routinely arrived at a volume of 30 ml. AMG1002, ¶119.

For these reasons, a POSA reading Hill '05, Bell, and Wang would have had

a reasonable expectation of successfully arriving at the claimed pharmaceutical composition recited in claims 1 and 3 for the same reasons discussed above in Ground 3. AMG1002, ¶¶115-120; *see* Section XI.

XIV. Ground 5: claims 1-3 would have been obvious over Bell, Wang, Bowdish, and Evans.

Discussed below, a POSA would have had a reason to combine Bell, Bowdish, Evans, and Wang with a reasonable expectation of successfully making the pharmaceutical composition of claims 1-3. AMG1002, ¶¶121-154. And no objective indicia weigh in favor of patentability. *See* Section XV.

A. Bell and Wang taught all the limitations of claims 1-3 except eculizumab's amino acid sequence.

Claims 1-3 are each directed to pharmaceutical compositions "for use in treating a patients afflicted with paroxysmal nocturnal hemoglobinuria (PNH)" (claim 1, or comprising an anti-C5 antibody (claims 2 and 3). Claims 1 and 3 also require that the pharmaceutical composition is "a sterile, preservative free, 300 mg single-use dosage form comprising 30 ml of a 10 mg/ml antibody [or anti-C5 antibody] solution." AMG1001, 39:2-8 and 13-16. Bell and Wang taught each of these elements of claims 1-3:

Bell taught a "[p]harmaceutical composition for use in treating a patients afflicted with paroxysmal nocturnal hemoglobinuria (PNH)"/ "anti-C5 antibody": Bell taught treating a patient suffering from PNH using a

pharmaceutical composition comprising eculizumab, a known anti-C5 antibody. AMG1005, ¶¶[0012], [0021], [0052], [0081]-[0096]; AMG1002, ¶¶128-132.

Bell taught "sterile, preservative free": Bell explains that its eculizumab compositions "*must be sterile* and non-pyrogenic," and that they "*may* contain … preservatives…" (i.e., preservatives are optional). AMG1005, ¶[0062]; AMG1002, ¶133. Dr. Balthasar further explains that a POSA would also have known that therapeutic antibodies are routinely prepared in sterile, preservative-free solutions. AMG1002, ¶133. Indeed, 100% of the 22 monoclonal antibodies approved by the FDA before March 15, 2007 were formulated in *sterile, preservative-free* solutions. AMG1002, ¶133; AMG 1056-AMG1076.

Bell and Wang taught a "300 mg single-use dosage form comprising 30 ml of a 10 mg/ml antibody solution" : Bell stated that eculizumab can be "administered in a variety of *unit dosage forms*."[21] AMG1005, ¶[0058]. As Dr. Balthasar explains, a POSA would have understood that a "unit dosage form" of an antibody as taught in Bell is formulated for use in a single patient and is therefore a **"single-use" dosage** form as claimed. AMG1002, ¶135; AMG1046, 2989; AMG1051, 1. As already discussed, a POSA would have generally known that antibody dosage forms should not be shared among patients or returned to storage

[21] As did Evans. AMG1007, 17:60-61

after opening, to prevent contamination and unnecessary health risks, and Alexion agrees. AMG1002, ¶135; AMG1046, 2989; AMG1026, 208-209.

Moreover, a POSA would have arrived at the **300 mg** strength through simple convenience and a logical application of knowledge in the art. AMG1002, ¶136. To accommodate Bell's dosages of 600 mg and 900 mg dosages (AMG1005, ¶[0082]), a 300 mg single-use unit dosage amount would have been the most obvious and convenient (and thus predictable) means to a POSA. It would have been easier to achieve 600 mg and 900 mg doses with two and three 300 mg dosages, respectively, than to separately manufacture those two strengths. AMG1002, ¶136. *See KSR*, 550 US at 416. Alexion has already admitted during a European opposition proceeding for a related EP patent that there is nothing critical about the claimed single-use dosage form beyond convenience: "*simple dilution* of product required by the label *conveniently* provides the required 600 or 900 mg administration dosages." AMG1027, 1104(¶5.3.9).

According to Dr. Balthasar, a POSA at the time of invention would have had a reasonable expectation of successfully preparing a 300 mg single-use dosage form because Bell taught methods for preparing unit dosage forms, and described successfully preparing and administering 600 mg and 900 mg doses. AMG1005, ¶¶[0058]-[0060], [0062], [0082]; AMG1002, ¶137.

Lastly, Wang taught liquid eculizumab formulations of between *1 and 30*

mg/ml, completely encompassing the claimed **10 mg/ml** concentration, rendering it obvious. AMG1028, Fig. 10, ¶¶[0170]-[0172]; AMG1002, ¶138. *In re Peterson*, 315 F.3d at 1330; *In re Wertheim*, 541 F.2d 257.

Wang's eculizumab concentrations are also consistent with the general knowledge in the art that 10 mg/ml was a common concentration used for formulating therapeutic antibodies. AMG1002, ¶138; AMG1029, Table 1; AMG1030, Table 1. Moreover, there is no evidence that the claimed 10 mg/ml concentration is in any way critical to the claimed method.[22]

Third, given the state of the art as discussed above, a POSA would have prepared the eculizumab formulation in **30 ml** single-use dosage forms based on simple arithmetic: a dose of 300 mg concentrated at 10 mg/ml must be in 30 ml of solution (10 mg/ml x 30 ml = 300 mg). AMG1002, ¶139.

Notably, the '880 patent specification mentions no newly discovered advantage of a 30 ml dosage volume to suggest inventiveness in any way. Alexion even acknowledged that the "low-volume liquid formulation … is *easy for the clinician to store and use*" and that the 30 ml size "*minimises the volume which must be stored* on site at each clinic." AMG1027, 1638(¶3.5), 1104(¶5.3.9). Easier

[22] As discussed at Section XV, Alexion's arguments in Europe that 10 mg/ml improved stability (AMG1027, 1104(¶5.3.8) are meritless.

storage for lower volumes compared to larger is common knowledge, not inventive. AMG1002, ¶139.

A POSA would have had a reasonable expectation of success here because formulating the doses taught in Bell into 300 mg single-use compositions of 30 ml of a 10 mg/ml sterile, preservative-free solution required mere conventional wisdom and routine skill, and eculizumab compositions of 10 mg/ml were already known in the art from Wang. AMG1002, ¶¶137-138.

B. Bowdish and Evans taught the claimed amino acid sequences.

Bowdish taught methods of making peptide-antibody chimeric proteins, and described using a humanized anti-C5 antibody as the starter "scaffold" antibody sequence for creating a recombinant TPO-mimetic. Bowdish provided the full antibody amino acid sequence except for the heavy chain CDR3 (HCDR3) sequence, which was replaced with the TPO-mimetic peptide sequence (LPIEGPTLRQWLAARAPV). AMG1006, ¶¶[0191]-[0193], Figs. 13A-13B, and SEQ ID NOs.:67 and 69; AMG1002, ¶¶140-145. As explained in detail below, the missing HCDR3 sequence from Bowdish was taught by Evans. AMG1002, ¶146. Dr. Balthasar's Figure 11 below depicts this combination of art providing the complete eculizumab sequence:

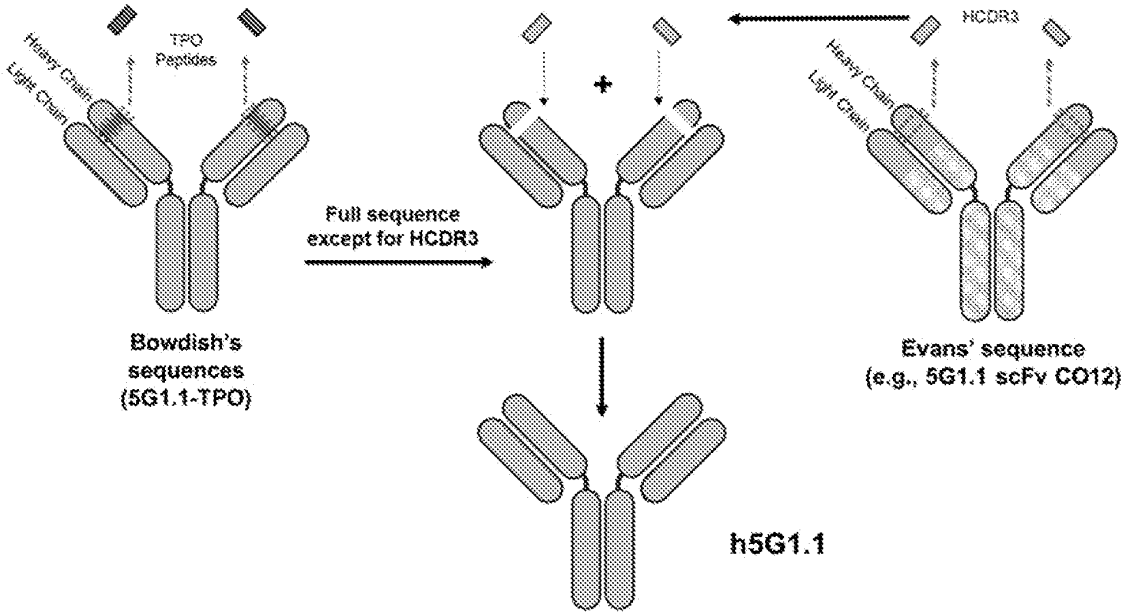


Figure 11.

AMG1002, ¶122.

Bowdish provides the complete sequences of the heavy and light chains of a recombinant TPO-mimetic-containing antibody ("5G1.1+TPO") as SEQ ID NO:67 ("5G1.1-TPO Heavy Chain") and SEQ ID NO:69 ("5G1.1 Light Chain") in Figures 13A and 13B, respectively. AMG1006, Figures 13A-13B; AMG1002, ¶143. Dr. Balthasar's Figure 3 below illustrates the structure of Bowdish's 5G1.1+TPO, showing the location of the TPO-mimetic peptide (blue) in the HCDR3 region of the polypeptide of SEQ ID NOs:67 (heavy chain), and the polypeptide of SEQ ID NO:69 (light chain), where green represents sequences 100 % identical to SEQ ID NO:2 and SEQ ID NO:4::

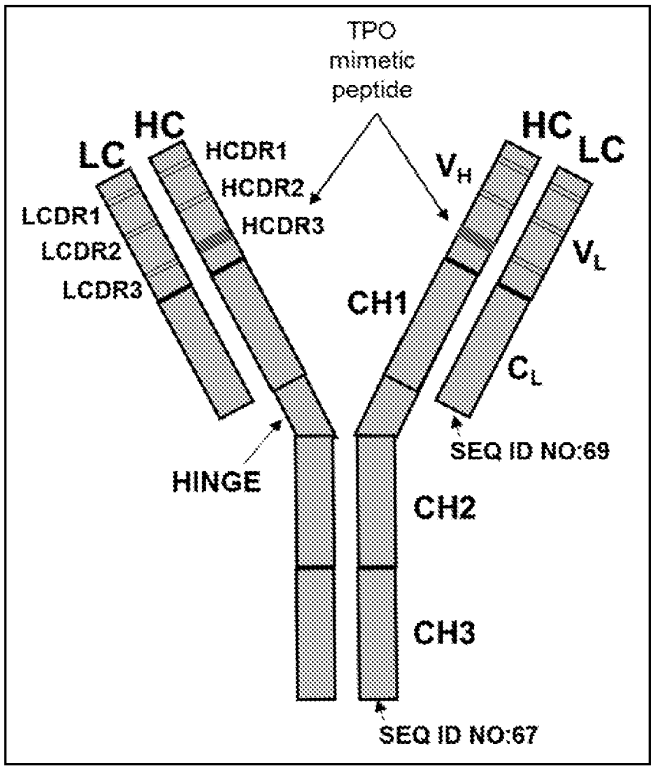


Figure 3.

AMG1002, ¶52; AMG1006, Figures 13A-13B.

As Dr. Balthasar explains, the mature portion of Bowdish's SEQ ID NO:69 (i.e., the light chain) is 100% identical to the claimed SEQ ID NO:4; and the mature portion of Bowdish's SEQ ID NO:67 (i.e., the heavy chain) is 100% identical to the claimed SEQ ID NO:2, with the exception of the HCDR3 sequence. AMG1002, ¶148.

Bowdish's sequences in SEQ ID NOs:69 and 67 each contain italicized portions, which Bowdish explicitly denoted as "leader sequence[s]." As Dr.

Balthasar explains, a POSA would have known that any leader sequence would be cleaved from the mature antibody sequence. AMG1002, ¶143. Thus, the POSA would have known that the leader sequences in Bowdish's Figures 13A-13B are not part of the mature antibody sequence[23]. AMG1006, Figures 13A-13B; AMG1002, ¶143; AMG1045, 582.

A POSA wanting to make a humanized anti-C5 antibody would have understood that the only portion of the heavy chain sequence missing from Bowdish is the HCDR3 sequence because Bowdish taught that "[t]he TPO mimetic peptide graft in Fab clone X4b has been *transplanted into the heavy chain CDR3* of another antibody framework, *5G1.1* … The sequence was *cloned into 5G1.1* in such a fashion as to *replace the native CDR3*." AMG1006, ¶[0191]; AMG1002, ¶¶144-145. The missing HCDR3 sequence, however, was taught in Evans, and Bowdish expressly directed a POSA to Evans. AMG1006, ¶[0191].

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[23] Alexion is estopped from arguing to the contrary. During prosecution, Alexion amended original SEQ ID NO:4 to *remove* the leader sequence, arguing that "the mature light chain sequence is an *inherent* portion of the precursor sequence … and could have been readily identified at the relevant filing date using well established rules and art-recognized techniques…." AMG1016, 179; *see Vitronics Corp.*, 90 F.3d at 1583; *Tyler Refrigeration*, 777 F.2d at 690.

Evans disclosed preparing humanized C5-binding antibodies, referred to therein as "5G1.1" antibodies. AMG1007, 19:47-49, 37:35-39:30, 40:31-45:4; AMG1002, ¶146. As Dr. Balthasar explains, a POSA would have understood that *each* of the 5G1.1 antibody heavy chain variable regions in Evans contain *the same CDR3 sequence*: YFFGSSPNWYFDV. AMG1007, Fig. 19, 43:13-14, 43:26-27, 43:33-34, 43:60-61, 44:2-3, 44:12-13, 44:21-22, 44:30-31, 44:39-40, 44:49-50, 44:59-60, 45:3-4; AMG1002, ¶146. Thus, regardless of which "version" of Evans' humanized 5G1.1 the POSA selected to combine with Bowdish, that heavy chain would contain the YFFGSSPNWYFDV CDR3 sequence. AMG1002, ¶146. Dr. Balthasar's Figure 11 above shows this, where Evans' HCDR3 sequence is extracted and inserted into Bowdish's construct. AMG1002, ¶122.[24] And a POSA would have expected that inserting Evans' YFFGSSPNWYFDV HCDR3 sequence in place of Bowdish's TPO peptide sequence (LPIEGPTLRQWLAARAPV) in SEQ ID NO:67 would provide a complete heavy chain of a humanized anti-C5 antibody that would bind C5. AMG1002, ¶¶147-148. Dr. Balthasar confirms by sequence alignment that this combination provides a sequence identical to the '880

[24] To be clear, a POSA would have known that this would be achieved by replacing DNA encoding Bowdish's TPO-mimetic (SEQ ID NO:65) with DNA encoding Evan's HCDR3. AMG1002, ¶123.

patent's SEQ ID NO: 2. AMG1002, ¶¶147-148.

C. A POSA would have had a reason to combine the references with a reasonable expectation of success.

Bell taught that targeting complement protein C5 with an anti-C5 antibody is. AMG1005, ¶¶[0083]-[0096]. Wang taught therapeutic antibody formulations, and explicitly taught formulating "*eculizumab* as the antibody therapeutic." AMG1028, ¶[0004]. Because neither Bell nor Wang expressly provides the amino acid sequence of its anti-c5 antibody, eculizumab, a POSA would have looked to other eculizumab teachings in the art, like Bowdish and Evans. AMG1002, ¶149.

A POSA is "presumed to be aware of all the pertinent prior art." *Standard Oil*, 774 F.2d at 454. A POSA, therefore, would have been well aware that eculizumab was also known in the art as humanized "5G1.1," "h5G1.1," or "h5G1.1-mAb" with a hybrid IgG2/IgG4 constant region. AMG1002, ¶150; AMG1005, ¶[0052]; AMG1034, 1279; *see also*, Section IV.D, *supra*. To possess the amino acid sequence of that antibody, a POSA would have consulted Bowdish because it taught using a humanized 5G1.1 antibody (including the hybrid IgG2/IgG4 heavy chain constant region) as the starter antibody sequence when creating the TPO-mimetic-containing antibody and further provided the amino acid sequence of the chimeric antibody. AMG1002, ¶¶150-151. Knowing that the only change made to the scaffold antibody in Bowdish was replacing its HCDR3 region

with a TPO-mimetic peptide, a POSA would have had a reason to restore the original HCDR3 region to complete the original anti-C5 antibody. AMG1002, ¶151.

The POSA would have looked to Evans for the missing HCDR3 sequence because both Bell and Bowdish explicitly direct the artisan there for information on how the h5G1.1 antibody was originally created. AMG1005, ¶[0052]; AMG1006, ¶[0191]; AMG1002, ¶151. For example, Bell stated: "[m]ethods for the preparation of *h5G1.1-mAb* [eculizumab] … are described in [Evans][25] … incorporated herein in [its] entirety …." AMG1005, ¶[0052]. And Bowdish similarly stated: "[c]onstruction of *5G1.1* is described in [Evans], incorporated herein by reference."[26] AMG1006, ¶[0191].

This combination of prior art would have led a POSA to make a simple substitution of one known element for another—i.e., replace the TPO-mimetic

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[25] Bell also cites Thomas. AMG1005, ¶[0052]. A POSA would have known that the humanized "h5G1.1 HuG4" antibody disclosed in Thomas is not eculizumab because Thomas's antibody is an IgG4 isotype—i.e., not a human hybrid IgG2/IgG4 isotype like eculizumab. AMG1002, ¶49; *see also*, Section IV.D, *supra.*

[26] *See* note 5, *supra.*

peptide sequence in Bowdish's antibody with the HCDR3 sequence from Evans—to yield predictable results: a complete humanized anti-C5 antibody. *KSR*, 550 US at 416 ("[A] combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results."). And, as Dr. Balthasar confirms by sequence alignment, the humanized anti-C5 antibody obtained from this combination of prior art comprises a heavy chain consisting of SEQ ID NO:2 and a light chain consisting of SEQ ID NO:4 as claimed. AMG1002, ¶¶147-148, 152.

A POSA would have had a reasonable expectation of successfully arriving at the composition of claim 1 because (i) making a humanized anti-C5 antibody would have required only basic molecular biology techniques to substitute Evans' HCDR3 sequence in place of Bowdish's TPO-mimetic peptide sequence (AMG1002, ¶153); (ii) antibody production methods were well-known in the art (see, e.g., AMG1006, ¶¶[0130]-[0131]; AMG1002, ¶153); (iii) Bell taught that a humanized anti-C5 antibody was a safe and effective treatment for PNH, and it provided methods and guidance on how to administer the treatment (AMG1005, ¶¶[0081]-[0096]); and (iv) Bell and Wang taught methods for preparing single-use dosage forms of humanized anti-C5 antibody compositions, including at 10 mg/ml concentrations. AMG1005, ¶¶[0058]-[0060], [0062]; AMG1028, Fig. 10, ¶¶[0170]-[0172]. This expectation is bolstered by numerous publications of

successful clinical trials involving administering pharmaceutical compositions comprising a humanized anti-C5 antibody for treating PNH. *See, e.g.,* AMG1004; AMG1042; AMG1047; AMG1011; AMG1005; AMG1012; AMG1013; AMG1002, ¶153.

XV. Ground 6: claims 1-3 would have been obvious over Bell, Wang, Evans, and Meuller.

A POSA would have had a reason to combine Bell, Evans, Mueller, and Wang with a reasonable expectation of successfully making the pharmaceutical composition of claims 1-3. AMG1002, ¶¶155-179. And no objective indicia weigh in favor of patentability. *See* § XV.

A. Bell and Wang taught all the limitations of claims 1-3 except eculizumab's amino acid sequence.

As shown in § XIII.A., Bell and Wang together taught all of the elements of claims 1-3 except for the claimed antibody sequences. AMG1002, ¶¶155, 159-163.

Bell taught methods of treating a patient suffering from PNH using a pharmaceutical composition comprising eculizumab, a known anti-C5 antibody. AMG1005, ¶¶[0012], [0021], [0052], [0058], [0081]-[0096]. Wang taught therapeutic antibody formulations, and explicitly taught formulating "*eculizumab* as the antibody therapeutic." AMG1028, ¶[0004]. In addition:

(i) Bell taught that the humanized antibody unit dose formulation "*must be sterile* and non-pyrogenic" and "*may* contain … preservatives…"

(i.e., preservatives are not required). AMG1005, ¶[0062]; AMG1002, ¶160. And a POSA would have known that therapeutic antibodies are routinely prepared in sterile, preservative-free solutions. AMG1002, ¶160; AMG1056-AMG1076.

(ii) Wang taught eculizumab formulations of between 1 to 30 mg/ml, encompassing the claimed 10 mg/ml concentration. AMG1028, Fig. 10, ¶[0067]. Wang's disclosure was also consistent with the general knowledge in the art. *See, e.g.,* AMG1029, Table 1; AMG1030, Table 1; AMG1002, ¶162.

(iii) A POSA would have prepared the eculizumab formulation in 30 ml single-use dosage forms using simple mathematics based on a 300 mg dosage unit concentrated at 10 mg/ml. AMG1002, ¶163. Again, there is nothing critical about a 30 ml volume. AMG1027, 1638(¶3.5), 1104(¶5.3.9). Accordingly, a POSA routinely arriving at a 300 mg single-use dose concentrated at 10 mg/ml would also have routinely arrived at a volume of 30 ml. AMG1002, ¶163.

B. Evans and Mueller taught eculizumab's amino acid sequences.

As discussed below, Evans disclosed the complete amino acid sequences of the heavy and light chain variable domains of a humanized anti-C5 antibody, . AMG1007, 44:4-13, SEQ ID NO:20; AMG1002, ¶¶170-175. And Mueller

disclosed the amino acid sequence of a light chain constant region and the hybrid IgG2/IgG4 heavy chain constant region. AMG1008, 58-61; AMG1002, ¶¶170-175. Dr. Balthasar's Figure 14 below depicts this combination of art providing the complete eculizumab sequence:

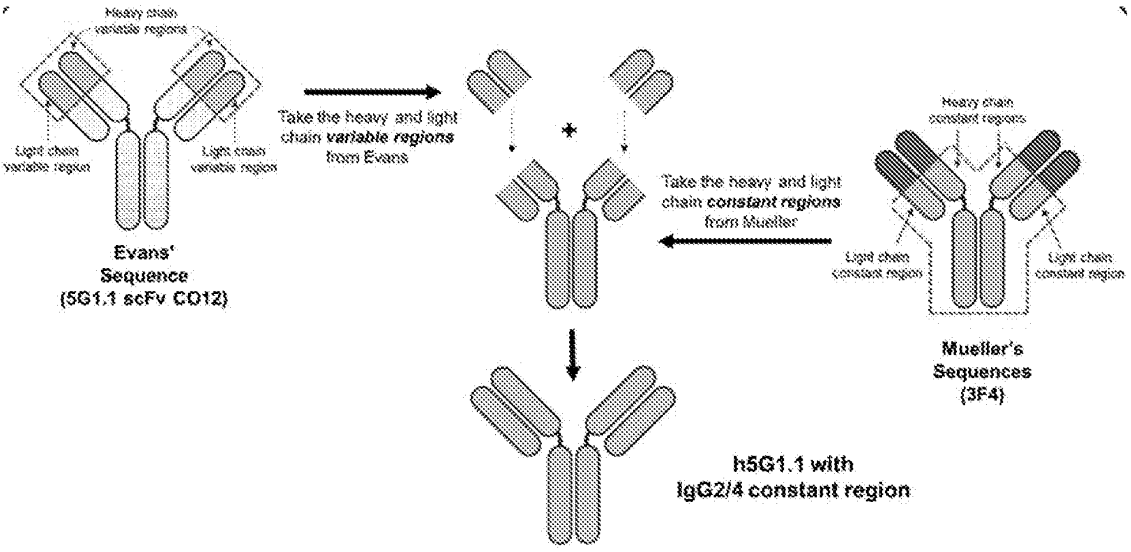


Figure 14.

See AMG1002, ¶156.

Evans—which Alexion previously said claims Soliris® and provides written description and enablement support for claims directed to eculizumab (AMG1009, 4; AMG1049, 838-839)—discloses making the original mouse 5G1.1 monoclonal antibody. AMG1007, 37:36-39:30 (Example 7); AMG1002, ¶¶164-165. Evans further described constructing a series of humanized 5G1.1 antibody constructs containing the heavy and light chain CDR sequences from the mouse 5G1.1

antibody inserted into a human framework. AMG1007, 42:58-45:4. In particular, Evans described nine different humanized 5G1.1 scFv constructs[27] along with their amino acid sequences. *Id.* (constructs 2 and 11-18); AMG1002, ¶¶165, 171. Evans also described combining the variable regions with constant domains—including hybrid IgG constant domains—to make a complete antibody. AMG1007, 45:24-33.

Knowing that chimeric IgG2/IgG4 constant regions were known not to activate the complement system (AMG1008, 7:28-31, 8:23-26, 12:27-32), a POSA reading Bell and Evans also would have looked to Mueller for "h5G1.1" sequence information. AMG1002, ¶¶166, 173. Mueller taught methods for making "chimeric antibodies containing the C1 and hinge region of human IgG2 and the C2 and C3 regions of human IgG4 … (HuG2/G4 mAb)." AMG1008, 12:27-30; *see also, id.,* 8:23-26. In particular, Mueller described a control antibody "h5G1.1 CO12 HuG2/G4 mAb," which a POSA would have readily identified as a humanized anti-C5 antibody because of the "h5G1.1" nomenclature coupled with the hybrid

[27] A POSA would have known that a scFv comprises light and heavy chain variable domains connected by a linker. *See, e.g.,* AMG1007, 6:39-41 ("single chain antibodies may include one each of only VH and VL domains, in which case they are *referred to as scFv antibodies*"); *see also,* AMG1040, 45-48; AMG1002, ¶165

IgG2/IgG4 constant region ("HuG2/G4"). AMG1008, 12:37, FIG. 15; AMG1005, ¶[0052]; AMG1034, 1279; AMG1049, 838-839; AMG1002, ¶¶166, 173-174.

As Dr. Balthasar explains, Mueller disclosed the amino acid sequence of a hybrid IgG2/IgG4 heavy chain constant region when Mueller disclosed the sequence of the chimeric anti-VCAM "3F4" antibody. AMG1002, ¶¶169, 173; AMG1008, 58-61. A POSA would have known that a chimeric antibody contains the variable region from a non-human antibody and the constant region from a human antibody, and therefore would have understood that Mueller's chimeric 3F4 HuG2/G4 mAb heavy chain contains the variable regions from murine antibody 3F4 (the blue portions in Dr. Balthasar's Figure 14 above) and the constant regions of human hybrid IgG2/IgG4 (the green portions in Dr. Balthasar's Figure 14). AMG1002, ¶¶156, 173; AMG1040, 29-30.

Mueller separately disclosed the amino acid sequences of the mature 3F4 heavy and light chain variable regions (i.e., the blue portion in Dr. Balthasar's Figure 14). AMG1008, Figure 9; AMG1002, ¶¶156, 173. A POSA aligning the 3F4 heavy and light chain variable region sequences from Figure 9 with the sequences of the 3F4 HuG2/G4 chimeric antibody would have identified the 3F4 variable regions (the regions a POSA would have excluded) as amino acids 20-137 of the 3F4 HuG2/G4 heavy chain and amino acids 20-131 of the 3F4 light chain. AMG1008, Figure 9, 52-53, 58-61; AMG1002, ¶173.

A POSA therefore would have immediately known that the remainder of the 3F4 HuG2/G4 heavy chain (amino acids 138-463) is the hybrid IgG2/IgG4 constant region of that antibody, and that the remainder of the 3F4 light chain (amino acids 132-238) is the light chain constant region of that antibody (i.e., the green portion in Dr. Balthasar's Figure 14). AMG1008, 52-53, 56-57; AMG1002, ¶173. Given that Mueller used this humanized anti-C5 antibody as an isotype control for 3F4 HuG2/G4, a POSA would have reasonably expected the disclosed heavy and light chain constant regions to be the same as those in the 3F4 HuG2/G4 chimeric antibody. AMG1002, ¶¶57, 166.

As Dr. Balthasar explains, overwhelming evidence in the art would have further confirmed a POSA's belief that the amino acid sequences of the heavy and light chain constant regions of Mueller's 3F4 HuG2/G4 antibody are the same as those in eculizumab. AMG1002, ¶¶47, 56, 169, 182. Publications such as Bowdish, Tacken, Mueller II, and Evans all disclosed portions of eculizumab constant regions that either overlapped with or were exact matches to the heavy and light chain constant regions of Mueller's 3F4 HuG2/G4 antibody. AMG1002, ¶¶47, 56, 169, 182; AMG1006, Figs. 13A-13B; AMG1034, 1279; AMG1031, Abstract, 448, Fig. 7; AMG1007, 43:50-55 (SEQ ID NO:15).

With the heavy and light chain constant region sequences obtained from Mueller, a POSA would have looked back to Evans to complete the amino acid

sequence. AMG1002, ¶¶167-168. Knowing that Mueller refers to the control antibody as "h5G1.1 CO12 HuG2/G4 mAb," the POSA would have referred to the series of humanized 5G1.1 scFvs taught in Evans and readily identified construct number 12 (SEQ ID NO:20) as the scFv of interest because Evans used the same "CO12" nomenclature as Mueller by designating it "5G1.1 scFv CO12." AMG1007, 44:4-13; AMG1002, ¶¶167-168. This is shown in Dr. Balthasar's Figure 14 above, where the green portions depict Evans' heavy and light chain variable regions. AMG1002, ¶156; AMG1007, 44:4-13, SEQ ID NO:20.

That Evans taught additional 5G1.1 scFv constructs is irrelevant because, "for an obviousness analysis, even the fact that 'a specific embodiment is taught to be preferred is not controlling, since all disclosures of the prior art, including unpreferred embodiments, must be considered.'" *In re Thomas*, 151 Fed. App'x. 930, 934 (Fed. Cir. 2005) (quoting *Merck & Co., Inc. v. Biocraft Labs., Inc.*, 874 F.2d 804, 807 (Fed. Cir. 1989). Here, any one of the combinations of Mueller's constant regions with Evans' variable regions would have been obvious, and does not make the combination with any one pair of variable regions any less obvious. *See Merck*, 874 F.2d at 807 ("That the [asserted prior art] discloses a multitude of effective combinations does not render any particular formulation less obvious.")

A POSA would have known that Evans' humanized 5G1.1 scFvs each contains a single polypeptide sequence comprising a humanized heavy chain

variable region of 5G1.1, a linker, and a humanized light chain variable region of 5G1.1. AMG1002, ¶172; AMG1007, 6:39-41; AMG1040, 45-48. As Dr. Balthasar explains, a POSA would have been able to readily identify the heavy and light chain variable regions within SEQ ID NO: 20 of Evans. AMG1002, ¶172.A POSA would have known that the linker in "5G1.1 scFv CO12" (SEQ ID NO:20) is amino acids 112-126 because this 15-amino acid sequence (GGGGSGGGGSGGGGS) was well known in the art as common a linker sequence in scFv antibodies. AMG1007, SEQ ID NO:20 (Certificate of Correction, 42-44); AMG1037, ¶¶[0021], [0097]; AMG1002, ¶172.

Alexion has also argued during prosecution that "the mature light chain sequence … could have been readily identified at the relevant filing date using well established rules and art-recognized techniques" and provided Adderson (AMG1048) as an example showing "the characteristic mature N-terminus (DIQ) of a light V kappa antibody light chain." AMG1014, 513; AMG1048, Fig. 6. Thus, the first two amino acids of Evans' SEQ ID NO:20 (MA) are a leader sequence, based on Alexion's admission that a mature kappa light chain starts with the sequence "DIQ" on its N terminus. AMG1002, ¶172; AMG1048, 734 (Fig. 6). Accordingly, a POSA would have understood that the mature light and heavy chain variable regions in Evans correspond to amino acids 3-111 and 127-248 of SEQ ID NO:20, respectively. AMG1007, 44:4-13; SEQ ID NO:20; AMG1002, ¶172.

Finally, a POSA would have expected that combining Evans' variable region sequences with Mueller's constant region sequences would provide complete heavy and light chain sequences of a humanized anti-C5 antibody.[28] *See* Dr. Balthasar's Figure 14 above; AMG1002, ¶¶156, 174-175.

As Dr. Balthasar shows by sequence alignment, the combination of heavy and light chain variable regions in Evans' SEQ ID NO:20 with Mueller's constant regions would make a humanized anti-C5 antibody having SEQ ID NOs:2 and 4, as claimed. AMG1002, ¶¶174-175.

C. A POSA would have had a reason to combine the references with a reasonable expectation of success.

Again, a POSA would have generally known that the humanized anti-C5 antibody eculizumab is a humanized 5G1.1 monoclonal antibody with a hybrid IgG2/IgG4 constant region, often referred to as "h5G1.1" in the art. AMG1005, ¶[0052]; AMG1034, 1279; AMG1049, 838-839; AMG1002, ¶¶44-47. A POSA reading Bell and Wang would have looked to Evans because Bell explicitly cited and incorporated by reference Evans for methods of making a humanized anti-C5 antibody. AMG1005, ¶[0052]; AMG1002, ¶¶164-165, 176-177. A POSA would

[28] To be clear, a POSA would have known that this would be achieved by combining DNA encoding Evans' variable region sequences with that encoding Mueller's constant region. AMG1002, ¶123.

have also looked to Mueller because Mueller disclosed an h5G1.1 antibody named "h5G1.1 CO12 HuG2/G4 mAb" with a hybrid IgG2/IgG4 heavy chain constant region, which a POSA would have readily understood to be the humanized anti-C5 antibody, eculizumab. AMG1002, ¶¶166, 177.

Evans and Mueller taught complementary, familiar elements of eculizumab's amino acid sequences. AMG1002, ¶177. Thus, by combining familiar elements in the art according to known methods, the artisan would have predictably arrived at a humanized anti-C5 antibody comprising a light chain consisting of SEQ ID NO:2 and a heavy chain consisting of SEQ ID NO:4 as claimed. *KSR*, 550 US at 416 ("a combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results."). A POSA would have easily confirmed this prediction by comparing the constructed sequences with another Alexion publication WO 2005/007809, which taught detailed descriptions of expression vectors designed for placing antibody variable regions in frame with chimeric IgG2/IgG4 heavy chain constant regions. AMG1032, 7, 28-32, FIG. 5; AMG1002, ¶177.

With eculizumab in hand, a POSA would have had a reasonable expectation of successfully arriving at the pharmaceutical composition of claims 1-3 because (i) making eculizumab would have required only basic molecular biology techniques to combine Evans' scFv variable regions with Mueller's constant

domains (AMG1002, ¶178); (ii) antibody production methods were well-known in the art (see, e.g., AMG1006, ¶¶[0130]-[0131]; AMG1002, ¶178); (iii) Bell taught that eculizumab was a safe and effective treatment for PNH, and it provided methods and guidance on how to administer the treatment (AMG1005, ¶¶[0081]-[0096]); and (iv) Bell and Wang taught methods for preparing single-use dosage forms of eculizumab compositions, including 10 mg/ml eculizumab. AMG1005, ¶¶[0058]-[0060], [0062]; AMG1028, Fig. 10, ¶¶[0170]-[0172]; AMG1002, ¶178. This expectation is bolstered by numerous publications of successful clinical trials involving administering pharmaceutical compositions comprising a humanized anti-C5 antibody for treating PNH. *See, e.g.,* AMG1004; AMG1042; AMG1047; AMG1011; AMG1005; AMG1012; AMG1013; AMG1002, ¶178.

XVI. Objective indicia do not support patentability.

"To be afforded substantial weight, the objective indicia of non-obviousness must be tied to the *novel elements* of the claim at issue." *Univ. Pierre et Marie Curie v. Focarino*, 738 F.3d 1337, 1347 (Fed. Cir. 2013). Objective evidence that is not "both claimed and *novel in the claim*" lacks nexus to the invention. *In re Kao*, 639 F.3d 1057, 1068 (Fed. Cir. 2011).

Alexion argued during prosecution of the related '504 patent that "the non-natural, protein-engineered, heavy chain of eculizumab" (i.e., the hybrid IgG2/IgG4 constant domain) provided "surprising and unpredictable" results such

as decreased effector function, reduced immunogenicity and increased half-life. AMG1014, 588, 593(¶8). Eculizumab's hybrid IgG2/IgG4 constant domain was well known in the art (e.g., AMG1034, 1279), however, and cannot be a "novel element." *Marie Curie*, 738 F.3d at 1347; *Kao*, 639 F.3d at 1068. Accordingly, the alleged "surprising and unpredictable" features of eculizumab have no nexus with the challenged claims and do not support non-obviousness. *Id.*

Moreover, Alexion's alleged results would not have been unexpected to a POSA. AMG1002, ¶¶180-182. Mueller II taught in 1997 that antibodies with a hybrid IgG2/IgG4 heavy chain "[do] not contain the antibody sequences necessary for FcR binding," and would not contain "any new epitopes that would likely be immunogenic." AMG1031, 448, 451. It was also well known that a hybrid IgG2/IgG4 heavy chain would "have increased half-life." *See, e.g.,* AMG1032, 5, 19; AMG1002, ¶182. There is nothing unexpected here.

Alexion also argued in Europe that the claimed 10 mg/ml concentration provided "improved stability compared with other concentrations." AMG1027, 1104(¶5.3.8). This argument also carries no weight because a 10 mg/ml concentration of an anti-C5 antibody is not a *novel feature* of the claims. Discussed above, numerous therapeutic antibodies were formulated at this concentration, *including eculizumab*. AMG1002, ¶¶105-106; AMG1028, Fig. 10. Further, data submitted in the opposition proceeding show that 10 mg/ml eculizumab did not

offer any improved stability over other tested concentrations, completely contradicting Alexion's assertion. AMG1027, 19-20, 89-100; AMG1002, ¶¶183-184.

Petitioner is not aware of any other alleged objective indicia, and reserves the right to rebut any evidence asserted by Alexion in this proceeding. *Amneal Pharms., LLC v. Supernus Pharms., Inc.*, IPR2013-00368, Paper 8, at 12-13 (Dec. 17, 2013); AMG1002, ¶185.

XVII. Certification that the Patent May Be Contested via *Inter Partes* Review by the Petitioner and Standing (37 C.F.R. §42.104(a)).

Amgen certifies that the '880 patent is available for IPR and Amgen is not barred or estopped from requesting IPR of any '880 patent claim.

XVIII. Mandatory Notices (37 C.F.R. §42.8(a)(1).

Real party-in-interest 37 C.F.R. §42.8(b)(1): Amgen Inc.

Related Matters (37 C.F.R. §42.8(b)(2)): Amgen has concurrently filed petitions for IPR of U.S. Patent Nos. 9,725,504 (IPR2019-00739) and 9,732,149 (IPR2019-00741), which are related to the '880 patent and also owned by Alexion.

Lead and back-up counsel (37 C.F.R. §42.8(b)(3)) for Amgen Inc. are

Lead Counsel	Back-Up Counsel

Deborah Sterling (Reg. No. 62,732) STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C. 1100 New York Avenue, NW Washington, DC 20005 202.772.8508 (telephone) 202.371.2540 (facsimile) dsterling-PTAB@skgf.com	David H. Holman (Reg. No. 61,205) Scott A. Schaller (Reg. No. 60,167) David W. Roadcap (Reg. No. 68,956) STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C. 1100 New York Avenue, NW Washington, DC 20005 202.371-2600 (telephone) 202.371.2540 (facsimile) dholman-PTAB@sternekessler.com sschalle-PTAB@ sternekessler.com droadcap-PTAB@sternekessler.com

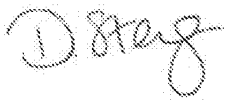
Notice of Service Information (37 C.F.R. §42.8(b)(4)): Please direct all correspondence regarding this Petition to counsel at the above addresses. Amgen consents to service by email at the addresses above.

Procedural Statements: This Petition is filed in accordance with 37 C.F.R. §42.106(a). Concurrently filed herewith are a Power of Attorney and Exhibit List under 37 C.F.R. §42.10(b) and §42.63(e), respectively. The required fee is paid through Deposit Acct. No. 19-0036 (Customer ID No. 45324). The Office is authorized to charge any fee deficiency, or credit any overpayment, to Deposit Acct. No. 19-0036 (Customer ID No. 45324).

XIX. Conclusion.

The challenged claims are unpatentable as anticipated or obvious and IPR is warranted.

Respectfully submitted,
STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C



Date: February 28, 2019
1100 New York Avenue, N.W.
Washington, D.C. 20005-3934
(202) 371-2600

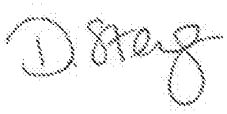
Deborah A. Sterling, Ph.D.
Registration No. 62,732
Lead Attorney for Petitioner

10498316.1

CERTIFICATE OF WORD COUNT (37 C.F.R. § 42.24(d))

I certify that Amgen Inc.'s Petition for *Inter Partes* Review for U.S. Patent No. 9,718,880 contains 13,703 words as counted by the word-processing program used to generate this response. This total does not include the table of contents, certificate of service, or this certificate of word count.

Respectfully submitted,
STERNE, KESSLER, GOLDSTEIN & FOX L.L.C.



Date: February 28, 2019
1100 New York Avenue, N.W.
Washington, D.C. 20005-3934
(202) 371-2600

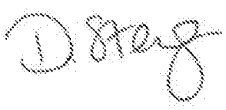
Deborah A. Sterling, Ph.D.
Registration No. 62,732
Lead Attorney for Petitioner

CERTIFICATE OF SERVICE (37 C.F.R. § 42.6(e)), §42.105(a))

I certify that the above-captioned "Petition for *Inter Partes* Review for U.S. Patent No. 9,718,880" was served in its entirety upon the Patent Owner on February 28, 2019, via FedEx, at the correspondence address of record indicated in the Patent Office's public PAIR system for U.S. Patent No. 9,718,880 B2:

Nelson Mullins Riley & Scarborough LLP/Alexion
One Post Office Square
30th Floor
Boston MA 02109-2127

STERNE, KESSLER, GOLDSTEIN & FOX L.L.C.



Date: February 28, 2019
1100 New York Avenue, N.W.
Washington, D.C. 20005-3934
(202) 371-2600

Deborah A. Sterling, Ph.D.
Registration No. 62,732
Lead Attorney for Petitioner

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

AMGEN INC.
Petitioner,

v.

ALEXION PHARMACEUTICALS
Patent Owner.

Case IPR2019-00739
U.S. Patent No. 9,725,504

PETITION FOR *INTER PARTES* REVIEW OF U.S. PATENT NO. 9,725,504

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TABLE OF CONTENTS

I. Statement of Precise Relief Requested and Reasons Therefor (37 C.F.R. §42.22(A)). 1

II. Introduction. 1

III. Summary. 2

IV. The '504 patent and its prosecution history. 7

V. State of the art before March 15, 2007. 11

A. Humanized monoclonal antibodies were well-known. 11
B. The prior art taught that PNH was associated with complement system dysregulation. 12
C. Eculizumab was a known, effective treatment for PNH. 13
D. The prior art taught that eculizumab is a humanized anti-C5 monoclonal antibody (h5G1.1) containing a hybrid IgG2/IgG4 constant region. 16
E. The art taught eculizumab's amino acid sequence. 18

VI. Person of ordinary skill in art. 21

VII. Claim construction. 22

VIII. Identification of the challenge (37 C.F.R. §42.104(b)). 23

IX. The same or substantially the same prior art or arguments were not previously presented to the Office. 24

X. Ground 1: Hillmen anticipates claims 1-3 and 7-10. 25

A. Hillmen anticipates claim 1. 25
1. Hillmen explicitly disclosed every element of claim 1, except for eculizumab's amino acid sequence. 26
2. Alexion admitted that Hillmen's eculizumab necessarily has the claimed sequence. 26
3. Hillmen's disclosure is enabling. 29
B. Hillmen anticipates claim 2. 32
C. Hillmen anticipates claim 3. 33
D. Hillmen anticipates claim 7. 34
E. Hillmen anticipates claim 8. 34
F. Hillmen anticipates claim 9 and 10. 35

XI. Ground 2: claims 4-5 would have been obvious over Hillmen and Bell. 37

XII. Ground 3: claim 6 would have been obvious over Hillmen, Bell, and Wang. 41

XIII. Grounds 4-7: claims 1-10 would have been obvious over Bell and prior-art disclosures of anti-C5 antibody sequences. 45

A. Ground 4: claims 1-5 and 7-10 would have been obvious over Bell, Bowdish, and Evans. 45

1. Claim 1 would have been obvious. 45

a) Bell expressly taught all the limitations of claim 1 except eculizumab's amino acid sequence 45

b) Bowdish and Evans taught the claimed amino acid sequences. 47

c) A POSA would have had a reason to combine the references with a reasonable expectation of success. 52

2. Claims 2-5 and 7-10 would have been obvious. 55

a) Claim 2: intravenous infusion 55

b) Claim 3: dosage level of between 5 mg/kg and 50 mg/kg. 55

c) Claim 8: dosing regimen 56

d) Claims 4 and 5: single unit dosage forms 57

e) Claim 7: patient is anemic. 58

f) Claims 9 and 10: immediate decrease in LDH levels 58

B. Ground 5: claim 6 would have been obvious over Bell, Bowdish, Evans, and Wang 60

C. Ground 6: claims 1-5 and 7-10 would have been obvious over Bell, Evans, and Mueller. 61

1. Claims 1-5 and 7-10 would have been obvious over Bell, Evans, and Mueller. 62

a) Claim 1 would have been obvious 62

(1) Bell expressly taught all the limitations of claim 1 except the eculizumab amino acid sequences 62

(2) Evans and Mueller taught eculizumab's amino acid sequences. 62

(3) A POSA would have had a reason to combine the references with a reasonable expectation of success. 69

b) Claims 2-5 and 7-10 would have been obvious. 71
D. Ground 7: claim 6 would have been obvious over Bell, Evans, Mueller, and Wang. 71
XIV. Objective indicia do not support patentability 71
XV. Certification that the Patent May Be Contested via *Inter Partes* Review by the Petitioner and Standing (37 C.F.R. §42.104(a)). 73
XVI. Mandatory Notices (37 C.F.R. §42.8(a)(1). 73
XVII. Conclusion. 75

EXHIBIT LIST

Exhibit #	*Description*
1001	Bell, L., *et al.*, "Treatment of Paroxysmal Nocturnal Hemoglobinuria Patients by an Inhibitor of Complement," U.S. Patent No. 9,725,504 B2 (filed September 9, 2016; issued August 8, 2017)
1002	Declaration of Joseph P. Balthasar, Ph.D.
1003	Curriculum Vitae of Joseph P. Balthasar, Ph.D.
1004	Hillmen, P., *et al.*, "Effect of Eculizumab on Hemolysis and Transfusion Requirements in Patients with Paroxysmal Nocturnal Hemoglobinuria," *N. Engl. J. Med.* 350:552-559 (2004)
1005	Bell, L. and Rother, R., "Method of Treating Hemolytic Disease," U.S. Patent Application Publication No. 2005/0191298 A1 (filed February 3,2005; published September 1, 2005)
1006	Bowdish, K., *et al.*, "Rationally Designed Antibodies," U.S. Patent Application Publication No. 2003/0232972 A1 (filed December 2, 2002; published December 18, 2003)
1007	Evans, M., *et al.*, "C5-Specific Antibodies for The Treatment of Inflammatory Diseases," U.S. Patent No. 6,355,245 B1 (filed June 7, 1995; issued March 12, 2002)
1008	Mueller, J., *et al.*, "Porcine Cell Interaction Proteins," International Patent Application Publication No. WO 97/11971 A1 (filed September 27 1996; published April 3, 1997)
1009	Patent Term Extension Application for U.S. Patent No. 6,355,245 B1
1010	Patent Term Extension Certificate for U.S. Patent No. 6,355,245 B1
1011	Hill, A. "Sustained Control of Hemolysis and Symptoms and Reduced Transfusion Requirements over a Period of 2 Years in Paroxysmal Nocturnal Hemoglobinuria (PNH) with Eculizumab Therapy," *Blood* 104: Abstract 2823 (2004)
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1013	Young, N., *et al.*, "Safety and Efficacy of the Terminal Complement Inhibitor Eculizumab in Patients with Paroxysmal Nocturnal Hemoglobinuria: Interim Shepherd Phase III Clinical Study," *Blood* 108: Abstract 971 (2006)
1014	File History for U.S. Patent No. 9,725,504 B2

Exhibit #	*Description*
1015	File History for U.S. Patent No. 9,732,149 B2
1016	File History for U.S. Patent No. 9,718,880 B2
1017	Opposition File History for European Patent No. 1720571
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1028	Wang, Y., "Nebulization of Monoclonal Antibodies for Treating Pulmonary Diseases," U.S. Patent Application Publication No. 2005/0271660 A1 (filed May 11,2005; published December 8, 2005)
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Exhibit #	***Description***
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1056	Genentech, Inc., Avastin 2004 Package Insert, available at https://www.accessdata.fda.gov/drugsatfda_docs/label/2004/125085lbl.pdf
1057	Corixa Corp., Bexxar 2003 Package Insert, available at https://www.accessdata.fda.gov/drugsatfda_docs/label/2003/tosicor062703lb.pdf
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Exhibit #	*Description*
1063	Genentech, Inc., Lucentis 2006 Package Insert, available at https://www.accessdata.fda.gov/drugsatfda_docs/label/2006/125156lbl.pdf
1064	Centocor Ortho Biotech, LP, Orthoclone OKT3 Package Insert, available at https://dailymed.nlm.nih.gov/dailymed/archives/fdaDrugInfo.cfm?archiveid=17321
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1073	Boehringer Ingelheim Int'l GmbH, Verluma 1996 Package Insert, available at https://www.accessdata.fda.gov/drugsatfda_docs/label/1996/nofedrk082096lb.pdf

Exhibit #	*Description*
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I. Statement of Precise Relief Requested and Reasons Therefor (37 C.F.R. §42.22(A)).

Amgen Inc. petitions for *Inter Partes* Review, seeking cancellation of claims 1-10 of U.S. Patent No 9,725,504 ("'504 patent"; AMG1001), assigned to Alexion Pharmaceuticals, Inc. The challenged claims are unpatentable under 35 U.S.C. §§ 102 and/or 103. This Petition is supported by the declaration of Dr. Joseph Balthasar (AMG1002), an expert in the development and evaluation of therapeutic monoclonal antibodies. *Id.*, ¶¶1-10.

II. Introduction.

The challenged claims encompass methods of treating paroxysmal nocturnal hemoglobinuria ("PNH") using a humanized anti-C5 antibody having a specified sequence. During prosecution of the '504 patent, Alexion asserted—incorrectly, yet repeatedly—that the claimed sequence "was not disclosed in the prior art; nor was it available to the public." AMG1014, 586-588, *see also* 739. Indeed, Alexion's argument that the claimed sequence was not publicly known was the only stated basis for withdrawing the rejections and issuing a notice of allowance: "none of the applied references in the rejections recite using an antibody [with the claimed sequence]." *Id.*, 790. But, as shown herein, long before the '504 patent's alleged priority date, Alexion repeatedly published the claimed method of using a humanized anti-C5 antibody to treat PNH.

Alexion admitted that it performed at least 17 clinical trials treating PNH with a humanized anti-C5 antibody ("eculizumab"), and that the antibody used in those trials had the claimed amino acid sequence. *Id.* 765-767. Thus, by Alexion's admission, prior art publications of those trials necessarily disclose the claimed sequence, inherently anticipating certain challenged claims. In addition, Alexion's own prior art publications of the sequence and structure of a humanized anti-C5 antibody, were such that the skilled artisan could and would have made and used an antibody as claimed, with a reasonable expectation of success. The challenged claims, therefore, offer nothing novel or inventive.

III. Summary.

Eculizumab (Soliris®) is a monoclonal antibody that binds complement protein C5 and inhibits C5 cleavage. Alexion obtained U.S. Patent No. 6,355,245 ("Evans"), which is prior art to the '504 patent, on March 12, 2002. Alexion contends that Evans "claims the approved product" (Soliris®; eculizumab), and provides both written description and enablement support for claims directed to eculizumab. AMG1009, 4; AMG1010, 2; AMG1049, 838-839.

The FDA approved Soliris® for treatment of patients with PNH on March 16, 2007. AMG1009, 2. Exactly *one day* before receiving FDA approval, Alexion

filed PCT Application No. PCT/US2007/006606[1] ("the '606 application") and began prosecuting a chain of new patents directed to eculizumab. This was no coincidence.

To secure new eculizumab patents from the '606 application, Alexion repeatedly told the USPTO that eculizumab's amino acid sequence—specifically its IgG2/IgG4 heavy chain constant region—was not available in the prior art. But Alexion did not inform the UPSTO that it had been repeatedly publishing on eculizumab, its amino acid sequence—including its engineered IgG2/IgG4 heavy chain— and its use for treating PNH years before March 15, 2007. Indeed, Alexion proudly boasted about widespread knowledge of the IgG2/IgG4 constant region in the art when it suited Alexion's interests, but remained silent about it to the USPTO. AMG1049, 838-839. Alexion's carefully timed pursuit of the '606 application, one day before product approval, improperly seeks to ensnare and monopolize that which is already in the public domain—through Alexion's own publications nonetheless.

Well before March 15, 2007, artisans were aware that eculizumab was safe and effective for treating PNH. Alexion itself admitted, prior to that date, that eculizumab was the subject of at least 17 different clinical trials, many of which

[1] The '504 patent claims priority to the '606 application.

were published (summarized below in Table 1). AMG1014, 765-767. Alexion also admitted that the eculizumab used in these trials has the claimed amino acid sequence. *Id.* Thus, by Alexion's admission, the humanized anti-C5 antibody administered in these published trials (eculizumab) necessarily has the claimed sequence.

In addition, and contrary to Alexion's misrepresentation, the amino acid sequence and structure of eculizumab *were* known in the art, and a skilled artisan would have had ample reasons, guidance, and direction to make and use eculizumab as claimed, rendering the challenged claims obvious. Indeed, it was Alexion who placed the claimed amino acid sequence into the public domain, yet failed to inform the examiner of this.

For example, Alexion's patent application publication US 2003/0232972 A1 ("Bowdish"), *not* raised by the examiner during prosecution, published in 2003, used eculizumab as the starter "scaffold" antibody for creating a recombinant thrombopoietin (TPO) peptide-antibody, and provided the full eculizumab amino acid sequence except for the heavy chain CDR3 ("HCDR3") sequence that it had replaced with the TPO peptide. AMG1006, ¶¶[0191]-[0193], Figs. 13A-13B, and SEQ ID NOs. 67 and 69. But the missing HCDR3 sequence was taught in Evans.

Bowdish explicitly incorporated by reference Evans[2] (another Alexion patent) for making eculizumab: "[c]onstruction of 5G1.1 [i.e., eculizumab] is described in U.S. Application Ser. No. 08/487,283, incorporated herein by reference."[3] *Id.*, ¶[0191]. As Dr. Balthasar explains, the skilled artisan would have readily identified the heavy chain CDR3 sequences in Evans, thereby obtaining the complete amino acid sequence of the claimed antibody. *See* AMG1002, ¶¶54-55.

The claimed amino acid sequence was also taught in the art through the combination of Evans and another Alexion publication, WO 97/11971 ("Mueller"; AMG1008). In addition to disclosing its heavy chain CDR3 sequence, Evans taught the amino acid sequences of eculizumab's light and heavy chain variable regions. AMG1007, 44:4-13; AMG1002, ¶¶55, 183-185. With the heavy and light chain variable region sequences in hand, the artisan would have needed only to identify the sequences for the light and heavy chain constant regions—information found in Evans (light chain) and Mueller (both the light and heavy chains).

Notably, Alexion *never* provided Mueller to the examiner. Published in

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[2] Alexion patent application publication US 2005/0191298 A1 ("Bell") also explicitly incorporates by reference Evans for preparing eculizumab. AMG1005, ¶[0052].

[3] Evans issued from U.S. Application No. 08/487,283. AMG1007, face.

1997, Mueller taught methods of creating recombinant antibodies with chimeric IgG2/IgG4 constant regions that were known not to activate the complement system. AMG1008, 7:28-31, 8:23-26, 12:27-32. Mueller further described using eculizumab—a humanized anti-C5 antibody with the same IgG2/IgG4 constant region as the experimental antibody—as a control antibody, and provided amino acid sequences of its IgG2/IgG4 heavy chain constant region and light chain constant region. *Id.*, 12:35-37, Fig. 15, 52-53, 58-61; AMG1002, ¶¶186-188. Dr. Balthasar explains that a skilled artisan also would have readily obtained the complete amino acid sequence of the humanized anti-C5 antibody that results from the combination of Evans and Mueller; a sequence that Alexion now claims to be novel. *See* AMG1002, ¶¶183-188.

Alexion admittedly placed methods of treating PNH using a humanized anti-C5 antibody, as claimed, squarely in the prior art, and it's numerous prior art publications of eculizumab clinical trials results would have given the artisan ample reason to use eculizumab to treat PNH. The prior art also supplied sufficient information about the sequence and structure of eculizumab such that the artisan could and would have made and used a humanized anti-C5 antibody, as claimed, with a reasonable expectation of success. The challenged claims, therefore, offer nothing novel or inventive over what was well known in the art to a POSA before March 15, 2007.

IV. The '504 patent and its prosecution history.

The '504 patent issued on August 8, 2017 from U.S. Appl. No. 15/260,888, and claims a priority date of March 15, 2007.[4] The '504 patent's sole independent claim recites:

> 1. A method of treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH) comprising administering to the patient a pharmaceutical composition comprising an antibody that binds C5, wherein the antibody comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4.

EX1001, 39:2-7.

Dependent claims 2-10 add certain limitations to this method. *Id.*, 39:8-32 Claim 2 requires administration by intravenous infusion ("IV"). Claim 3 requires dosing between 5 and 50 mg/kg. Claims 4-6 respectively require a single unit dosage form (claim 4) of 300 mg (claim 5), or a 300 mg single-use formulation of 30 ml of a 10 mg/ml sterile, preservative-free solution (claim 6). Claim 7 requires that the patient is anemic. Claim 8 requires a specific dosing schedule. Claims 9 and 10 require that the treatment results in a specified decrease in lactate

[4] Petitioner does not concede that the '504 patent is entitled to any of its claimed priority dates.

dehydrogenase levels.

During prosecution, the examiner initially rejected Alexion's claims as anticipated by Hillmen, which disclosed methods of using eculizumab for treating PNH, in view of the general knowledge in the art of eculizumab's sequence, as reflected in references such as Thomas (AMG1023). AMG1014, 557-558. The examiner also rejected the claims as obvious over (1) Hillmen, Thomas, and Evans or (2) Hillmen, Thomas, Evans, and US2005/0271660 ("Wang"). *Id.*, 558-561.

In response, Alexion asserted—incorrectly—that the complete structure of eculizumab "was not disclosed in the prior art; nor was it available to the public" and that "none of these references even suggests the unique non-naturally occurring, protein-engineered heavy chain of eculizumab, which confers important, unexpected functional effects." *Id.*, 586-588. Indeed, Alexion's argument that eculizumab's amino acid sequence was not publicly known was the only stated basis for withdrawing the rejections and issuing a notice of allowance: "none of the applied references in the rejection recite using an antibody [with the claimed sequence]." *Id.*, 790.

As shown herein, the examiner was misled. And Alexion has repeatedly made these same misleading arguments to obtain additional eculizumab patents

related to the '504 patent[5] that claim subject matter already in the public domain. For example, when prosecuting U.S. 9,732,149 (claiming eculizumab), Alexion argued that "[n]either eculizumab nor its complete sequence … was in the public domain prior to the March 15, 2007 effective filing date." AMG1015, 709-712. And when prosecuting U.S. 9,718,880 (claiming eculizumab formulations), Alexion again argued that "the complete structure of eculizumab was not disclosed in the prior art; nor was it available to the public." AMG1016, 179-180, 720.

Meanwhile, Alexion was saying the exact opposite in a European opposition proceeding over an eculizumab-related EP patent being challenged for sufficiency of disclosure. There—contrary to what it was telling the USPTO—Alexion stated that "the sequence for eculizumab was publicly available prior to the [February 3, 2004] priority and filing date" and "a sequence for eculizumab was submitted to Chemical Abstract Services (CAS) and entered into their STN database on 14 February 1999…."[6] AMG1017, 277, 291(¶5.1.2). And during prosecution of

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[5] Prosecution of parent applications is considered part of the file history of the child application. *Omega Engg, Inc., v. Raytek Corp.*, 334 F.3d 1314, 1333 (Fed. Cir. 2003); *see also*, *Microsoft Corp. v. Multi-Tech Sys., Inc.*, 357 F.3d 1340, 1349 (Fed. Cir. 2004).

[6] Alexion later tried to take this statement back during prosecution of a

related U.S. Application No. 11/127,438, Alexion argued that its provisional applications provided written description for claims to "eculizumab" and an antibody containing a "mutated Fc portion" because the provisional applications incorporated by reference the Evans prior art:

> Applicant respectfully disagrees and asserts that the priority applications provide ample written support for the claimed descriptions. For example, the priority documents each describe that "Particularly useful anti-C5 antibodies are h5G1.1, h5G1.1-scFv and functional fragments of h5G1.1 are *described in U.S. Patent No. 6,355,245*, the disclosures of which are incorporated herein in their entirely [*sic*] by this reference … Applicant submits that *h5G1.1* … [was] *well-known to one of ordinary skill in the art as eculizumab* … at the time of filing of priority applications.

AMG1049, 838-839 (emphasis added).[7] Alexion cannot have it both ways. Indeed, the European Opposition Division has already revoked at least two of Alexion's

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different European application, arguing that the eculizumab sequence information submitted in February 1999 had unintentional errors in it and therefore was not a public disclosure of the true eculizumab amino acid sequence, notwithstanding Alexion's intent to disclose it to the public. AMG1054, 247-254, 292-293.

[7] Unless otherwise stated, emphasis has been added throughout this Petition.

European eculizumab-related patents. AMG1017, 368-378; AMG1027, 2667-2685.

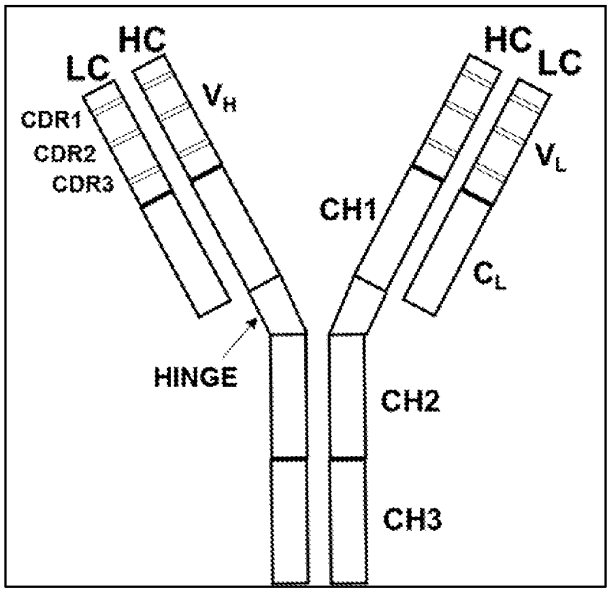
V. State of the art before March 15, 2007.

A. Humanized monoclonal antibodies were well-known.

Before March 15, 2007, the structure of humanized monoclonal antibodies was well understood in the art. AMG1002, ¶¶21-27. Antibodies in general were known to be Y-shaped proteins made up of two identical "heavy chain" polypeptides and two identical "light chain" polypeptides. AMG1018, 7. The art taught that these heavy and light chains comprise a **variable region**—denoted as V_L (for the light chain) and V_H (for the heavy chain)—and a **constant region**—denoted as C_L (for the light chain) and C_H (for the heavy chain). AMG1018, 11-12.

The V_L and V_H regions each contain three "complementarity-determining regions" ("CDRs"), which provide the antibody with its antigen-binding specificity. *Id.* The term "humanized" refers to an antibody having a human framework, into which CDR regions from a non-human monoclonal antibody (*e.g.*, mouse) are inserted. AMG1007, 5:57-67.

This diagram depicts the basic antibody structure:



See AMG1002, ¶25.

B. The prior art taught that PNH was associated with complement system dysregulation.

The complement system comprises a series of proteins that interact with one another in a cascade fashion as part of an immune response, serving a "complementary" role alongside the antibody immune response. AMG1022, R259. Before March 15, 2007, complement was well-understood to play a "central role in host defence against many micro-organisms and in the modulation of inflammatory reactions." *Id.* Complement system dysregulation was known to result in different pathological conditions, including PNH. AMG1023, 1389; AMG1002, ¶¶28-34.

PNH arises from a genetic mutation that depletes one or more cyto-protective proteins that prevent destruction of red blood cells ("RBCs"), platelets,

and other blood cells from complement-mediated attack. AMG1004, 553. PNH is characterized by hemolytic anemia (a decreased number of RBCs due to cell lysis), hemoglobinuria (hemoglobin in the urine due to RBC lysis), and hemoglobinemia (free hemoglobin in the bloodstream due to RBC lysis). AMG1005, ¶[0007]; AMG1002, ¶32.

C. Eculizumab was a known, effective treatment for PNH.

Eculizumab was already known to treat PNH. Alexion itself acknowledged that there were at least "17 different clinical studies using eculizumab that occurred prior to March 16, 2007." AMG1014, 764(¶3). Indeed, numerous Alexion publications described clinical studies using eculizumab for successfully treating PNH. AMG1004, Abstract, 554; AMG1042, Abstract; AMG1047, Abstract, 2560; AMG1011, Abstract; AMG1005, ¶¶[0081]-[0096]; AMG1012, Abstract, 1235; AMG1013, Abstract. Each of the publications described IV infusion of eculizumab in weekly 600 mg doses for four weeks followed by a 900 mg dose one week later, then biweekly 900 mg maintenance doses (summarized below). Thus, the art taught not only that eculizumab was safe and effective for treating PNH, but also taught an effective dosing regimen. AMG1002, ¶¶35-42.

Table 1 Clinical Trials Using Eculizumab to Treat PNH

Study	Alexion Study Number[8]	Dosing Regimen	Outcome
Hillmen (AMG1004) Phase 2 Pilot Study[9] 11 patients	C02-001	"infusions of 600 mg of eculizumab weekly for four weeks, followed one week later by a 900-mg dose and then by a dose of 900 mg every other week through week 12" AMG1004, 554.	"safe and well tolerated in patients with PNH" "improvement in the quality of life in patients with PNH" AMG1004, Abstract.
Hill '05 (AMG1047) Phase 2 Pilot Study Extension #1 11 patients	E02-001	"maintenance dose of 900 mg intravenously every 14 days" for 52 weeks (64 weeks total) AMG1047, 2560.	"sustained reductions in hemolysis and blood transfusions and continued improvement in quality of life." AMG1047, 2565.
Hill '04 (AMG1011) Phase 2 Pilot Study Extension #2	X03-001	"10 of the 11 patients from the initial 3 month study have continued to receive 900 mg eculizumab every other week for 2 years."	"eculizumab is well tolerated and has persisting efficacy … as well as improvement in the symptoms of PNH, for over 2 years of

[8] Study numbers as identified in Alexion's statements made to the USPTO during prosecution. *See, e.g.*, AMG1014, 757, 765.

[9] Alexion also disclosed results from what appears to be the same pilot study in an Abstract published by Hillmen et al. in 2003. AMG1042 ("Hillmen '03").

Table 1 Clinical Trials Using Eculizumab to Treat PNH

Study	Alexion Study Number[8]	Dosing Regimen	Outcome
10 patients		AMG1011, Abstract.	therapy." AMG1011, Abstract.
Bell (AMG1005) Summary of Pilot Study and Extensions 11 patients	N/A	"weekly 600 mg intravenous infusion of [eculizumab] … Patients received 900 mg of eculizumab 1 week later then 900 mg on a bi-weekly basis." AMG1005, ¶[0082].	"reduction in adverse symptoms associated with PNH" AMG1005, ¶[0096].
Hillmen '06 (AMG1012) Phase 3 "TRIUMPH" study 87 patients	C04-001	"infusions of 600 mg of eculizumab or placebo every week (±2 days) for 4 weeks, followed 1 week (±2 days) later by 900 mg of eculizumab or placebo, and then by a maintenance dose of 900 mg of eculizumab or placebo every 2 weeks (±2 days) through week 26." AMG1012, 1235.	"Eculizumab is an effective therapy for PNH." AMG1012, 1233.
Young (AMG1013) Phase 3 "SHEPHERD" study	C04-002	"Eculizumab was dosed as follows: 600 mg IV every 7 days x 4; 900 mg 7 days later; and then 900 mg every 14±2 days."	"eculizumab treatment markedly reduces intravascular hemolysis, thereby providing clinical benefit to treated [PNH] patients."

Table 1 Clinical Trials Using Eculizumab to Treat PNH

Study	Alexion Study Number[8]	Dosing Regimen	Outcome
97 patients		AMG1013, Abstract.	AMG1013, Abstract.

D. The prior art taught that eculizumab is a humanized anti-C5 monoclonal antibody (h5G1.1) containing a hybrid IgG2/IgG4 constant region.

Before March 15, 2007, artisans knew that "eculizumab" was more than just a name; it was a known humanized anti- C5 monoclonal antibody derived from the mouse monoclonal antibody "5G1.1," and was frequently referred to as "h5G1.1" or "h5G1.1-mAb." *See* AMG1005, ¶[0052] ("[t]he antibody *h5G1.1-mAb* is currently undergoing clinical trials under the trade name *eculizumab*."); AMG1034, 1279 ("*h5G1.1-mAb*" is synonymous with "*5G1.1, eculizumab*, Alexion Pharmaceuticals."); and AMG1002, ¶¶43-45.[10] Moreover, Alexion has admitted that "*h5G1.1* … [was] well-known to one of ordinary skill in the art *as eculizumab*…." AMG1049, 838.

[10] *See also*, AMG1019, 56 ("*Eculizumab (5G1.1*; Alexion Pharmaceuticals) is a humanized monoclonal antibody."); AMG1020, 2123 ("*Eculizumab (5G1.1)*, the humanized anti-C5 mAb."); AMG1021, 1017 ("*Synonyms 5G1.1, h5G1.1*, C5 complement inhibitor (Alexion), h5G 1.1 scFv").

The prior art also taught structural aspects of eculizumab, including that eculizumab contains a hybrid IgG2/IgG4 constant region. AMG1034, 1279; AMG1049, 838-839; AMG1002, ¶¶46-49. For example, Tacken explicitly described using "h5G1.1-mAb (5G1.1, *eculizumab*; Alexion Pharmaceuticals)" containing an "*IgG2/IgG4 constant region*." AMG1034, 1279. Likewise, when prosecuting a related application, Alexion told the USPTO that "it was well-known to one of ordinary skill in the art at the time of filing of priority applications [in 2002] that eculizumab has a G2/G4 Fc portion, *i.e.*, a mutated Fc portion."[11] AMG1049, 838-839. It was also well known before March 15, 2007, that antibodies with a hybrid IgG2/IgG4 constant region carried certain benefits, such as a reduced ability to elicit unwanted inflammatory events and lessened propensity to activate the complement system. AMG1032, 11, 19, 28; AMG1031 ("Mueller II"), 451; AMG1002, ¶¶47, 57.

[11] A POSA would have readily distinguished another "humanized 5G1.1" published in Thomas et al. from eculizumab because Thomas' antibody, was an "IgG4 isotype" called "h5G1.1 HuG4," and not an IgG2/IgG4 isotype. The artisan would have been able to readily distinguish eculizumab from them based on whether a hybrid IgG2/IgG4 constant region was present. AMG1023, 1389, 1396; AMG1002, ¶48.

E. The art taught eculizumab's amino acid sequence.

Before March 15, 2007, the art taught eculizumab's amino acid sequence. AMG1002, ¶¶50-60. In 2003, Bowdish described a peptide-antibody recombinant protein using eculizumab as the starter antibody and TPO-mimetic as the agonistic peptide inserted in place of eculizumab's heavy chain CDR3 sequence. AMG1006, ¶¶[0191]-[0193]. Bowdish provided the full amino acid sequence for the TPO-mimetic-eculizumab antibody, and thus taught eculizumab's amino acid sequence with the exception of its heavy chain CDR3 sequence (HCDR3).[12] *Id*, Figures 13A-13B (SEQ ID NOs: 67 and 69); AMG1002, ¶¶51-53. But that HCDR3 sequence was also known in the art from Evans. Indeed, Bowdish cites and incorporates by reference Evans for making eculizumab. AMG1006, ¶[0191] ("[c]onstruction of 5G1.1 is described in [Evans], incorporated herein by reference.")[13].

Evans disclosed all six CDR regions of the original mouse 5G1.1 antibody, which are underlined in the sequences in Evans' Figures 18 and 19. AMG1007,

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[12] As Dr. Balthasar explains, a skilled artisan would have understood that the italicized portions of the sequences in Bowdish's Figures 13A-13B are "leader sequences" that are cleaved off during antibody maturation. AMG1002, ¶52; AMG1006, Figs. 13A-13B; AMG1045, 582.

[13] *See* note 5.

9:65-10:20, Figures 18-19. As Dr. Balthasar confirms, a POSA would have had a reason to replace the TPO mimetic from Bowdish's TPO-eculizumab antibody with Evans' HCDR3 to generate eculizumab for use in treating PNH. AMG1002, ¶¶139-142. When combined, the "scaffold" sequences in Bowdish and eculizumab HCDR3 in Evans together form a humanized anti-C5 antibody having the claimed sequence. AMG1002, ¶¶130-138.

Another Alexion publication, Mueller, also provided complementary pieces of the eculizumab amino acid sequence, along with direction and guidance for making and using the antibody. AMG1002, ¶¶57-59. Mueller published in 1997 and disclosed the amino acid sequence of eculizumab's hybrid IgG2/IgG4 constant region and eculizumab's light chain constant region. AMG1008, 52-53, 58-61; AMG1002, ¶¶57-59. Mueller taught methods of developing recombinant antibodies to reduce immune-mediated organ transplant rejection, including antibodies comprising a hybrid IgG2/IgG4 constant region. *Id.*, 8:23-26, 12:27-30. Mueller described using eculizumab (referred to as "h5G1.1 CO12 HuG2/G4 mAb") as a control antibody that shares that same hybrid IgG2/IgG4 constant region as the experimental antibodies. *Id.*, 12:35-37, Figure 15. In these disclosures, Mueller provided the amino acid sequence of the hybrid IgG2/IgG4 constant region used in its antibodies—*i.e.*, the amino acid sequence of eculizumab's hybrid IgG2/IgG4 constant region. *Id.*, 58-61; AMG1002, ¶¶57-59.

Dr. Balthasar's Figure 10 below schematically shows which portions of eculizumab were disclosed in the prior art.[14]

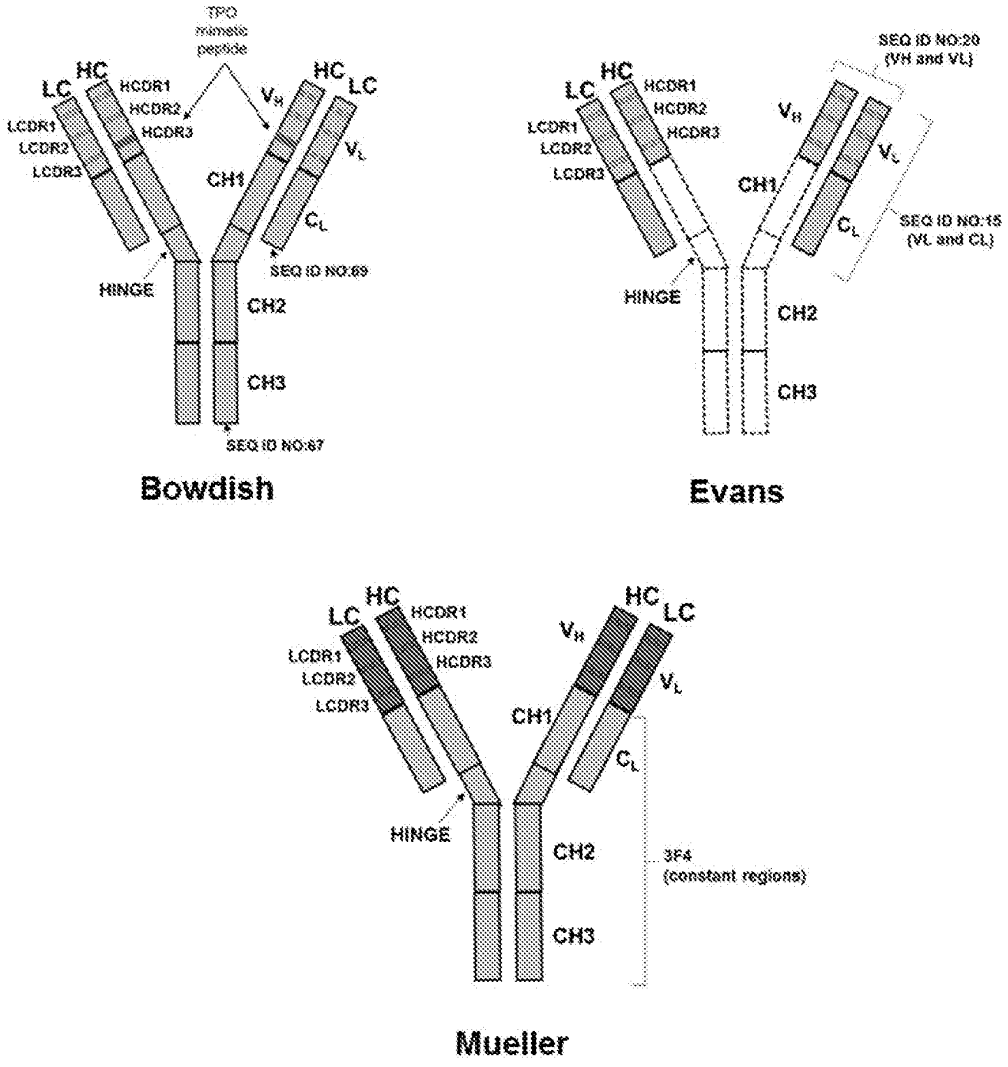


Figure 10.

[14] Green represents eculizumab sequences disclosed in the reference; blue represents non-eculizumab sequences in each reference. AMG1002, ¶60.

See AMG1002, ¶60.

Though different portions of eculizumab's amino acid sequence were taught in different references, the POSA is presumed to be knowledgeable of all the pertinent art—*i.e.*, all the portions of eculizumab and its general structure. *Standard Oil Co. v. American Cyanamid Co*., 774 F.2d 448, 454 (Fed. Cir. 1985).

VI. Person of ordinary skill in art.

A person of ordinary skill in the art (POSA) is a hypothetical person, presumed to be aware of all pertinent art, who thinks along conventional wisdom in the art, and is a person of ordinary creativity. *KSR Int'l Co. v. Teleflex Inc.*, 550 US 398, 421 (2007); *Standard Oil*, 774 F.2d at 454. A POSA in the field of the '504 patent had knowledge of the scientific literature and have skills relating to the design and generation of antibodies, the complement system, and the application of antibodies as therapeutics before March 15, 2007. AMG1002, ¶20. A POSA also had knowledge of laboratory techniques and strategies used in immunology research, including practical applications of the same. *Id.* Typically, a POSA would have had an M.D. and/or a Ph.D. in immunology, biochemistry, cell biology, molecular biology, pharmaceutics, or a related discipline, with at least two years of experience in the field. *Id.* Also, a POSA may have worked as part of a multidisciplinary team and drawn upon not only his or her own skills, but also taken advantage of certain specialized skills of others on the team, *e.g.*, to solve a

given problem; for example, a clinician and a formulation chemist may have been part of a team. *Id.*

VII. Claim construction.

Claims must be given their ordinary and customary meaning in light of the specification—"the meaning that the term would have to a person of ordinary skill in the art in question at the time of the invention." *Phillips v. AWH Corp.*, 415 F.3d 1303, 1312-1313 (Fed. Cir. 2005) (en banc); *see also*, 37 C.F.R. §42.100(b); 83 Fed. Reg. 51340, 51358 (Oct. 11, 2018).

"Immediate." Claim 9 recites that the claimed method "results in an *immediate* and sustained decrease in mean levels of lactate dehydrogenase (LDH)." AMG1001, claim 9. The '504 patent specification teaches "[t]he impact of terminal complement inhibition with eculizumab on chronic intravascular hemolysis in PNH patients was demonstrated in this study by an *immediate (one week)* and sustained decrease in mean levels of LDH." AMG1001, 22:22-25. Claim 10 depends from claim 9 and further requires that "the immediate decrease occurs *within one week* of administration of the antibody." AMG1001, claim 10. Thus, as Dr. Balthasar explains, a POSA would have understood the term "immediate" to include a decrease beginning "within one week" after administering the eculizumab. AMG1002, ¶¶64-65.

The meaning of all other claim terms in the '504 patent are plain on their

face and require no further construction. AMG1002, ¶65. Amgen reserves the right to rebut any claim construction arguments Alexion might raise.

VIII. Identification of the challenge (37 C.F.R. §42.104(b)).

Amgen requests IPR based on the grounds summarized below.

Ground	35 U.S.C. Section (pre-AIA)	Claims	References
1	§102(b)	1-3, 7-10	Hillmen
2	§103(a)	4-5	Hillmen and Bell
3	§103(a)	6	Hillmen, Bell, and Wang
4	§103(a)	1-5, 7-10	Bell, Bowdish, and Evans
5	§103(a)	6	Bell, Bowdish, Evans, and Wang
6	§103(a)	1-5, 7-10	Bell, Evans, and Mueller
7	§103(a)	6	Bell, Evans, Mueller, and Wang

- Hillmen et al., *N. Engl. J. Med.* 350(6):552-559 (2004) ("**Hillmen**") published February 5, 2004. AMG1004, 552.

- US 2005/0191298 A1 ("**Bell**"), published September 1, 2005. AMG1005, face.

- US 2005/0271660 A1 ("**Wang**") published December 8, 2005. AMG1028, face.

- US 2003/0232972 A1 ("**Bowdish**"), published December 18, 2003. AMG1006, face.

- U.S. Patent No. 6,355,245 B1 ("**Evans**"), issued March 12, 2002. AMG1007, face.

- WO 97/11971 ("**Mueller**"), published April 3, 1997. AMG1008, face.

These references are prior art under 35 U.S.C. §102(b) because each published more than one year before March 15, 2007, the '504 patent's earliest claimed priority date.

IX. The same or substantially the same prior art or arguments were not previously presented to the Office.

The arguments and evidence presented herein were not before the examiner during prosecution and, therefore, do not constitute "the same or substantially the same prior art or arguments" under 35 U.S.C. §325(d).

During prosecution, the examiner rejected Alexion's claims as (i) anticipated by Hillmen in view of Thomas; (ii) obvious over Hillmen, Thomas and Evans; and (iii) obvious over Hillmen, Thomas, Evans, and Wang. AMG1014, 557-561. Those rejections rested solely on disclosures in Thomas and Evans for eculizumab sequence information. *Id.* The examiner later allowed the '504 patent claims mistakenly believing—because of Alexion's mischaracterization of the art—that the sequence and structure of eculizumab were not already known.

Though Hillmen, Evans, and Wang were referenced by the examiner during prosecution, this Petition presents them in a different light, along with new references—Bell, Bowdish, and Mueller, which teach the IgG2/IgG4 constant region missing from the art combination raised during prosecution.

Bell and a parent application to Bowdish (US 2003/0049683 A1) was cited but not relied upon during prosecution, and Mueller was not cited at all. The art combinations here, which were not raised by the examiner during prosecution, provide the complete sequence of eculizumab, thereby teaching the very thing the examiner mistakenly concluded was missing from the prior art. Consequently, this Petition is not the same/substantially the same as or cumulative of any previous arguments and § 325(d) does not preclude instituting this Petition.

X. Ground 1: Hillmen anticipates claims 1-3 and 7-10.

Hillmen anticipates claims 1-3 and 7-10. A reference anticipates when it discloses each and every claim limitation "either expressly or inherently." *In re Crish*, 393 F.3d 1253, 1256 (Fed. Cir. 2004). "Under the principles of inherency, if the prior art necessarily … includes[] the claimed limitations, it anticipates." *MEHL/Biophile Int'l Corp. v. Milgraum*, 192 F.3d 1362, 1363 (Fed. Cir. 1999).

A. Hillmen anticipates claim 1.

Dr. Balthasar confirms that Hillmen discloses all the limitations of claim 1, either expressly or inherently, and is enabling. AMG1002, ¶¶75-84.

1. Hillmen explicitly disclosed every element of claim 1, except for eculizumab's amino acid sequence.

Hillmen taught the claimed "method of treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH)" by disclosing "[e]leven transfusion-dependent *patients with PNH* received infusions of eculizumab…." AMG1004, Abstract. Hillmen also disclosed that "*eculizumab treatment* significantly reduced transfusion requirements, even though the levels of hemoglobin did not change significantly." *Id*, 558.

Hillmen also taught the claimed "administering to the patient a pharmaceutical composition comprising an antibody that binds C5" by disclosing administering pharmaceutical compositions of eculizumab (a known antibody that binds C5) to patients: "*patients* with PNH *received infusions of eculizumab*…." *Id*, Abstract. Hillmen further disclosed that "[t]his *antibody against terminal complement protein C5* reduces intravascular hemolysis, hemoglobinuria, and the need for transfusion…." *Id*, Abstract; *see also, id.*, 554 ("Patients *received infusions* of 600 mg *of eculizumab*….").

2. Alexion admitted that Hillmen's eculizumab necessarily has the claimed sequence.

Hillmen's antibody necessarily "comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4" because Alexion admitted that Hillmen's eculizumab possesses those very amino acid sequences.

During prosecution, Alexion submitted a list of eculizumab clinical studies—including Hillmen's Phase 2 Pilot Study (Study "C02-001")—and stated that "the antibody (eculizumab) used in each of the studies … *contained the heavy and light chain sequences of SEQ ID NOs: 2 and 4*." AMG1014, 767(¶6); *see also, id.*, 765 (study number "C02-001"). This admission is binding on Alexion. *See, e.g., Vitronics Corp. v. Conceptronic, Inc.*, 90 F.3d 1576, 1583 (Fed. Cir. 1996); *Tyler Refrigeration v. Kysar Indus. Corp.*, 777 F.2d 687, 690 (Fed. Cir. 1985).

The FDA Medical Review in Alexion's approval package for Soliris® confirmed that "Study C02-001" was published in "Hillmen, P et al. … NEJM. 2004; 350:552-558" (i.e., Hillmen). AMG1024, 109. Similarly, Australia's Pharmaceutical Benefits Advisory Committee ("PBAC") produced a public summary document for Soliris® showing that clinical trial "C02-001 (Pilot Study)" was published in "N Engl J Med 2004, 350:552-559." AMG1025, 2.

Because Alexion's admission confirms that the "eculizumab" disclosed in Hillmen necessarily comprised SEQ ID NO:2 and SEQ ID NO:4, Hillmen inherently discloses the claimed sequences.

Despite admitting to the Office that the eculizumab disclosed in Hillmen necessarily comprised SEQ ID NO:2 and SEQ ID NO:4, Alexion misleadingly argued during prosecution that its contribution over the art was the specific amino acid sequence of eculizumab. AMG1014, 586-587, 738-744. However, as *Crish*

makes clear, "just as the discovery of properties of a known material does not make it novel, *the identification and characterization of a prior art material also does not make it novel*." *Crish*, 393 F.3d at 1258. There is "[a] long line of cases confirm[ing] that one cannot establish novelty by claiming a known material by its properties." *Id.*

In *Crish*, the applicant claimed an hINV promoter region based on its nucleotide sequence. The court stated that the pertinent inquiry for its anticipation analysis is "whether the claimed [invention] was new," and determined that:

> The promoter region of hINV *was not new* … hINV was *known and used years before* … The only arguable contribution to the art that Crish's application makes is the identification of the nucleotide sequence of the promoter region of hINV. However, just as the discovery of properties of a known material does not make it novel, *the identification and characterization of a prior art material also does not make it novel*.

Id.; *see also, Atlas Powder Co. v. IRECO Inc.,* 190 F.3d 1342, 1347 (Fed. Cir. 1999 ("the discovery of a previously unappreciated property of a prior art composition … does not render the old composition patentably new to the discoverer."); *Abbott Labs. v. Baxter Pharms.*, 471 F. 3d 1363, 1368 (Fed. Cir. 2006) (lack of knowledge of a compound's property is "wholly irrelevant to the

question of whether [a patent] claims something 'new' over the disclosure of the [prior art]."). This precedent is squarely applicable here and compels finding anticipation. The '504 patent's mere claim to an amino acid sequence that Alexion admits was a property of a prior art compound (eculizumab) contributes nothing over the prior art.

3. Hillmen's disclosure is enabling.

Hillmen would have enabled a POSA to practice the method of claim 1 without undue experimentation. An anticipatory publication "must be capable, *when taken in conjunction with the knowledge of those skilled in the art* to which it pertains, of placing that invention in the possession of the public." *In re Donohue*, 632 F.2d 123, 125 (CCPA 1980) ("*Donohue I*"); *see also*, *In re Donohue*, 766 F.2d 531, 533 (Fed. Cir. 1985) ("*Donohue II*") (public possession "is effected if one of ordinary skill in the art could have *combined the publication's description* of the invention *with his own knowledge* to make the claimed invention."); and *Elan Pharms. v. Mayo Found.*, 346 F.3d 1051, 1054 (Fed. Cir. 2003).

To determine whether experimentation would be undue, one must examine "(1) the quantity of experimentation; (2) the amount of direction or guidance present; (3) the presence or absence of working examples; (4) the nature of the invention; (5) the state of the prior art; (6) the relative skill of those in the art; (7) the predictability or unpredictability of the art; and (8) the breadth of the claims."

Impax Labs. v. Aventis Pharms., 545 F.3d 1312, 1314-1315 (Fed. Cir. 2008) (citing *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988)). Applying these factors, Dr. Balthasar explains that Hillmen, coupled with the general knowledge in the art, would have enabled a POSA to practice the method of claim 1. AMG1002, ¶¶81-84.

The alleged invention and claims are directed to a method of treatment using eculizumab. This method was expressly taught by Hillmen. AMG1004, 553-554. And the general knowledge in the prior art was replete with disclosures, direction, and guidance of eculizumab's structure and amino acid sequence. AMG1002, ¶¶81-84.

Dr. Balthasar's Figure 10 above illustrates the art disclosing eculizumab amino acid sequences. *See* Section IV.E, *supra*; AMG1002, ¶60. Dr. Balthasar explains two independent ways in which a POSA would have obtained eculizumab's amino acid sequence:

(1) A POSA would have known that **Bowdish** disclosed the entire amino acid sequence of eculizumab with the exception of the heavy chain CDR3 region, and that **Evans** disclosed the eculizumab heavy chain CDR3 region (the missing piece from Bowdish). AMG1006, ¶¶[0191]-[0193], Figure 13A-13B (SEQ ID NOs:67 and 69); AMG1007, 44:4-13 (SEQ ID NO:20); AMG1002, ¶82.

(2) A POSA also would have known that **Evans** disclosed the amino acid sequences of eculizumab's heavy and light chain variable domains, and **Mueller** disclosed the hybrid IgG2/IgG4 heavy chain and light chain constant regions of eculizumab. AMG1007, 44:4-13 (SEQ ID NO:20); AMG1008, 52-53, 58-61; AMG1002, ¶82.

And given the high level of skill in the relevant field, the POSA would have readily obtained eculizumab's sequences as claimed from the art using only routine experimentation. AMG1002, ¶83

Armed with the general knowledge in the relevant field, a POSA reading Hillmen would not have "needed to experiment unduly to gain possession of the invention." *Impax*, 545 F.3d at 1315-1316. Here, just as in *In re Donohue*, "the primary reference named a composition falling within the scope of the claims and indicated that it had previously been made and tested; additional references showed that a method of making this composition would have been within the knowledge of one of ordinary skill in the art." *Donohue I* at 126.

The law compels finding anticipation here. The claimed methods were not new—Hillmen taught them. Alexion's admission that Hillmen's eculizumab inherently possesses the claimed sequences is binding under *Vitronics. Crish* dictates that Alexion cannot later claim the amino acid sequence it admits was inherent to an antibody in the prior art. And under *Donohue I* and *Donohue II*,

Hillmen is enabling.

B. Hillmen anticipates claim 2.

Claim 2 depends from claim 1, and further requires that the pharmaceutical composition be "administered by intravenous infusion." Claim 2 is anticipated because Hillmen expressly discloses that "[p]atients received infusions" of eculizumab. AMG1004, 554, Abstract. That Hillmen does not explicitly say "intravenous" is irrelevant. Other Alexion publications confirm that Hillmen administered eculizumab *via* intravenous infusion. *See* AMG1042 (2003), Abstract (disclosing that 11 PNH patients "completed a 3-month pilot study receiving 600 mg eculizumab by *intravenous infusion*…."); AMG1043 (2007), 4124 (disclosing Hillmen as administering eculizumab "via *intravenous infusion*" in the "12-week phase 2 pilot study."). AMG1002, ¶¶85-86.There can be no dispute that Hillmen's eculizumab "infusions" were intravenous. Indeed, the '504 patent itself refers to intravenous infusions of eculizumab as "infusions." *See e.g.,* AMG1001, 26:47 ("patients experienced headache within 48 hours of *infusion*").

Hillmen is enabling for the method of claim 2 for the same reasons provided for claim 1. AMG1002, ¶87. Moreover, preparing and administering pharmaceutical compositions by intravenous infusion was well within the routine skills of a POSA. *Id.*

C. Hillmen anticipates claim 3.

Claim 3 depends from claim 1 and further requires that "the antibody is administered to the patient at a dosage level of between 5 mg per kg and 50 mg per kg per patient per treatment." Hillmen anticipates claim 3 because Hillmen disclosed administering eculizumab dosages of 600 mg and 900 mg to adult men and women. AMG1004, Abstract, 554; AMG1002, ¶¶88-91.

As Dr. Balthasar explains, the amount of eculizumab in mg per kg administered to a patient will vary depending on the patient's body weight. AMG1002, ¶¶89-90. The FDA's Pharmacometrics Review in the Soliris® approval package provides the minimum and maximum patient body weights from 209 patients in six different eculizumab clinical trials, *including the Hillmen pilot study C02-001*, and definitively shows that Hillmen's 11 patients weighed between 48.5 kg and 157.2 kg. AMG1044, 8-9. Even with these lower and upper limits for body weight, the 11 patients treated in Hillmen necessarily received at least one dose of eculizumab of between 5 to 50 mg/kg:[15]

[15] According to the CDC, the average body weight for adult females and males in the U.S. is 74 kg and 87 kg, respectively. AMG1050, 61. Administering Hillmen's 600 and 900 mg doses of eculizumab to patients with these body weights would provide doses ranging from 6.9 to 12.2 mg/kg. AMG1002, ¶90.

Body weight (kg)	Dose (mg/kg) for 600 mg	Dose (mg/kg) for 900 mg
Minimum 48.5	12.4	18.6
Mean 85.4	7.0	10.5
Maximum 157.2	3.8	5.7

See AMG1044, 8-9; AMG1002, ¶89. Thus, Hillmen's disclosure of administering 600 mg or 900 mg eculizumab anticipates claim 3 because every patient in Hillmen's trial received at least one dose of eculizumab that would necessarily have fallen within the claimed range. AMG1004, 553; AMG1002, ¶¶89-90.

Hillmen is enabling for the method of claim 3 for the same reasons already discussed. AMG1004, 554; AMG1002, ¶91. Moreover, preparing and administering pharmaceutical compositions based on a patient's body weight (mg per kg) was well within the routine skills of a POSA. AMG1002, ¶91.

D. Hillmen anticipates claim 7.

Claim 7 depends from claim 1 and further requires that the "patient is anemic." Claim 7 is anticipated because Hillmen expressly discloses treating patients who had "received a diagnosis of aplastic *anemia*." AMG1004, 555. Hillmen is enabling for the method of claim 7 for the same reasons already discussed. AMG1002, ¶92.

E. Hillmen anticipates claim 8.

Claim 8 depends from claim 1 and further requires that the patient is dosed with "600 mg of the antibody via intravenous infusion every 7 ± 1 days for 4

doses; followed by 900 mg of the antibody via intravenous infusion 7± 1 days later; followed by a maintenance dose of 900 mg of the antibody via intravenous infusion every 14 ± 2 days."

Hillmen expressly taught this dosing regimen:

> Patients received infusions of 600 mg of eculizumab weekly for four weeks, followed one week later by a 900-mg dose and then by a dose of 900 mg every other week through week 12.

AMG1004, 554; *see also*, AMG1002. ¶93. Hillmen is enabling for the method of claim 8 for the same reasons already discussed. AMG1002, ¶93.

F. Hillmen anticipates claim 9 and 10.

Claim 9 depends from claim 1 and further requires that "administration of the antibody results in an immediate and sustained decrease in mean levels of lactate dehydrogenase (LDH)." Claim 10 depends from claim 9 and further requires that "the immediate decrease occurs within one week of administration of the antibody." Claims 9 and 10 are anticipated because Hillmen taught that administration of eculizumab resulted in an immediate and sustained decrease in LDH levels. AMG1004, Fig. 2.

Discussed above in Section VI, a POSA would have understood that "immediate" decrease includes a decrease beginning within one week after

administering the eculizumab. AMG1002, ¶65; AMG1001, 22:22-25. Hillmen disclosed that "[t]he decrease in lactate dehydrogenase began after a single dose of eculizumab in all patients" and that "[LDH] levels remained within or just above the normal range for the duration of the study." AMG1004, 555; AMG1002, ¶95. Dr. Balthasar confirms that Hillmen's data show an immediate and sustained decrease in LDH levels beginning within one week of administering eculizumab:

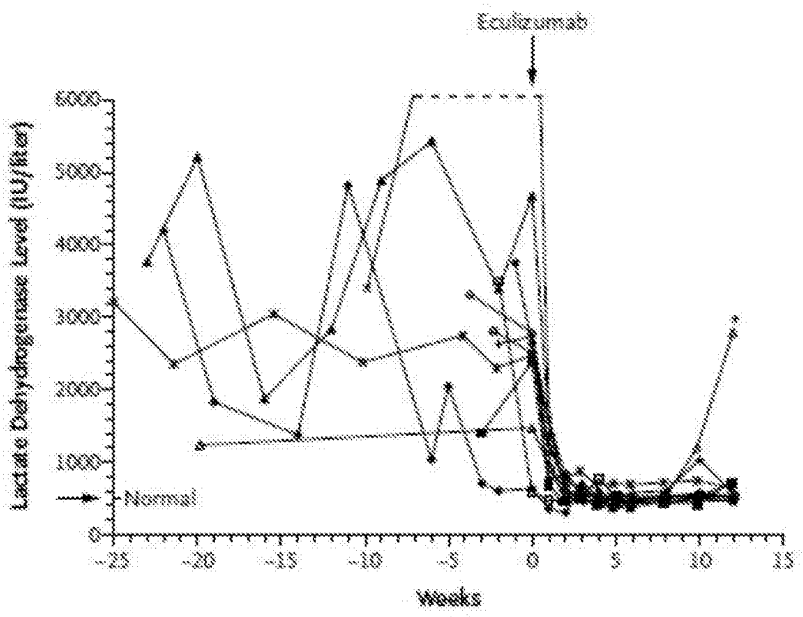


Figure 2. **Analysis of Lactate Dehydrogenase Levels, a Biochemical Indicator of Hemolysis, in 11 Patients with Paroxysmal Nocturnal Hemoglobinuria up to 25 Weeks before and during 12 Weeks of Eculizumab Treatment.**
The first dose of eculizumab is indicated by an arrow, as is the upper limit of the normal range of lactate dehydrogenase at the Leeds Teaching Hospital. The data point identified at week 12 by the asterisk represents a reading that was obtained from a duplicate serum sample since the original sample was lost. The dashed line represents off-scale points from one patient with a peak value of 12,100 IU per liter.

See AMG1004, Fig. 2; *see also*, AMG1002, ¶¶94-95.

Hillmen is enabling for the method of claims 9 and 10 for the same reasons already discussed. AMG1002, ¶95.

XI. Ground 2: claims 4-5 would have been obvious over Hillmen and Bell.

Hillmen taught all the elements of claim 1. While Hillmen might not explicitly disclose additional limitations of claims 4-5, those limitations were taught in Bell. A POSA would have had reason to combine Bell with Hillmen to arrive at claims 4-5, with a reasonable expectation of success. AMG1002, ¶¶97-109. And no objective indicia support patentability. *See* Section XIII.

Like Hillmen, Bell taught treating a patient suffering from PNH using a pharmaceutical composition comprising eculizumab. AMG1005, ¶¶[0012], [0052], [0081]-[0096]. Indeed, Bell disclosed a Phase 2 Pilot Study involving 11 PNH patients treated with eculizumab over a period of 12 weeks—identical to that disclosed in Hillmen. *Compare* AMG1005, ¶¶[0081]-[0082] *with* AMG1004, Abstract and 553-554. As Dr. Balthasar explains, Bell and Hillmen shared the following identical features:

- antibody (eculizumab);
- patient pool (6 male, 5 female);
- administration route (intravenous);

- dosing regimens;[16] and
- resulting data.

AMG1002, ¶¶39, 98; AMG1005, ¶¶[0081]-[0096].[17] Given the substantial overlap, a POSA would have had ample reason to combine Hillmen and Bell, with a reasonable expectation of success at achieving the claimed subject matter. No objective indicia of nonobviousness support patentability. *See* Section XIII.

Claim 4 depends from claim 1, and requires that "the pharmaceutical composition is in a *single unit dosage form*." Claim 5 depends from claim 4 and

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[16] 600 mg eculizumab weekly for four weeks, followed by a 900 mg dose, then 900 mg eculizumab every other week through week 12.

[17] Because the clinical study taught in Bell is the same C02-001 study disclosed in Hillmen, which discloses the eculizumab amino acid sequences of SEQ ID NOs: 2 and 4, Bell, too, therefore anticipates claims 1-4 and 7-10 for the same reasons discussed above for Hillmen. AMG1014, 767(¶6); *see also, id.*, 765 (study number "C02-001"); AMG1005, ¶[0082]; AMG1002, ¶120, n9. Hill '05, Hill '04, Hillmen '06, and Young '06 also anticipate claims 1-3 and 7-10 for the same reasons. AMG1014, 767(¶6), 765 (study numbers E02-001, X03-001, C04-001, and C04-002, respectively); AMG1047, 2559-2560; AMG1011, Abstract; AMG1012, 1235; AMG1013, Abstract.

requires that "the single unit dosage form is a *300 mg single unit dosage form*." AMG1001, 39:13-16. Bell taught these limitations, stating that eculizumab can be "administered in a variety of *unit dosage forms*. AMG1005, ¶[0058].

As Dr. Balthasar explains, a POSA would have understood that a "unit dosage form" of an antibody as taught in Bell is formulated for use in a single patient and is therefore a "single" unit dosage form as claimed. AMG1002, ¶¶102-103; AMG1046, 2989; AMG1051, 1. For example, a POSA would have generally known that antibody dosage forms should not be shared among patients or returned to storage after opening, to prevent contamination and unnecessary health risks. AMG1002, ¶103; AMG1046, 2989. Alexion agrees, telling the USPTO during prosecution[18] that skilled artisans "would have been well aware … that single-dose vials are intended for a single procedure or injection and should always be discarded at the end of the procedure/injection. They should never be stored for later use or subsequent procedures, even if for the same patient." AMG1026, 208-209 (internal quotation omitted).

Thus, Bell would have motivated a POSA to prepare eculizumab formulations as single unit dosage forms as claimed in claim 4 using routine

[18] U.S. Appl. No. 13/426,973 is the abandoned grandparent application of the '504 patent. AMG1001, face.

formulation preparation techniques that were standard and well-known in the art, particularly for intravenous therapeutic antibody formulations. AMG1002, ¶103. Indeed, the '504 patent mentions no "newly discovered" advantages of the single-unit-dosage-form claim limitation.

Moreover, a POSA would have arrived at the 300 mg strength (claim 5) through simple convenience and a logical application of knowledge in the art. AMG1002, ¶¶104-109. Both Hillmen and Bell explicitly taught administering eculizumab in doses of 600 mg and 900 mg (AMG1004, 554; AMG1005, ¶[0082]), thus prompting a POSA, as Dr. Balthasar explains, to administer those same doses because they were each shown to successfully treat PNH. AMG1004, 558-559; AMG1005, ¶¶[0089]-[0096]; AMG1002, ¶105.

To accommodate 600 mg and 900 mg dosages, a 300 mg single unit dosage amount would have been the most obvious and convenient (and thus predictable) means to a POSA. It would have been easier to achieve 600 mg and 900 mg doses with two and three 300 mg dosages, respectively, than to separately manufacture those two strengths. AMG1002, ¶¶106-107. *See KSR*, 550 US at 416 ("When a work is available in one field, design incentives and other market forces can prompt variations of it … If a person of ordinary skill in the art can implement a predictable variation, and would see the benefit of doing so, § 103 likely bars its patentability.") Alexion confirmed during a European opposition proceeding for a

related EP patent[19] that there is nothing critical about the claimed single unit dosage form beyond convenience: "*simple dilution* of product required by the label *conveniently* provides the required 600 or 900 mg administration dosages." AMG1027, 1104(¶5.3.9).

According to Dr. Balthasar, a POSA at the time of invention would have had a reasonable expectation of successfully preparing a 300 mg single unit dosage form because doing so would have been a simple, conventional process of reconstitution and dilution. AMG1002, ¶108; AMG1053, ¶[0043]. Moreover, Bell taught methods for preparing unit dosage forms, and both Hillmen and Bell described successfully preparing and administering 600 mg and 900 mg doses. AMG1004, 553-554; AMG1005, ¶¶[0058]-[0060], [0062], [0082]; AMG1002, ¶108.

XII. Ground 3: claim 6 would have been obvious over Hillmen, Bell, and Wang.

Claim 6 depends from claim 1 and further requires formulating eculizumab as "a 300 mg single-use[20] formulation of 30 ml of a 10 mg/ml sterile, preservative

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[19] EP Patent No. 2359834 is a European counterpart to the '504 patent.

[20] As explained above, a single unit dosage form is understood to be for a single use. AMG1002, ¶¶102-103; AMG1046, 2989. Alexion agrees. AMG1026, 208-209.

free solution." AMG1001, claim 6. The only added limitation in claim 6 over claim 5 is a "30 ml of a 10 mg/ml sterile, preservative free solution." This limitation would have been obvious over Hillmen, Bell, and Wang. AMG1002, ¶¶110-118. The teachings in Hillmen and Bell are discussed in Ground 2. Wang taught therapeutic antibody formulations, and explicitly taught formulating "*eculizumab* as the antibody therapeutic."[21] AMG1028, ¶[0004]. With all three references explicitly teaching aspects of eculizumab, a POSA would have had ample reason to combine Hillmen, Bell, and Wang with a reasonable expectation of success at achieving the claimed subject matter. AMG1002, ¶¶117.

First, Bell explains that its eculizumab formulations "*must be sterile* and non-pyrogenic," and that they "*may* contain … preservatives…" (i.e., preservatives are optional). AMG1005, ¶[0062]. Dr. Balthasar further explains that a POSA would also have generally known that therapeutic antibodies are routinely prepared in sterile, preservative-free solutions. AMG1002, ¶112. In fact, of 22 monoclonal

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[21] Wang's nebulization formulation is equally relevant to intravenous infusion because at bottom, Wang taught that liquid formulations of eculizumab at 1-30 mg/ml would be sufficiently stable and maintain its biological activity. AMG1028, ¶[0173]; AMG1002, ¶113.

antibodies approved by the FDA before March 15, 2007—and of which the POSA would have been aware—100% of them were formulated in *sterile, preservative-free* solutions. *Id.*; AMG1056-AMG1076.

Second, Wang taught eculizumab formulations of between *1 and 30 mg/ml*, completely encompassing the claimed 10 mg/ml concentration. AMG1028, Fig. 10, ¶¶[0170]-[0172]; AMG1002, ¶113. "[A] prior art reference that discloses a range encompassing a somewhat narrower claimed range is sufficient to establish a *prima facie* case of obviousness." *In re Peterson*, 315 F.3d 1325, 1330 (Fed. Cir. 2003); *In re Wertheim*, 541 F.2d 257 (CCPA 1976) (finding when claimed values "overlap or lie inside ranges disclosed by the prior art," a *prima facie* case of obviousness exists.).

Wang's eculizumab concentrations are also consistent with the general knowledge in the art that 10 mg/ml was a common concentration used for formulating therapeutic antibodies. AMG1002, ¶114; AMG1029, Table 1; AMG1030, Table 1. The law is settled: "[w]here the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experimentation." *In re Aller*, 220 F.2d 454, 456 (CCPA 1955). Moreover, there is no evidence that the claimed 10 mg/ml

concentration is in any way critical to the claimed method.[22] AMG1002, ¶115.

Third, given the state of the art as discussed above, a POSA would have prepared the eculizumab formulation in 30 ml single-use dosage forms based on simple arithmetic. As discussed, 300 mg dosage units would be the easiest way to have one dosage form achieve 600 mg and 900 mg dosages. Likewise, 10 mg/mL was the prevailing standard concentration for this class of drug. Therefore, a dose of 300 mg concentrated at 10 mg/ml must be in 30 ml of solution (10 mg/ml x 30 ml = 300 mg). AMG1002, ¶116.

Notably, the '504 patent specification mentions no newly discovered advantage of a 30 ml dosage volume to suggest inventiveness in any way. Alexion even stated that the "low-volume liquid formulation … is *easy for the clinician to store and use*" and that the 30 ml size "*minimises the volume which must be stored* on site at each clinic." AMG1027, 1638(¶3.5), 1104(¶5.3.9). Easier storage for lower volumes compared to larger is common knowledge, not inventive. AMG1002, ¶116.

A POSA would have had a reasonable expectation of success here because formulating the doses into 300 mg single-use formulations of 30 ml of a 10 mg/ml

[22] Alexion argued in Europe that 10 mg/ml improved stability (AMG1027, 1104(¶5.3.8)), but as discussed at Section XIII, Alexion's arguments are meritless.

sterile, preservative-free solution required mere conventional wisdom and routine skill. AMG1002, ¶117. And no objective indicia support patentability. *See* Section XIII.

XIII. Grounds 4-7: claims 1-10 would have been obvious over Bell and prior-art disclosures of anti-C5 antibody sequences.

Dr. Balthasar explains that a POSA reading Bell would have constructed a humanized 5G1.1 anti-C5 antibody, like eculizumab at least two different ways: (i) by combining the sequences taught in Bowdish and Evans **(Grounds 4-5)**; or (ii) by combining the sequences taught in Evans and Mueller **(Grounds 6-7)**. AMG1002, ¶¶119-209. And a POSA would have had reasons to combine these references with a reasonable expectation of success in arriving at the claimed subject matter. *Id.*. And no objective indicia support patentability. *See* Section XIII.

A. Ground 4: claims 1-5 and 7-10 would have been obvious over Bell, Bowdish, and Evans.

Discussed below, a POSA would have had a reason to combine Bell, Bowdish, and Evans with a reasonable expectation of successfully practicing the method of claims 1-5 and 7-10. AMG1002, ¶¶119-124.

1. Claim 1 would have been obvious.

a) Bell expressly taught all the limitations of claim 1 except eculizumab's amino acid sequence.

Bell taught the claimed method of treating PNH by disclosing "a method of

treating a hemolytic disease such as, for example, *paroxysmal nocturnal hemoglobinuria ("PNH")*" and that "*[p]aroxysmal nocturnal hemoglobinuria ("PNH")* and other hemolytic diseases *are treated* in accordance with this disclosure." AMG1005, ¶¶[0003], [0012]; AMG1002, ¶¶125-129. Bell also taught the claimed treatment composition comprising an antibody that binds C5 by disclosing "an *anti-C5 antibody* selected from the group consisting of *h5G1.1-mAb (eculizumab)…*" suitable for treating PNH, and also that the antibody "h5G1.1-mAb" was "undergoing clinical trials under the tradename *eculizumab*." AMG1005, ¶¶[0012], [0052]. Bell further taught methods of preparing eculizumab compositions comprising "pharmaceutically effective carriers" and preparing "[f]ormulations suitable for injection." AMG1005, ¶¶ [0060], [0062]; AMG1002, ¶127.

Bell taught that eculizumab successfully treated patients suffering from PNH. AMG1005, ¶¶[0081]-[0096]; AMG1002, ¶128. Bell described an eculizumab clinical trial involving a 12-week "pilot study," followed by a 52-week extension study for a total of 64 weeks, followed by a second extension study for a total of two years.[23] AMG1005, ¶[0082]. Bell concluded that "[p]atients in the two

[23] As already discussed, Bell's studies are identical to Hillmen, Hill '05, and Hill '04. *See* note 15, *supra*; *see also,* AMG1002, ¶39; AMG1014, 757, 765.

year study experienced a reduction in adverse symptoms associated with PNH." AMG1005, ¶[0096]. Thus, Bell would have motivated a POSA to practice the claimed method of treating PNH with an anti-C5 antibody like eculizumab. The only information arguably missing from Bell is the antibody amino acid sequences (SEQ ID NOs: 2 and 4 as claimed), but they were readily found in Bowdish and Evans.

b) Bowdish and Evans taught the claimed amino acid sequences.

Bowdish taught methods of making peptide-antibody chimeric proteins, and described using a humanized anti-C5 antibody as the starter "scaffold" antibody sequence for creating a recombinant TPO-mimetic. Bowdish provided the full antibody amino acid sequence except for the heavy chain CDR3 (HCDR3) sequence, which was replaced with the TPO-mimetic peptide sequence (LPIEGPTLRQWLAARAPV). AMG1006, ¶¶[0191]-[0193], Figs. 13A-13B, and SEQ ID NOs.:67 and 69; AMG1002, ¶¶130-135. As explained in detail below, the missing HCDR3 sequence from Bowdish was taught by Evans. Dr. Balthasar's Figure 11 below depicts this combination of art providing the complete eculizumab sequence:

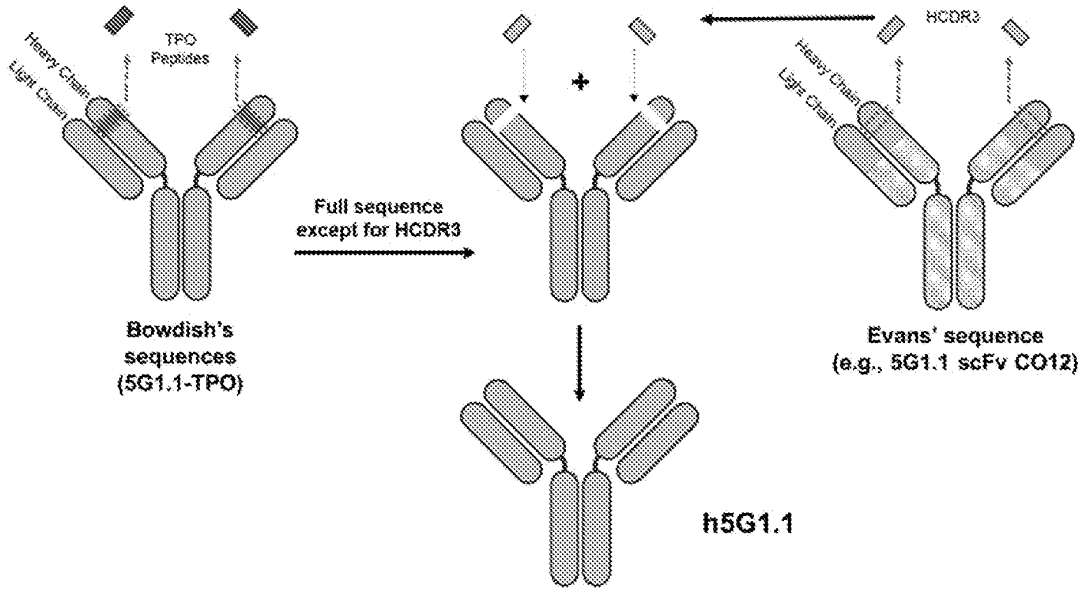


Figure 11.

AMG1002, ¶120.

Bowdish provides the complete sequences of the heavy and light chains of a recombinant TPO-mimetic-containing antibody ("5G1.1+TPO") as SEQ ID NO:67 ("5G1.1-TPO Heavy Chain") and SEQ ID NO:69 ("5G1.1 Light Chain") in Figures 13A and 13B, respectively. AMG1006, Figures 13A-13B; AMG1002, ¶¶130-133. Dr. Balthasar's Figure 3 below illustrates the structure of Bowdish's 5G1.1+TPO, showing the location of the TPO-mimetic peptide (blue) in the HCDR3 region of the polypeptide of SEQ ID NOs:67 (heavy chain), and the polypeptide of SEQ ID NO:69 (light chain), where green represents sequences 100 % identical to SEQ ID NO:2 and SEQ ID NO:4::

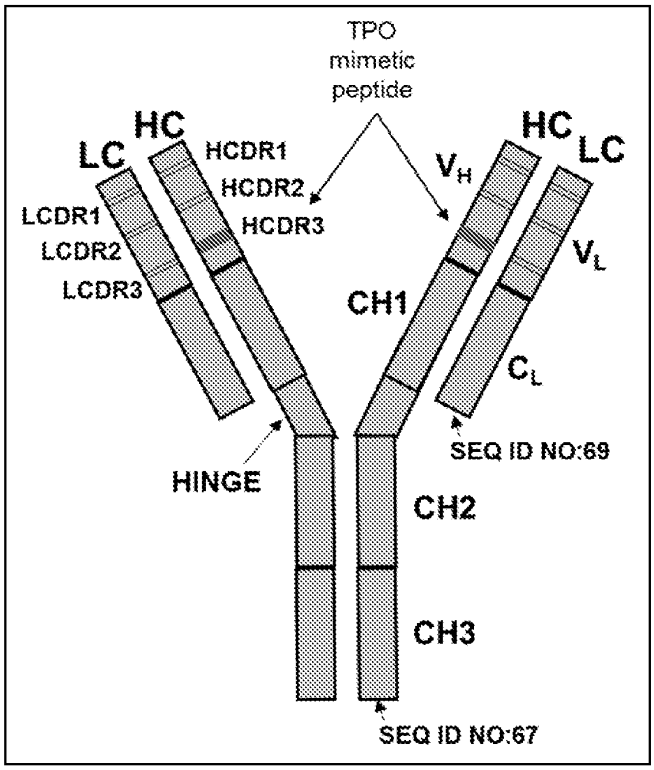


Figure 3.

AMG1002, ¶¶51-53; AMG1006, Figures 13A-13B.

As Dr. Balthasar explains, the mature portion of Bowdish's SEQ ID NO:69 (i.e., the light chain) is 100% identical to the claimed SEQ ID NO:4; and the mature portion of Bowdish's SEQ ID NO:67 (i.e., the heavy chain) is 100% identical to the claimed SEQ ID NO:2, with the exception of the HCDR3 sequence. AMG1002, ¶¶133-138.

Bowdish's sequences in SEQ ID NOs:69 and 67 each contain italicized portions, which Bowdish explicitly denoted as "leader sequence[s]." As Dr.

Balthasar explains, a POSA would have known that any leader sequence would be cleaved from the mature antibody sequence. AMG1002, ¶133. Thus, the POSA would have known that the leader sequences in Bowdish's Figures 13A-13B are not part of the mature antibody sequence[24]. AMG1006, Figures 13A-13B; AMG1002, ¶133; AMG1045, 582.

A POSA wanting to make a humanized anti-C5 antibody would have understood that the only portion of the heavy chain sequence missing from Bowdish is the HCDR3 sequence because Bowdish taught that "[t]he TPO mimetic peptide graft in Fab clone X4b has been *transplanted into the heavy chain CDR3* of another antibody framework, *5G1.1* … The sequence was *cloned into 5G1.1* in such a fashion as to *replace the native CDR3*." AMG1006, ¶[0191]; AMG1002, ¶¶137. The missing HCDR3 sequence, however, was taught in Evans, and Bowdish expressly directed a POSA to Evans. AMG1006, ¶[0191].

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[24] Alexion is estopped from arguing to the contrary. During prosecution Alexion amended original SEQ ID NO:4 to *remove* the leader sequence, arguing that "the mature light chain sequence is an *inherent* portion of the precursor sequence … and could have been readily identified at the relevant filing date using well established rules and art-recognized techniques…." AMG1014, 513; *see Vitronics Corp.*, 90 F.3d at 1583; *Tyler Refrigeration*, 777 F.2d at 690.

Evans disclosed preparing humanized C5-binding antibodies, referred to therein as "5G1.1" antibodies. AMG1007, 19:47-49, 37:35-39:30, 40:31-45:4; AMG1002, ¶136. As Dr. Balthasar explains, a POSA would have understood that *each* of the 5G1.1 antibody heavy chain variable regions in Evans contain *the same CDR3 sequence*: YFFGSSPNWYFDV. AMG1007, Fig. 19, 43:13-14, 43:26-27, 43:33-34, 43:60-61, 44:2-3, 44:12-13, 44:21-22, 44:30-31, 44:39-40, 44:49-50, 44:59-60, 45:3-4; AMG1002, ¶136. Thus, regardless of which "version" of Evans' humanized 5G1.1 the POSA selected to combine with Bowdish, that heavy chain would contain the YFFGSSPNWYFDV CDR3 sequence. AMG1002, ¶136. Dr. Balthasar's Figure 11 above shows this, where Evans' HCDR3 sequence is extracted and inserted into Bowdish's construct. AMG1002, ¶120.[25] And a POSA would have expected that inserting Evans' YFFGSSPNWYFDV HCDR3 sequence in place of Bowdish's TPO peptide sequence (LPIEGPTLRQWLAARAPV) in SEQ ID NO:67 would provide a complete heavy chain of a humanized anti-C5 antibody that would bind C5. AMG1002, ¶¶137-138. Dr. Balthasar confirms by sequence alignment that this combination provides a sequence identical to the '504

[25] To be clear, a POSA would have known that this would be achieved by replacing DNA encoding Bowdish's TPO-mimetic (SEQ ID NO:65) with DNA encoding Evan's HCDR3. AMG1002, ¶121.

patent's SEQ ID NO: 2. *Id.*.

c) A POSA would have had a reason to combine the references with a reasonable expectation of success.

Bell taught that targeting complement protein C5 with an anti-C5 antibody is safe and effective for treating PNH patients. AMG1005, ¶¶[0083]-[0096]. Because Bell does not expressly provide the amino acid sequence of its antibody, a POSA would have looked to other teachings in the art, like Bowdish and Evans. AMG1002, ¶¶139-142.

A POSA is "presumed to be aware of all the pertinent prior art." *Standard Oil*, 774 F.2d at 454. A POSA, therefore, would have been well aware that Bell's anti-C5 antibody, eculizumab was also known in the art as humanized "5G1.1," "h5G1.1," or "h5G1.1-mAb" with a hybrid IgG2/IgG4 constant region. AMG1002, ¶¶43-45; AMG1005, ¶[0052]; AMG1034, 1279; *see also*, Section IV.D, *supra*. To possess the amino acid sequence of that antibody, a POSA would have consulted Bowdish because it taught using a humanized 5G1.1 antibody (including the hybrid IgG2/IgG4 heavy chain constant region) as the starter antibody sequence when creating the TPO-mimetic-containing antibody and further provided the amino acid sequence of the chimeric antibody. AMG1002, ¶140. Knowing that the only change made to the scaffold antibody in Bowdish was replacing its HCDR3 region with a TPO-mimetic peptide, a POSA would have had a reason to restore

the original HCDR3 region to complete the original anti-C5 antibody. AMG1002, ¶141.

The POSA would have looked to Evans for the missing HCDR3 sequence because both Bell and Bowdish explicitly direct the artisan there for information on how the h5G1.1 antibody was originally created. AMG1005, ¶[0052]; AMG1006, ¶[0191]; AMG1002, ¶142. For example, Bell stated: "[m]ethods for the preparation of *h5G1.1-mAb* [eculizumab] … are described in [Evans][26] … incorporated herein in [its] entirety …." AMG1005, ¶[0052]. And Bowdish similarly stated: "[c]onstruction of *5G1.1* is described in [Evans], incorporated herein by reference."[27] AMG1006, ¶[0191].

This combination of prior art would have led a POSA to make a simple substitution of one known element for another—i.e., replace the TPO-mimetic peptide sequence in Bowdish's antibody with the HCDR3 sequence from Evans—

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[26] Bell also cites Thomas. AMG1005, ¶[0052]. A POSA would have known that the humanized "h5G1.1 HuG4" antibody disclosed in Thomas is not eculizumab because Thomas's antibody is an IgG4 isotype—i.e., not a human hybrid IgG2/IgG4 isotype like eculizumab. AMG1002, ¶48; *see also*, Section IV.D, *supra.*

[27] *See* note 5, *supra.*

to yield predictable results: a humanized anti-C5 antibody. *KSR*, 550 US at 416 ("[A] combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results."). And, as Dr. Balthasar confirms by sequence alignment, the humanized anti-C5 antibody obtained from this combination comprises a heavy chain consisting of SEQ ID NO:2 and a light chain consisting of SEQ ID NO:4 as claimed. AMG1002, ¶¶52-53, 137-138.

A POSA would have reasonably expected to successfully perform the method of claim 1 because (i) making a humanized anti-C5 antibody would have required only basic molecular biology techniques to substitute Evans' HCDR3 sequence for Bowdish's TPO-mimetic peptide sequence; (ii) antibody production methods were well-known in the art (see, e.g., AMG1006, ¶¶[0130]-[0131]); and (iii) Bell taught that a humanized anti-C5 antibody was a safe and effective treatment for PNH, and provided methods and guidance on how to administer the treatment (AMG1005, ¶¶[0081]-[0096]). AMG1002, ¶¶143-144). This expectation is bolstered by numerous publications of successful clinical trials involving treating PNH with the humanized anti-C5 antibody, eculizumab. *See, e.g.,* AMG1004; AMG1042; AMG1047; AMG1011; AMG1005; AMG1012; AMG1013; AMG1002, ¶143.

2. Claims 2-5 and 7-10 would have been obvious.

Claims 2-10 all depend directly or indirectly from claim 1. All of the above arguments above regarding claim 1 also apply to these dependent claims.

a) Claim 2: intravenous infusion.

Bell and Evans both taught "intravenous infusion." Bell taught that eculizumab can be administered "via *intravenous infusion* by injection" and included a working example in which patients "received a weekly 600 mg *intravenous infusion* of anti-C5 antibody." AMG1005, ¶¶[0060], [0082]. Evans discloses that "[a]dministration of the anti-C5 antibodies will generally be performed by an intravascular route, e.g., via *intravenous infusion* by injection." AMG1007, 18:29-31; *see also, id.,* 142:53-59, 143:5-8.

A POSA would have administered the eculizumab composition intravenously because (i) both Bell and Evans expressly taught this route and (ii) intravenous infusion was standard for therapeutic antibodies. AMG1002, ¶¶145-146. And a POSA would have had a reasonable expectation of success because Bell described successful results using this method. AMG1005, ¶¶[0081]-[0096]; AMG1002, ¶146.

b) Claim 3: dosage level of between 5 mg/kg and 50 mg/kg.

Evans taught claim 3's dosage: "[d]osage levels of the antibodies for human subjects are … preferably between about *5 mg per kg and about 50 mg per kg per*

patient per treatment." AMG1007, 18:7-11. Moreover, as explained above in the anticipation analysis for claim 3, administering 600 mg or 900 mg eculizumab to adult men and women—as taught in Bell—results in patients receiving doses within claim 3's dosage range. *See* Section IX.C, *supra*; AMG1005, ¶[0082]; AMG1002, ¶¶89-90, 148.

A POSA would have administered eculizumab at a dosage level of between 5 mg/kg and 50 mg/kg per patient per treatment with a reasonable expectation of success because Evans expressly taught this dosing range and Bell taught that doses within this range could successfully treat PNH. AMG1007, 18:7-11; AMG1005, ¶¶[0081]-[0096]; AMG1002, ¶¶147-149.

c) Claim 8: dosing regimen.

Bell disclosed a regimen claimed in claim 8, treating PNH patients with "a weekly 600 mg intravenous infusion" of eculizumab for four weeks, followed by "900 mg of eculizumab 1 week later then 900 mg on a bi-weekly basis." AMG1005, ¶[0082]. Bell also taught that this treatment regimen was effective for treating PNH patients. AMG1005, ¶¶[0081]-[0096].

Thus, Bell disclosed a regimen having all of the limitations of claim 8 and would have provided a POSA with a reason to administer a humanized anti-C5 antibody as taught, with a reasonable expectation of success as discussed above. AMG1002, ¶¶150-152.

d) Claims 4 and 5: single unit dosage forms.

As discussed in Ground 2, Bell taught that eculizumab can be "administered in a variety of *unit dosage forms*." AMG1005, ¶[0058]; AMG1002, ¶¶153-154. Evans provided the same teaching *verbatim*. AMG1007, 17:60-61.

A POSA would have understood that a "unit dosage form" of an antibody as taught in Bell and Evans is formulated for use in a single patient and is therefore a "single" unit dosage form as claimed. AMG1002, ¶154; AMG1046, 2989; AMG1051, 1. A POSA reading Bell and Evans would have therefore prepared the a humanized anti-C5 antibody formulation as a single unit dosage form using routine formulation preparation. Single unit dosage forms were routinely used for intravenous therapeutic antibody formulations. AMG1002, ¶154.

Also discussed above, a POSA would have arrived at a single unit dosage form of 300 mg as claimed in claim 5 through simple convenience and logic. AMG1002, ¶158. A POSA would have had incentive to design a 300 mg single unit dosage amount as claimed because 300 mg would have been the most obvious and convenient means for administering the 600 mg and 900 mg doses taught by Bell. AMG1002, ¶¶156-158. And Alexion admits there is nothing critical about the claimed single unit dosage. AMG1027, 1104(¶5.3.9).

A POSA reading Bell and Evans would have had a reasonable expectation of successfully preparing a 300 mg single unit dosage form for the same reasons

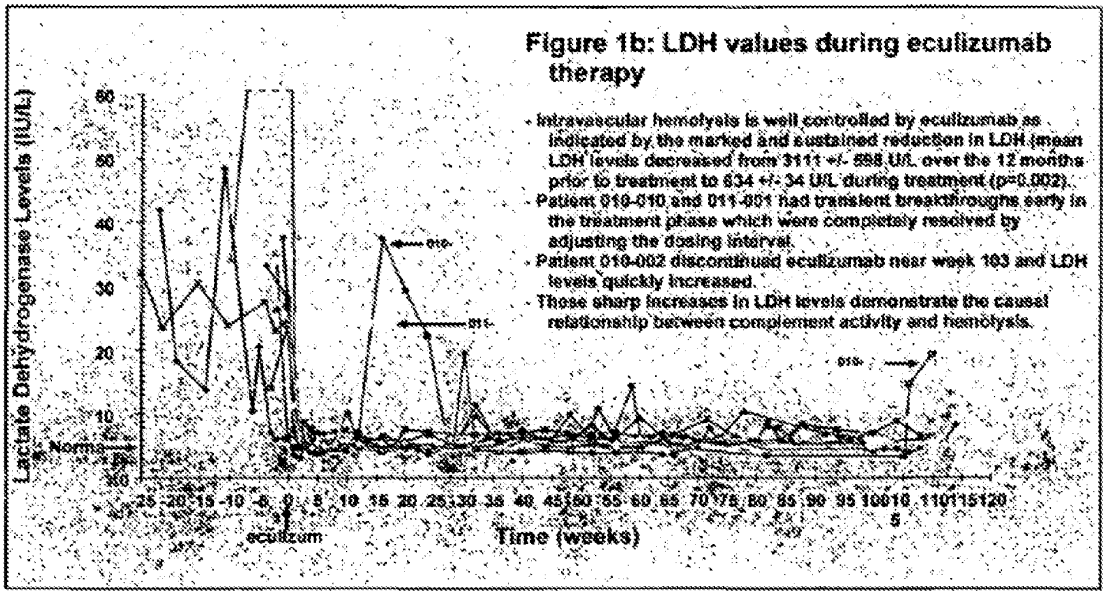
discussed above in Ground 2. AMG1002, ¶¶155, 159; *see* Section X.

e) Claim 7: patient is anemic.

Bell taught that, by administering eculizumab to a patient suffering from PNH, "the effects of PNH and other hemolytic diseases (including symptoms *such as anemia…*) are eliminated or decreased." AMG1005, ¶[0037]; *see also*, *id.*, ¶¶[0014], [0066], [0076]-[0077]. Thus, Bell would have given the POSA a reason to administer a humanized anti-C5 antibody to a PNH patient that is anemic, with a reasonable expectation of success, because Bell expressly taught this method and showed successful results of treating PNH patients. AMG1005, ¶¶[0081]-[0096]; AMG1002, ¶¶160-161.

f) Claims 9 and 10: immediate decrease in LDH levels

Bell taught the clinical outcomes required by claims 9 and 10. AMG1002, ¶¶162-164; AMG1005, FIG. 1B. Bell taught that "[t]he *LDH levels were decreased* by greater than 80% as compared to pre-treatment levels." AMG1005, ¶[0085]. And as Dr. Balthasar explains, Bell's data show an immediate decrease in LDH beginning within one week of administering eculizumab, followed by a sustained decrease:



AMG1005, FIG. 1b; AMG1002, ¶164. Bell described the data in Fig. 1b as showing a "*marked and sustained reduction* in LDH (*mean LDH levels decreased…*)." *Id.; see also,* AMG1005, ¶[0085].

A POSA would have understood that Bell's decrease in mean LDH levels is an "immediate decrease" because Bell shows a decrease in LDH levels beginning within one week of administration of eculizumab administration. AMG1005, FIG. 1B; AMG1002, ¶164. A POSA also would have understood that this decrease is "sustained" because Bell shows the LDH levels remaining decreased throughout two years (105 weeks) of treatment. *Id.*

A POSA reading Bell, therefore, would have had reason to administer a humanized anti-C5 antibody to a patient to reduce the mean LDH levels with a

reasonable expectation of success because Bell taught (i) that "LDH levels are used as an objective indicator of hemolysis" – i.e., reduced LDH levels are clinically beneficial in PNH patients; and (ii) that administering eculizumab caused an immediate and sustained decrease in LDH levels. AMG1005, ¶[0085], FIG. 1B; AMG1002, ¶165.

B. Ground 5: claim 6 would have been obvious over Bell, Bowdish, Evans, and Wang.

As discussed in Ground 3, claim 6 would have been obvious over Hillmen, Bell, and Wang. *See* Section XI. Claim 6 also would have been obvious over Bell, Bowdish, Evans, and Wang for the same reasons, namely:

(i) Bell taught that the humanized antibody unit dose formulation "*must be sterile* and non-pyrogenic" and "*may* contain … preservatives…" (i.e., preservatives are not required). AMG1005, ¶[0062]; AMG1002, ¶¶167-168. And a POSA would have known that therapeutic antibodies are routinely prepared in sterile, preservative-free solutions. AMG1002, ¶¶167-168; AMG1056-AMG1076.

(ii) Wang taught eculizumab formulations of between 1 to 30 mg/ml, encompassing the claimed 10 mg/ml concentration. AMG1028, Fig. 10, ¶[0067]. Wang's disclosure was also consistent with the general knowledge in the art. *See, e.g.,* AMG1029, Table 1; AMG1030, Table

1; AMG1002, ¶168.

(iii) A POSA would have prepared the a humanized anti-C5 antibody formulation in 30 ml single-use dosage forms using simple mathematics based on a 300 mg dosage unit concentrated at 10 mg/ml. AMG1002, ¶168. Again, there is nothing critical about a 30 ml volume. AMG1027, 1638(¶3.5), 1104(¶5.3.9).

Accordingly, a POSA routinely arriving at a 300 mg single-use dose concentrated at 10 mg/ml would also have routinely arrived at a volume of 30 ml. AMG1002, ¶168. A POSA reading Bell, Bowdish, Evans, and Wang would have had a reasonable expectation of successfully practicing the method of claim 6 for the same reasons discussed above in Ground 3. AMG1002, ¶¶169-170; *see* Section XI. And no objective indicia support patentability. *See* Section XIII.

C. Ground 6: claims 1-5 and 7-10 would have been obvious over Bell, Evans, and Mueller.

Claims 1-5 and 7-10 also would have been obvious in view of Bell, Evans, and Mueller. AMG1002, ¶¶171-205. Discussed below, a POSA would have had a reason to combine these references with a reasonable expectation of successfully practicing the claimed method. And no objective indicia support patentability. *See* Section XIII.

1. Claims 1-5 and 7-10 would have been obvious over Bell, Evans, and Mueller.

Discussed below, a POSA would have had a reason to combine Bell, Evans, and Mueller with a reasonable expectation of successfully practicing the method of claims 1-5 and 7-10. And no objective indicia weigh in favor of patentability. *See* Section XIII.

a) Claim 1 would have been obvious.

(1) Bell expressly taught all the limitations of claim 1 except the eculizumab amino acid sequences

Bell's teachings discussed in Grounds 2 and 3 also apply in Ground 4. As already discussed, the only element of claim 1 not expressly taught in Bell is that its humanized anti-C5 antibody "comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4." This information was, however, taught in Evans and Mueller. AMG1002, ¶¶171-176.

(2) Evans and Mueller taught eculizumab's amino acid sequences.

As discussed below, Evans disclosed the complete amino acid sequences of the heavy and light chain variable domains of a humanized anti-C5 antibody, . AMG1007, 44:4-13, SEQ ID NO:20; AMG1002, ¶¶172-173, 183-185. And Mueller disclosed the amino acid sequence of a light chain constant region and the hybrid IgG2/IgG4 heavy chain constant region. AMG1008, 58-61; AMG1002, ¶¶172-173, 186. Dr. Balthasar's Figure 14 below depicts this combination of art

providing the complete eculizumab sequence:

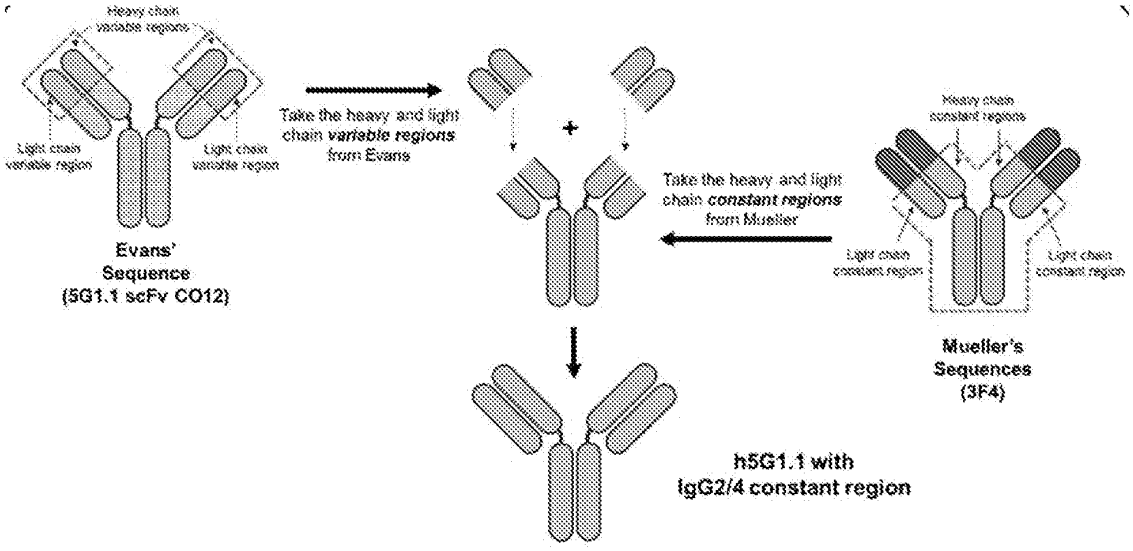


Figure 14.

See AMG1002, ¶173.

Evans—which Alexion previously said claims Soliris® and provides written description and enablement support for claims directed to eculizumab (AMG1009, 4; AMG1049, 838-839)—discloses making the original mouse 5G1.1 monoclonal antibody. AMG1007, 37:36-39:30 (Example 7); AMG1002, ¶¶183-184. Evans further described constructing a series of humanized 5G1.1 antibody constructs containing the heavy and light chain CDR sequences from the mouse 5G1.1 antibody inserted into a human framework. AMG1007, 42:58-45:4. In particular, Evans described nine different humanized 5G1.1 scFv constructs[28] along with their

[28] A POSA would have known that a scFv comprises light and heavy chain

amino acid sequences. *Id.* (constructs 2 and 11-18); AMG1002, ¶¶178, 185. Evans also described combining the variable regions with constant domains—including hybrid IgG constant domains—to make a complete antibody. AMG1007, 45:24-33.

Knowing that chimeric IgG2/IgG4 constant regions that were known not to activate the complement system (AMG1008, 7:28-31, 8:23-26, 12:27-32), a POSA reading Bell and Evans also would have looked to Mueller for "h5G1.1" sequence information. Mueller taught methods for making "chimeric antibodies containing the C1 and hinge region of human IgG2 and the C2 and C3 regions of human IgG4 … (HuG2/G4 mAb)." AMG1008, 12:27-30; *see also, id.,* 8:23-26. In particular, Mueller described a control antibody "h5G1.1 CO12 HuG2/G4 mAb," which a POSA would have readily identified as a humanized anti-C5 antibody because of the "h5G1.1" nomenclature coupled with the hybrid IgG2/IgG4 constant region ("HuG2/G4"). AMG1008, 12:37, FIG. 15; AMG1005, ¶[0052]; AMG1034, 1279; AMG1049, 838-839; AMG1002, ¶190.

As Dr. Balthasar explains, Mueller disclosed the amino acid sequence of a

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variable domains connected by a linker. *See, e.g.,* AMG1007, 6:39-41 ("single chain antibodies may include one each of only VH and VL domains, in which case they are *referred to as scFv antibodies*"); *see also,* AMG1040, 45-48; AMG1002, ¶178.

hybrid IgG2/IgG4 heavy chain constant region when Mueller disclosed the sequence of the chimeric anti-VCAM "3F4" antibody. AMG1002, ¶186; AMG1008, 58-61. A POSA would have known that a chimeric antibody contains the variable region from a non-human antibody and the constant region from a human antibody, and therefore would have understood that Mueller's chimeric 3F4 HuG2/G4 mAb heavy chain contains the variable regions from murine antibody 3F4 (the blue portions in Dr. Balthasar's Figure 14 above) and the constant regions of human hybrid IgG2/IgG4 (the green portions in Dr. Balthasar's Figure 14). AMG1002, ¶186; AMG1040, 29-30.

Mueller separately disclosed the amino acid sequences of the mature 3F4 heavy and light chain variable regions (i.e., the blue portion in Dr. Balthasar's Figure 14). AMG1008, Figure 9; AMG1002, ¶186. A POSA aligning the 3F4 heavy and light chain variable region sequences from Figure 9 with the sequences of the 3F4 HuG2/G4 chimeric antibody would have identified the 3F4 variable regions (the regions a POSA would have excluded) as amino acids 20-137 of the 3F4 HuG2/G4 heavy chain and amino acids 20-131 of the 3F4 light chain. AMG1008, Figure 9, 52-53, 58-61; AMG1002, ¶186.

A POSA therefore would have immediately known that the remainder of the 3F4 HuG2/G4 heavy chain (amino acids 138-463) is the hybrid IgG2/IgG4 constant region of that antibody, and that the remainder of the 3F4 light chain

(amino acids 132-238) is the light chain constant region of that antibody (i.e., the green portion in Dr. Balthasar's Figure 15). AMG1008, 52-53, 56-57; AMG1002, ¶186. Given that Mueller used this humanized anti-C5 antibody as an isotype control for 3F4 HuG2/G4, a POSA would have reasonably expected the disclosed heavy and light chain constant regions to be the same as those in the 3F4 HuG2/G4 chimeric antibody. AMG1002, ¶179.

As Dr. Balthasar explains, overwhelming evidence in the art would have further confirmed a POSA's belief that the amino acid sequences of the heavy and light chain constant regions of Mueller's 3F4 HuG2/G4 antibody are the same as those in eculizumab. AMG1002, ¶¶56-57, 179. Publications such as Bowdish, Tacken, Mueller II, and Evans all disclosed portions of eculizumab constant regions that either overlapped with or were exact matches to the heavy and light chain constant regions of Mueller's 3F4 HuG2/G4 antibody. *Id.*; AMG1006, Figs. 13A-13B; AMG1034, 1279; AMG1031, Abstract, 448, Fig. 7; AMG1007, 43:50-55 (SEQ ID NO:15).

With the heavy and light chain constant region sequences obtained from Mueller, a POSA would have looked back to Evans to complete the amino acid sequence. Knowing that Mueller refers to the control antibody as "h5G1.1 CO12 HuG2/G4 mAb," the POSA would have referred to the series of humanized 5G1.1 scFvs taught in Evans and readily identified construct number 12 (SEQ ID NO:20)

as the scFv of interest because Evans used the same "CO12" nomenclature as Mueller by designating it "5G1.1 scFv CO12." AMG1007, 44:4-13; AMG1002, ¶¶180-182. This is shown in Dr. Balthasar's Figure 14 above, where the green portions depict Evans' heavy and light chain variable regions. AMG1002, ¶173; AMG1007, 44:4-13, SEQ ID NO:20.

That Evans taught additional 5G1.1 scFv constructs is irrelevant because, "for an obviousness analysis, even the fact that 'a specific embodiment is taught to be preferred is not controlling, since all disclosures of the prior art, including unpreferred embodiments, must be considered.'" *In re Thomas*, 151 Fed. App'x. 930, 934 (Fed. Cir. 2005) (quoting *Merck & Co., Inc. v. Biocraft Labs., Inc.*, 874 F.2d 804, 807 (Fed. Cir. 1989). Here, any one of the combinations of Mueller's constant regions with Evans' variable regions would have been obvious, and does not make the combination with any one pair of variable regions any less obvious. *See Merck*, 874 F.2d at 807 ("That the [asserted prior art] discloses a multitude of effective combinations does not render any particular formulation less obvious.")

A POSA would have known that Evans' humanized 5G1.1 scFvs each contains a single polypeptide sequence comprising a humanized heavy chain variable region of 5G1.1, a linker, and a humanized light chain variable region of 5G1.1. AMG1002, ¶184; AMG1007, 6:39-41; AMG1040, 45-48. As Dr. Balthasar explains, a POSA would have been able to readily identify the heavy and light

chain variable regions within SEQ ID NO: 20 of Evans. AMG1002, ¶185. A POSA would have known that the linker in "5G1.1 scFv CO12" (SEQ ID NO:20) is amino acids 112-126 because this 15-amino acid sequence (GGGGSGGGGSGGGGS) was well known in the art as common a linker sequence in scFv antibodies. AMG1007, SEQ ID NO:20 (Certificate of Correction, 42-44); AMG1037, ¶¶[0021], [0097]; AMG1002, ¶185.

Alexion has also argued during prosecution that "the mature light chain sequence … could have been readily identified at the relevant filing date using well established rules and art-recognized techniques" and provided Adderson (AMG1048) as an example showing "the characteristic mature N-terminus (DIQ) of a light V kappa antibody light chain." AMG1014, 513; AMG1048, Fig. 6. Thus, the first two amino acids of Evans' SEQ ID NO:20 (MA) are a leader sequence, based on Alexion's admission that a mature kappa light chain starts with the sequence "DIQ" on its N terminus. AMG1002, ¶185; AMG1048, 734 (Fig. 6). Accordingly, a POSA would have understood that the mature light and heavy chain variable regions in Evans correspond to amino acids 3-111 and 127-248 of SEQ ID NO:20, respectively. AMG1007, 44:4-13; SEQ ID NO:20; AMG1002, ¶185.

Finally, a POSA would have expected that combining Evans' variable region sequences with Mueller's constant region sequences would provide complete heavy

and light chain sequences of a humanized anti-C5 antibody.[29] *See* Dr. Balthasar's Figure 14 above; AMG1002, ¶¶186-188.

As Dr. Balthasar shows by sequence alignment, the combination of heavy and light chain variable regions in Evans' SEQ ID NO:20 with Mueller's constant regions would make a humanized anti-C5 antibody having SEQ ID NOs:2 and 4, as claimed. AMG1002, ¶¶187-188.

(3) A POSA would have had a reason to combine the references with a reasonable expectation of success.

Again, a POSA would have known that the humanized anti-C5 antibody eculizumab is a humanized 5G1.1 monoclonal antibody with a hybrid IgG2/IgG4 constant region, often referred to as "h5G1.1" in the art. AMG1005, ¶[0052]; AMG1034, 1279; AMG1049, 838-839; AMG1002, ¶¶189-190. A POSA reading Bell would have looked to Evans because Bell explicitly cited and incorporated by reference Evans for methods of making a humanized anti-C5 antibody. AMG1005, ¶[0052]; AMG1002, ¶¶189-190. A POSA would have also looked to Mueller because Mueller disclosed an h5G1.1 antibody named "h5G1.1 CO12 HuG2/G4

[29] To be clear, a POSA would have known that this would be achieved by combining DNA encoding Evans' variable region sequences with that encoding Mueller's constant region. AMG1002, ¶121.

mAb" with a hybrid IgG2/IgG4 heavy chain constant region, which a POSA would have readily understood to be the humanized anti-C5 antibody, eculizumab. AMG1002, ¶190.

Evans and Mueller taught complementary, familiar elements of eculizumab's amino acid sequences. AMG1002, ¶¶191-192. Thus, by combining familiar elements in the art according to known methods, the artisan would have predictably arrived at a humanized anti-C5 antibody comprising a light chain consisting of SEQ ID NO:2 and a heavy chain consisting of SEQ ID NO:4 as claimed. *KSR*, 550 US at 416 ("a combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results."). A POSA would have easily confirmed this prediction by comparing the constructed sequences with another Alexion publication WO 2005/007809, which taught detailed descriptions of expression vectors designed for placing antibody variable regions in frame with chimeric IgG2/IgG4 heavy chain constant regions. AMG1032, 7, 28-32, FIG. 5; AMG1002, ¶192.

A POSA would have had a reasonable expectation of successfully practicing the method of claim 1 because (i) making a humanized anti-C5 antibody would have required only basic molecular biology techniques to combine Evans' scFv variable regions with Mueller's constant regions; (ii) antibody production methods already were well-known (see, e.g., AMG1006, ¶¶[0130]-[0131]); and (iii) Bell

taught that a humanized anti-C5 antibody was a safe and effective treatment for PNH, and provided methods and guidance on how to administer the treatment (AMG1005, ¶¶[0081]-[0096]). AMG1002, ¶192. This expectation is bolstered by numerous publications of successful clinical trials involving treating PNH with eculizumab. *See, e.g.,* AMG1004; AMG1042; AMG1047; AMG1011; AMG1005; AMG1012; AMG1013.

b) Claims 2-5 and 7-10 would have been obvious.

The additional elements required in claims 2-5 and 7-10 in Ground 6 are disclosed in Bell and Evans (and general knowledge in the art). Those same disclosures and arguments are presented above in Ground 4. Therefore, the rationales and conclusions presented in Ground 4 for claims 2-5 and 7-10 also apply to Ground 6 for the same reasons. AMG1002, ¶¶193-205.

D. Ground 7: claim 6 would have been obvious over Bell, Evans, Mueller, and Wang.

The additional elements required in claim 6 in Ground 7 are disclosed in Wang (and general knowledge in the art). Those same disclosures and arguments are presented above in Grounds 3 and 5. Therefore, the rationales and conclusions presented in Grounds 3 and 5 for claim 6 also apply to Ground 7 for the same reasons. AMG1002, ¶¶206-209.

XIV. Objective indicia do not support patentability.

"To be afforded substantial weight, the objective indicia of non-obviousness

must be tied to the *novel elements* of the claim at issue." *Univ. Pierre et Marie Curie v. Focarino*, 738 F.3d 1337, 1347 (Fed. Cir. 2013). Objective evidence that is not "both claimed and *novel in the claim*" lacks nexus to the invention. *In re Kao*, 639 F.3d 1057, 1068 (Fed. Cir. 2011).

Alexion argued during prosecution that "the non-natural, protein-engineered, heavy chain of eculizumab" (i.e., the hybrid IgG2/IgG4 constant region) provided "surprising and unpredictable" results such as decreased effector function, reduced immunogenicity, and increased half-life. AMG1014, 588, 593(¶8). Eculizumab's hybrid IgG2/IgG4 constant region was well known in the art (e.g., AMG1034, 1279), however, and cannot be a "novel element." *Marie Curie*, 738 F.3d at 1347; *Kao*, 639 F.3d at 1068. Accordingly, the alleged "surprising and unpredictable" features of eculizumab have no nexus with the challenged claims and do not support non-obviousness. *Id.*

Moreover, Alexion's alleged results would not have been unexpected to a POSA. AMG1002, ¶¶210-212. Mueller II taught in 1997 that antibodies with a hybrid IgG2/IgG4 heavy chain "[do] not contain the antibody sequences necessary for FcR binding," and would not contain "any new epitopes that would likely be immunogenic." AMG1031, 448, 451. It was also well known that a hybrid IgG2/IgG4 heavy chain would "have increased half-life." *See, e.g.,* AMG1032, 5, 19; AMG1002, ¶212. There is nothing unexpected here.

Alexion also argued in Europe that the claimed 10 mg/ml concentration provided "improved stability compared with other concentrations." AMG1027, 1104(¶5.3.8). This argument also carries no weight because a 10 mg/ml concentration is not a *novel feature* of the claims. Discussed above, numerous therapeutic antibodies were formulated at this concentration, *including eculizumab*. AMG1002, ¶¶113-114; AMG1028, Fig. 10. Further, data submitted in the opposition proceeding show that 10 mg/ml eculizumab did not offer any improved stability over other tested concentrations, completely contradicting Alexion's assertion. AMG1027, 19-20, 89-100; AMG1002, ¶¶213-214.

Petitioner is not aware of any other alleged objective indicia, and reserves the right to rebut any evidence Alexion asserts. *Amneal Pharms., LLC v. Supernus Pharms., Inc.*, IPR2013-00368, Paper 8, at 12-13 (Dec. 17, 2013); AMG1002, ¶215.

XV. Certification that the Patent May Be Contested via *Inter Partes* Review by the Petitioner and Standing (37 C.F.R. §42.104(a)).

Amgen certifies that the '504 patent is available for IPR and Amgen is not barred or estopped from requesting IPR of any '504 patent claim.

XVI. Mandatory Notices (37 C.F.R. §42.8(a)(1).

Real party-in-interest 37 C.F.R. §42.8(b)(1): Amgen Inc.

Related Matters (37 C.F.R. §42.8(b)(2)): Amgen has concurrently filed

petitions for IPR of U.S. Patent Nos. 9,718,880 (IPR2019-00740) and 9,732,149 (IPR2019-00741), which are related to the '504 patent and also owned by Alexion.

Lead and back-up counsel (37 C.F.R. §42.8(b)(3)) for Amgen Inc. are

Lead Counsel	Back-Up Counsel
Deborah Sterling (Reg. No. 62,732) STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C. 1100 New York Avenue, NW Washington, DC 20005 202.772.8508 (telephone) 202.371.2540 (facsimile) dsterling-PTAB@skgf.com	David H. Holman (Reg. No. 61,205) Scott A. Schaller (Reg. No. 60,167) David W. Roadcap (Reg. No. 68,956) STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C. 1100 New York Avenue, NW Washington, DC 20005 202.371-2600 (telephone) 202.371.2540 (facsimile) dholman-PTAB@sternekessler.com sschalle-PTAB@ sternekessler.com droadcap-PTAB@sternekessler.com

Notice of Service Information (37 C.F.R. §42.8(b)(4)): Please direct all correspondence regarding this Petition to counsel at the above addresses. Amgen consents to service by email at the addresses above.

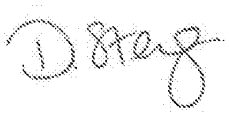
Procedural Statements: This Petition is filed in accordance with 37 C.F.R. §42.106(a). Concurrently filed herewith are a Power of Attorney and Exhibit List under 37 C.F.R. §42.10(b) and §42.63(e), respectively. The required fee is paid through Deposit Acct. No. 19-0036 (Customer ID No. 45324). The Office is authorized to charge any fee deficiency, or credit any overpayment, to Deposit

Acct. No. 19-0036 (Customer ID No. 45324).

XVII. Conclusion.

The challenged claims are unpatentable as anticipated or obvious and IPR is warranted.

Respectfully submitted,
STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C



Date: February 28, 2019
1100 New York Avenue, N.W.
Washington, D.C. 20005-3934
(202) 371-2600

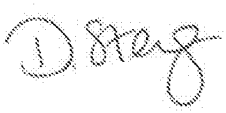
Deborah A. Sterling, Ph.D.
Registration No. 62,732
Lead Attorney for Petitioner

10498316.1

CERTIFICATE OF WORD COUNT (37 C.F.R. § 42.24(d))

I certify that Amgen Inc.'s Petition for *Inter Partes* Review for U.S. Patent No. 9,725,504 contains 13,974 words as counted by the word-processing program used to generate this response. This total does not include the table of contents, certificate of service, or this certificate of word count.

Respectfully submitted,
STERNE, KESSLER, GOLDSTEIN & FOX L.L.C.



Date: February 28, 2019
1100 New York Avenue, N.W.
Washington, D.C. 20005-3934
(202) 371-2600

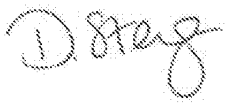
Deborah A. Sterling, Ph.D.
Registration No. 62,732
Lead Attorney for Petitioner

CERTIFICATE OF SERVICE (37 C.F.R. § 42.6(e)), §42.105(a))

I certify that the above-captioned "Petition for *Inter Partes* Review for U.S. Patent No. 9,725,504" was served in its entirety upon the Patent Owner on February 28, 2019, via FedEx, at the correspondence address of record indicated in the Patent Office's public PAIR system for U.S. Patent No. 9,725,504:

Nelson Mullins Riley & Scarborough LLP/Alexion
One Post Office Square
30th Floor
Boston MA 02109-2127

STERNE, KESSLER, GOLDSTEIN & FOX L.L.C.



Date: February 28, 2019
1100 New York Avenue, N.W.
Washington, D.C. 20005-3934
(202) 371-2600

Deborah A. Sterling, Ph.D.
Registration No. 62,732
Lead Attorney for Petitioner

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

AMGEN INC.
Petitioner,

v.

ALEXION PHARMACEUTICALS
Patent Owner.

Case IPR2019-00741
U.S. Patent No. 9,732,149

PETITION FOR *INTER PARTES* REVIEW OF U.S. PATENT NO. 9,732,149

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TABLE OF CONTENTS

I. Statement of the Precise Relief Requested and the Reasons Therefor (37 C.F.R. §42.22(A)). 1

II. Introduction. 1

III. Summary. 2

IV. The '149 Patent and its prosecution history. 9

V. State of the art before March 15, 2007. 13

A. Humanized monoclonal antibodies were well-known. 13

B. The prior art taught that eculizumab is a humanized anti-C5 monoclonal antibody (h5G1.1) containing a hybrid IgG2/IgG4 constant region. 14

C. The art taught eculizumab's amino acid sequence. 16

VI. Person of ordinary skill in art. 20

VII. Claim construction. 21

VIII. Identification of the challenge (37 C.F.R. §42.104(b)). 21

IX. The same or substantially the same prior art or arguments were not previously presented to the Office. 23

X. Ground 1: Hillmen anticipates claim 1. 24

A. Alexion admitted that Hillmen's eculizumab necessarily has the claimed sequence. 24

B. Hillmen's disclosure is enabling. 28

XI. Ground 2: Hill '05 anticipates claim 1. 31

A. Alexion admitted that Hill '05's eculizumab necessarily has the claimed sequence 31

B. Hill '05's disclosure is enabling. 33

XII. Ground 3: Bowdish anticipates claim 1. 35

A. Bowdish disclosed the claimed antibody. 35

B. Bowdish's disclosure is enabling. 40

XIII. Ground 4: claim 1 would have been obvious over Bell, Bowdish, and Evans. 41

A. Claim 1 would have been obvious. 41

1. Bell expressly taught all the limitations of claim 1 except eculizumab's amino acid sequence. 41
2. Bowdish and Evans taught the claimed amino acid sequences. 42
3. A POSA would have had a reason to combine the references with a reasonable expectation of success. 45

XIV. Ground 5: Claim 1 would have been obvious over Evans and Mueller..... 48

A. Claim 1 would have been obvious. 48
1. Evans and Mueller disclose the claimed amino acid sequences. 48
2. A POSA would have had a reason to combine the references with a reasonable expectation of success. 55
B. Objective indicia do not support patentability. 57

XV. Certification that the Patent May Be Contested via *Inter Partes* Review by the Petitioner and Standing (37 C.F.R. §42.104(a)). 58

XVI. Mandatory Notices (37 C.F.R. §42.8(a)(1). 58

XVII. Conclusion. 60

EXHIBIT LIST

Exhibit #	*Description*
1001	Bell, L., *et al.*, "Treatment of Paroxysmal Nocturnal Hemoglobinuria Patients by an Inhibitor of Complement," U.S. Patent No. 9,732,149 B2 (filed October 3, 2016; issued August 15, 2017)
1002	Declaration of Joseph P. Balthasar, Ph.D.
1003	Curriculum Vitae of Joseph P. Balthasar, Ph.D.
1004	Hillmen, P., *et al.*, "Effect of Eculizumab on Hemolysis and Transfusion Requirements in Patients with Paroxysmal Nocturnal Hemoglobinuria," *N. Engl. J. Med.* 350:552-559 (2004)
1005	Bell, L. and Rother, R., "Method of Treating Hemolytic Disease," U.S. Patent Application Publication No. 2005/0191298 A1 (filed February 3,2005; published September 1, 2005)
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1009	Patent Term Extension Application for U.S. Patent No. 6,355,245 B1
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1014	File History for U.S. Patent No. 9,725,504 B2

Exhibit #	***Description***
1015	File History for U.S. Patent No. 9,732,149 B2
1016	File History for U.S. Patent No. 9,718,880 B2
1017	Opposition File History for European Patent No. 1720571
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1039	Matis, L. and Rollins, S., "Complement-specific antibodies: Designing novel anti-inflammatories," *Nature Medicine* 1: 839-842 (1995)

Exhibit #	***Description***
1040	King, Chapter 1: Preparation, Structure and Function of Monoclonal Antibodies, Chapter 2: Antibody Engineering: Design for Specific Applications, and Chapter 3: Monoclonal Antibodies in Research and Diagnostic Applications in *Applications and Engineering of Monoclonal Antibodies* (1998)
1041	Winter, G. and Harris, W., "Humanized Antibodies," *TiPS* 14:139–143 (1993)
1042	Hillmen, P., *et al*., "Eculizumab, a C5 complement blocking antibody, is the first therapy to reduce transfusion requirements in patients with paroxysmal nocturnal haemoglobinuria (PNH)," *British Journal of Haematology - Supplement* 121:87, Abstract 257 (2003)
1043	Hillmen, P., *et al*., "Effect of the complement inhibitor eculizumab on thromboembolism in patients with paroxysmal nocturnal hemoglobinuria," *Blood* 110: 4123-4128 (2007)
1044	FDA Pharmacometrics Review for Application Number 125166 (eculizumab), available at https://www.accessdata.fda.gov/drugsatfda_docs/nda/2007/125166s000TOC.cfm
1045	Alberts, Chapter 12: Intracellular Compartments and Protein Sorting, in *Molecular Biology of the Cell* (1994)
1046	US Pharmacopeia, Chapter 1136: Packaging Unit of Use, in USP29–NF24 (2006)
1047	Hill, A., *et al*., "Sustained response and long-term safety of eculizumab in paroxysmal nocturnal Hemoglobinuria," *Blood* 106: 2559-2565 (2005)
1048	Adderson, E., *et al*., "Immunoglobulin Light Chain Variable Region Gene Sequences for Human Antibodies to *Haemophilus influenzae* Type b Capsular Polysaccharide Are Dominated by a Limited Number of V_κ and V_λ Segments and V J Combinations," *J. Clin. Invest*. 89: 729-738 (1992)
1049	File History for U.S. Application No. 11/127,438
1050	Health, United States, 2005 with Chartbook and Trends in the Health of Americans, U.S. Department of Health and Human Services, Centers for Disease Control and Prevention (2005)
1051	"ASHP statement on unit dose drug distribution," *Am. J. Hosp. Pharm*. 46:2346 (1989)

Exhibit #	*Description*
1052	Note for Guidance on Excipients, Antioxidant and Microbial Preservatives in the Dossier for Application for Marketing Authorisation of a Medicinal Product, European Agency for the Evaluation of Medicinal Products (2003)
1053	Rother, R., "Stratification of patient populations having or suspected of having rheumatoid arthritis ," U.S. Patent Application Publication No. 2005/0221382 A1 (filed March 17, 2003, published October 6, 2005)
1054	File History for European Patent No. 3167888 A1
1055	ICH Harmonised Tripartite Guideline Q6B, Step 4 Version (March 10, 1999), available at https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q6B/Step4/Q6B_Guideline.pdf

I. Statement of the Precise Relief Requested and the Reasons Therefor (37 C.F.R. §42.22(A)).

Amgen Inc. petitions for *Inter Partes* Review, seeking cancellation of claim 1, the sole claim, of U.S. Patent No 9,732,149 ("the '149 patent"; AMG1001), assigned to Alexion Pharmaceuticals, Inc. ("Alexion"). Claim 1 is unpatentable under 35 U.S.C. §§ 102 and/or 103. This Petition is supported by the expert declaration of Dr. Joseph Balthasar (AMG1002), an expert in the development and evaluation of therapeutic monoclonal antibodies. *Id.*, ¶¶1-10. Because the petition demonstrates a reasonable likelihood that claim 1 is unpatentable, institution is warranted.

II. Introduction.

Claim 1 of the '149 patent claims an antibody having a specific sequence that binds C5. During prosecution of the parent patent, Alexion asserted—incorrectly—that the claimed sequence "was not disclosed in the prior art; nor was it available to the public." AMG1014, 586-588. Indeed, Alexion's argument that the claimed sequence was not publicly known was the only stated basis for withdrawing the rejections and issuing a notice of allowance: "none of the applied references in the rejections recite using an antibody [with the claimed sequence]." AMG1015, 772. But, as shown herein long before the '149 patent's alleged priority date, Alexion repeatedly published the claimed anti-C5 antibody.

Alexion admitted that it performed at least 17 clinical trials treating paroxysmal nocturnal hemoglobinuria ("PNH") with a humanized anti-C5 antibody ("eculizumab"), and that the antibody used in those trials had the claimed amino acid sequence. *Id.* 735-738. Thus, by Alexion's admission, prior art publications of those trials necessarily disclose the claimed sequence, inherently anticipating the challenged claim. In addition, Alexion's own prior art publications of the sequence and structure of a humanized anti-C5 antibody, were such that the skilled artisan could and would have made and used an antibody as claimed, with a reasonable expectation of success. The challenged claim, therefore, offers nothing novel or inventive.

III. Summary.

Eculizumab (Soliris®) is a monoclonal antibody that binds complement protein C5 and inhibits C5 cleavage. Alexion obtained U.S. Patent No. 6,355,245 to Evans et al. ("Evans"), which is prior art to the '149 patent, on March 12, 2002. Alexion contends that Evans "claims the approved product" (Soliris®; eculizumab), and provides both written description and enablement support for claims directed to eculizumab. AMG1009, 4; AMG1010, 2; AMG1049, 838-839.

The FDA approved Soliris® for treatment of patients with PNH on March 16, 2007. AMG1009, 2. Exactly *one day* before receiving FDA approval, Alexion

filed PCT Application No. PCT/US2007/006606[1] ("the '606 application") and began prosecuting a chain of new patents directed to eculizumab. This was no coincidence.

To secure new eculizumab patents from the '606 application, Alexion repeatedly told the USPTO that eculizumab's amino acid sequence—specifically its IgG2/IgG4 heavy chain constant region—was not available in the prior art. But meanwhile Alexion did not inform the UPSTO that it had been repeatedly publishing on eculizumab, methods of using it to treat PNH, and its amino acid sequence—including its engineered IgG2/IgG4 heavy chain—years before filing the '606 application. Indeed, Alexion proudly boasted about widespread knowledge of the IgG2/IgG4 constant region in the art when it suited Alexion's interests, but it remained silent about it to the USPTO. AMG1049, 838-839. Thus, Alexion's carefully timed pursuit of the '606 application one day before product approval improperly seeks to ensnare and monopolize that which is already in the public domain, through Alexion's own publications nonetheless.

Well before March 15, 2007, artisans were aware of eculizumab. Indeed, Alexion itself admitted, prior to that date, that eculizumab was the subject of at least 17 different clinical trials, many of which were published. The table below

[1] The '149 patent claims priority to the '606 application.

summarizes Alexion's prior art public disclosures of the claimed antibody:

Table 1 Publications Disclosing the Claimed Antibody (Eculizumab)

Study	Alexion Study Number[2]	Exemplary disclosure
Hillmen (AMG1004) Phase 2 Pilot Study[3] 11 patients	C02-001	"In this trial, we investigated whether *eculizumab* could reduce the incidence of intravascular hemolysis, hemoglobinuria, and transfusion requirements in patients with PNH." AMG1004, 553.
Hill '05 (AMG1047) Phase 2 Pilot Study Extension #1 11 patients	E02-001	"Here we report the results of a 1 year follow-up study designed to assess the long-term efficacy and safety of *eculizumab* in patients with PNH." AMG1047, 2559.
Hill '04 (AMG1011) Phase 2 Pilot Study Extension #2 10 patients	X03-001	"We now report that 10 of the 11 patients from the initial 3 month study have continued to receive 900 mg *eculizumab* every other week for 2 years." AMG1011, Abstract.

[2] Study numbers as identified in Alexion's statements made to the USPTO during prosecution. *See, e.g.*, AMG1015, 728, 736.

[3] Alexion also disclosed results from what appears to be the same pilot study in an Abstract published by Hillmen et al. in 2003. AMG1042 ("Hillmen '03").

Table 1 □Publications Disclosing the Claimed Antibody (Eculizumab)

Study	Alexion Study Number[2]	Exemplary disclosure
Bell (AMG1005) Summary of Pilot Study and Extensions 11 patients	N/A	"The specific anti-C5 antibody used in the study was *eculizumab*." AMG1005, ¶[0082].
Hillmen '06 (AMG1012) Phase 3 "TRIUMPH" study 87 patients	C04-001	"We report the results of the phase 3 Transfusion Reduction Efficacy and Safety Clinical Investigation, a Randomized, Multicenter, Double-Blind, Placebo-Controlled, Using *Eculizumab* in Paroxysmal Nocturnal Hemoglobinuria (TRIUMPH) study…." AMG1012, 1234.
Young (AMG1013) Phase 3 "SHEPHERD" study 97 patients	C04-002	"SHEPHERD, an open-label, non-placebo controlled 52-week phase III clinical stud y, is underway to evaluate the safety and efficacy of *eculizumab* in a broader PNH population including patients with significant thrombocytopenia and/or lower transfusion requirements." AMG1013, Abstract.

Alexion also admitted to the USPTO that the eculizumab used in these trials necessarily has the claimed amino acid sequence. AMG1015, 738(¶6), 736. Thus,

by Alexion's admission, the humanized anti-C5 antibody administered in these published trials (eculizumab) necessarily has the claimed sequence, and the published eculizumab trials anticipate claim 1.

In addition, and contrary to Alexion's misrepresentation, the amino acid sequence and structure of eculizumab *were* known in the art, and a skilled artisan would have had ample reasons, guidance, and direction to make eculizumab as claimed, rendering claim 1 obvious. Indeed, it was Alexion who placed the claimed amino acid sequence into the public domain, yet failed to inform the examiner of this.

For example, Alexion's patent application publication US 2003/0232972 A1 ("Bowdish"), *not* raised by the examiner during prosecution, published in 2003 and used eculizumab as the starter "scaffold" antibody for creating a recombinant thrombopoietin (TPO) mimetic peptide-antibody, and provided the full eculizumab amino acid sequence except for the heavy chain CDR3 ("HCDR3") sequence that it had replaced with the TPO peptide sequence, LPIEGPTLRQWLAARAPV. AMG1006, ¶¶[0191]-[0193], Figs. 13A-13B, and SEQ ID NOs. 67 and 69. But the missing HCDR3 sequence was taught in Evans.

Bowdish explicitly incorporated by reference Evans[4] (another Alexion

[4] Alexion patent application publication US 2005/0191298 A1 ("Bell") also

patent) for more information on making eculizumab: "[c]onstruction of 5G1.1 [i.e., eculizumab] is described in U.S. Application Ser. No. 08/487,283, incorporated herein by reference."[5] *Id.*, ¶[0191]. As Dr. Balthasar explains, the skilled artisan would have readily identified the eculizumab heavy chain CDR3 sequences in Evans, thereby obtaining the complete amino acid sequence of the claimed antibody. *See* AMG1002, ¶¶52-53.

The claimed amino acid sequence was also taught in the art through the combination of Evans and another Alexion publication, WO 97/11971 ("Mueller"; AMG1008). In addition to disclosing its heavy chain CDR3 sequence, Evans taught the amino acid sequences of eculizumab's light and heavy chain variable regions, and that they can be combined with hybrid IgG constant domains to form a complete antibody. AMG1007, 44:4-13, 45:24-33; AMG1002, ¶¶14, 123-135. With the heavy and light chain variable region sequences in hand, the artisan would have needed only to identify the sequences for the light and heavy chain constant regions—information found in Evans (light chain) and Mueller (both the light and heavy chains).

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explicitly cites and incorporates by reference Evans for preparing eculizumab. AMG1005, ¶[0052].

[5] Evans issued from U.S. Application No. 08/487,283. AMG1007, face.

Notably, Alexion *never* provided Mueller to the examiner. Published in 1997, Mueller taught methods of creating recombinant antibodies with chimeric IgG2/IgG4 constant regions that were known not to activate the complement system. AMG1008, 7:28-31, 8:23-26, 12:27-32. Mueller further described using eculizumab—a humanized anti-C5 antibody with the same IgG2/IgG4 constant region as the experimental antibodies—as a control antibody. *Id.*, 12:35-37, Fig. 15. Mueller provided the amino acid sequences of eculizumab's IgG2/IgG4 heavy chain constant region and light chain constant region. *Id.*, 52-53, 58-61; AMG1002, ¶55. Further, artisans knew well before March 15, 2007, that eculizumab contained a hybrid IgG2/IgG4 constant region because Alexion expressly disclosed this feature to the public at least as early as 2005. AMG1034, 1279. Dr. Balthasar explains that a skilled artisan also would have readily obtained the complete amino acid sequence of the humanized anti-C5 antibody that results from the combination of Evans and Mueller, a sequence that Alexion now claims to be novel. *See* AMG1002, ¶¶123-135.

Alexion admittedly placed the anti-C5 antibody as claimed squarely in the prior art, and it's numerous prior art publications of eculizumab clinical trials results would have given the artisan ample reason to make the anti-C5 antibody as claimed. The prior art also supplied sufficient information about the sequence and structure of eculizumab such that the artisan could and would have made the

antibody as claimed with a reasonable expectation of success. The challenged claim, therefore, offers nothing novel or inventive over what was well known in the art to a POSA before March 15, 2007.

The examiner was not aware that eculizumab's amino acid sequence was already in the prior art. The Board here has the benefit of a more complete record, and should take the opportunity to correct the examiner's error by determining that claim 1 of the '149 patent is unpatentable as anticipated and/or obvious.

IV. The '149 Patent and its prosecution history.

The '149 patent issued on August 15, 2017 and claims a priority date of March 15, 2007.[6] The '149 patent's sole claim recites:

> 1. An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4.

AMG1001, 39:2-4.

The '149 patent issued from U.S. Appl. No. 15/284,015, filed on October 3, 2016. During prosecution, the examiner initially rejected Alexion's claim as anticipated by Hillmen, which disclosed methods of using eculizumab for treating PNH, in view of the general knowledge in the art of eculizumab's sequence, as

[6] Petitioner does not concede that the '149 patent is entitled to any of its claimed priority dates.

reflected in references such as Thomas (AMG1023). AMG1015, 596-598. The examiner also rejected the claims as anticipated by (1) Appel et al., *Kidney International* 70, S45-S50, (2006), and (2) Wang et al., US2005/0271660. *Id.*, 598.

In response, Alexion asserted—incorrectly—that "[n]either eculizumab nor its complete sequence, including the sequence of its unique, non-naturally occurring, protein-engineered heavy chain, was in the public domain prior to the March 15, 2007 effective filing date of the present application." *Id.*, 709. Indeed, Alexion's argument that eculizumab's amino acid sequence was not publicly known was the only stated basis for withdrawing the rejections and issuing a notice of allowance: "none of the applied references in the rejections recite using an antibody [with the claimed sequence]." *Id.*, 772.

As shown herein, the examiner was misled. And Alexion has repeatedly made these same misleading arguments to obtain additional eculizumab patents related to the '149 patent[7] that claim subject matter already in the public domain. For example, when prosecuting U.S. 9,725,504 (claiming methods of using

[7] Prosecution of parent applications is considered part of the file history of the child application. *Omega Engg, Inc., v. Raytek Corp.*, 334 F.3d 1314, 1333 (Fed. Cir. 2003); *see also*, *Microsoft Corp. v. Multi-Tech Sys., Inc.*, 357 F.3d 1340, 1349 (Fed. Cir. 2004).

eculizumab formulations), Alexion argued that the "complete structure of eculizumab was not disclosed in the prior art; nor was it available to the public" and that "none of these references even suggests the unique non-naturally occurring, protein-engineered heavy chain of eculizumab, which confers important, unexpected functional effects." AMG1014, 586-588. And when prosecuting U.S. 9,718,880 (claiming eculizumab formulations), Alexion again argued that "the complete structure of eculizumab was not disclosed in the prior art, nor was it available to the public." AMG1016, 179-180, 720.

Meanwhile, Alexion was saying the exact opposite in a European opposition proceeding over an eculizumab-related EP patent being challenged for sufficiency of disclosure. There—contrary to what it was telling the USPTO—Alexion stated that "the sequence for eculizumab was publicly available prior to the [February 3, 2004] priority and filing date" and "a sequence for eculizumab was submitted to Chemical Abstract Services (CAS) and entered into their STN database on 14 February 1999…."[8] AMG1017, 277, 291(¶5.1.2). And during prosecution of

[8] Alexion later tried to take this statement back during prosecution of a different European application, arguing that the eculizumab sequence information submitted in February 1999 had unintentional errors in it and therefore was not a public disclosure of the true eculizumab amino acid sequence, notwithstanding

related U.S. Application No. 11/127,438, Alexion argued that its provisional applications provided written description for claims to "eculizumab" and an antibody containing a "mutated Fc portion" because the provisional applications incorporated by reference the Evans prior art:

> Applicant respectfully disagrees and asserts that the priority applications provide ample written support for the claimed descriptions. For example, the priority documents each describe that "Particularly useful anti-C5 antibodies are h5G1.1, h5G1.1-scFv and functional fragments of h5G1.1 are *described in U.S. Patent No. 6,355,245*, the disclosures of which are incorporated herein in their entirely [*sic*] by this reference … Applicant submits that *h5G1.1* … [was] *well-known to one of ordinary skill in the art as eculizumab* … at the time of filing of priority applications.

AMG1049, 838-839 (emphasis added).[9] Alexion cannot have it both ways. Indeed, the European Opposition Division has already revoked at least two of Alexion's European eculizumab-related patents. AMG1017, 368-378; AMG1027, 2667-2685.

Alexion's intent to disclose it to the public. AMG1054, 247-254, 292-293.

[9] Unless otherwise stated, emphasis has been added throughout this Petition.

V. State of the art before March 15, 2007.

A. Humanized monoclonal antibodies were well-known.

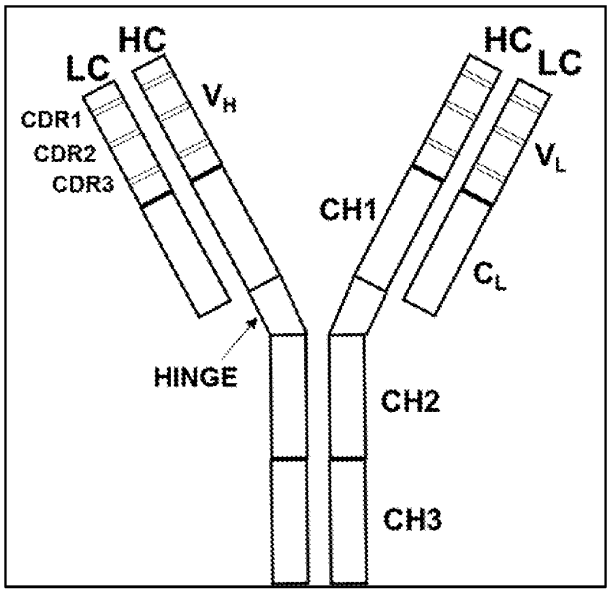
Before March 15, 2007, the structure of humanized monoclonal antibodies was well understood in the art. AMG1002, ¶¶20-26. Antibodies in general were known to be Y-shaped proteins made up of two identical "heavy chain" polypeptides and two identical "light chain" polypeptides. AMG1018, 7. The art taught that these heavy and light chains comprise a **variable region**—denoted as V_L (for the light chain) and V_H (for the heavy chain)—and a **constant region**—denoted as C_L (for the light chain) and C_H (for the heavy chain). AMG1018, 11-12.

The V_L and V_H regions each contain three "complementarity-determining regions" ("CDRs") which provide the antibody with its antigen-binding specificity. *Id.* The term "humanized" is used to refer to an antibody having a human framework into which CDR regions from a non-human monoclonal antibody (*e.g.*, a mouse antibody) are inserted into a human antibody framework. AMG1007, 5:57-67.

The constant regions of the antibody heavy chain provide different functional characteristics to the antibody. AMG1018, 8. Different antibody subclasses (e.g., IgG2, IgG4) can provide different functions based on the Fc receptors to which they bind. For example, IgG4 was known for its low propensity to activate the complement system. AMG1023, 1399. And recombinant antibodies

with a hybrid IgG2/IgG4 constant region have both a reduced ability to elicit unwanted inflammatory events and lessened propensity to activate complement. AMG1032, 11, 19, 28; AMG1031 ("Mueller II"), 451; AMG1002, ¶22.

A diagram depicting the basic antibody structure is shown below:



See AMG1002, ¶24.

B. The prior art taught that eculizumab is a humanized anti-C5 monoclonal antibody (h5G1.1) containing a hybrid IgG2/IgG4 constant region.

Before March 15, 2007, artisans knew that "eculizumab" was more than just a name; it was well known as a humanized anti-complement (C5) monoclonal antibody derived from the mouse monoclonal antibody "5G1.1," and thus was frequently referred to as "h5G1.1" or "h5G1.1-mAb" in the art:

- Bell taught in 2005 that "[t]he antibody *h5G1.1-mAb* is currently

undergoing clinical trials under the trade name *eculizumab*." AMG1005, ¶[0052]; and

- Tacken taught in 2005 that the antibody "*h5G1.1-mAb*" is synonymous with "*5G1.1, eculizumab*, Alexion Pharmaceuticals." AMG1034, 1279; AMG1002, ¶¶27-48.[10]

Moreover, Alexion has admitted on the record that "*h5G1.1* … [was] well-known to one of ordinary skill in the art *as eculizumab*…." AMG1049, 838.

The prior art also taught structural aspects of eculizumab, including that eculizumab contains a hybrid IgG2/IgG4 constant region. AMG1034, 1279; AMG1049, 838-839; AMG1002, ¶¶42-48. Whereas therapeutic monoclonal antibodies are typically based on a particular antibody isotype constant region (*e.g.*, IgG2 or IgG4; *see e.g.,* AMG1040, 55), eculizumab was known to contain a hybrid constant region with portions of both IgG2 and IgG4. For example, Tacken explicitly described using "h5G1.1-mAb (5G1.1, *eculizumab*; Alexion Pharmaceuticals)" containing an "*IgG2/IgG4 constant region*." AMG1034, 1279.

[10] *See also*, AMG1019, 56 ("*Eculizumab (5G1.1*; Alexion Pharmaceuticals) is a humanized monoclonal antibody."); AMG1020, 2123 ("*Eculizumab (5G1.1)*, the humanized anti-C5 mAb."); AMG1021, 1017 ("*Synonyms 5G1.1, h5G1.1*, C5 complement inhibitor (Alexion), h5G 1.1 scFv").

Likewise, when prosecuting a related application, Alexion told the USPTO that "it was well-known to one of ordinary skill in the art at the time of filing of priority applications [in 2002] that eculizumab has a G2/G4 Fc portion, *i.e.*, a mutated Fc portion." AMG1049, 838-839. As already discussed above, artisans knew that antibodies with a hybrid IgG2/IgG4 constant region carried certain benefits, such as a reduced ability to elicit unwanted inflammatory events and lessened propensity to activate the complement system. AMG1032, 11, 19, 28; AMG1031 ("Mueller II"), 451; AMG1002, ¶22.

Even though the prior art contained other "humanized 5G1.1" antibodies, the skilled artisan would have been able to readily distinguish eculizumab from them based on whether a hybrid IgG2/IgG4 constant region was present. AMG1002, ¶47. For example, in 1996, Thomas *et al.* disclosed creating a humanized 5G1.1 antibody containing the same CDR regions as the original murine 5G1.1 antibody used to make eculizumab. AMG1023, 1389. Thomas' antibody, however, was an "IgG4 isotype" called "h5G1.1 HuG4." AMG1023, 1396. A skilled artisan would have known that Thomas's "h5G1.1 HuG4" antibody, having an IgG4 constant region, was different than eculizumab, with its hybrid IgG2/IgG4 constant region. AMG1002, ¶47.

C. The art taught eculizumab's amino acid sequence.

Before March 15, 2007, the art taught eculizumab's full amino acid

sequence. AMG1002, ¶¶49-58. In 2003, Bowdish described a peptide-antibody recombinant protein using eculizumab as the starter antibody and a TPO agonistic peptide (mimetic) inserted in place of eculizumab's heavy chain CDR3 sequence. AMG1006, ¶¶[0191]-[0193]. Bowdish provided the full amino acid sequence for the TPO-mimetic+eculizumab antibody, and thus taught the entire eculizumab amino acid sequence with the exception of the eculizumab heavy chain CDR3 sequence (HCDR3).[11] AMG1006, ¶¶[0191]-[0193], Figures 13A-13B (SEQ ID NOs: 67 and 69); AMG1002, ¶¶50-51. But that HCDR3 sequence—the only piece of eculizumab not expressly described in Bowdish—was known in the art from Evans. Indeed, Bowdish explicitly cites and incorporates by reference Evans for methods of making eculizumab, stating: "[c]onstruction of 5G1.1 is described in [Evans], incorporated herein by reference."[12] AMG1006, ¶[0191].

Evans disclosed all six CDR regions of the original mouse 5G1.1 antibody, which are underlined in the sequences provided in Evans' Figures 18 and 19.

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[11] As Dr. Balthasar explains, a skilled artisan would have understood that the italicized portions of the sequences in Bowdish's Figures 13A-13B are "leader sequences" that are cleaved off during antibody maturation. AMG1002, ¶51; AMG1006, Figs. 13A-13B; AMG1045, 582.

[12] *See* note 5, *supra*.

AMG1007, 9:65-10:20, Figures 18-19. As Dr. Balthasar confirms, a POSA would have had a reason to replace the TPO mimetic from Bowdish's TPO-eculizumab antibody with Evans' HCDR3 to generate eculizumab. AMG1002, ¶¶108-117. When combined, the "scaffold" sequences for the TPO-mimetic+h5G1.1 antibody in Bowdish and eculizumab HCDR3 in Evans together form an anti-C5 antibody that has the claimed sequence. AMG1002, ¶¶51, 99, 108-113.

Another Alexion publication, Mueller, also provided complementary pieces of the eculizumab amino acid sequence, along with direction and guidance for making and using the antibody. AMG1002, ¶¶55-57. Mueller published in 1997 and disclosed the amino acid sequence of eculizumab's hybrid IgG2/IgG4 constant region and eculizumab's light chain constant region. AMG1008, 52-53, 58-61; AMG1002, ¶55. Mueller taught methods of developing recombinant antibodies to reduce immune-mediated organ transplant rejection, including antibodies comprising a hybrid IgG2/IgG4 constant region. *Id.*, 8:23-26, 12:27-30. Mueller described using eculizumab (referred to as "h5G1.1 CO12 HuG2/G4 mAb") as a control antibody that shares that same hybrid IgG2/IgG4 constant region as the experimental antibodies. *Id.*, 12:35-37, Figure 15. In these disclosures, Mueller provided the amino acid sequence of the hybrid IgG2/IgG4 constant region used in its antibodies—*i.e.*, the amino acid sequence of eculizumab's hybrid IgG2/IgG4 constant domain. *Id.*, 58-61; AMG1002, ¶¶55-57.

Dr. Balthasar's Figure 10 below schematically shows which portions of eculizumab were disclosed in the prior art.

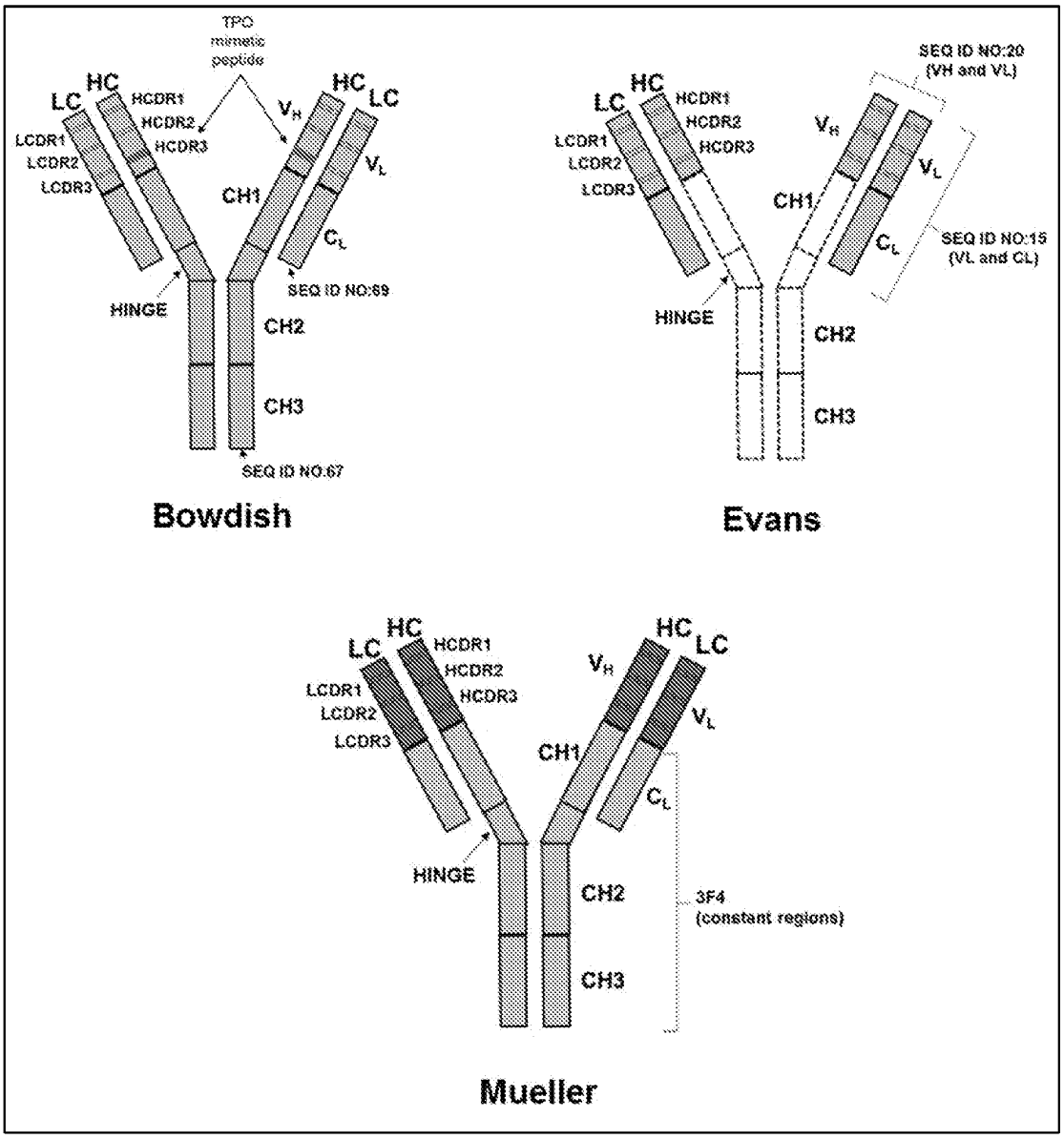


Figure 10.

See AMG1002, ¶58. In the above diagram, green represents the eculizumab

sequences disclosed in the reference, and blue represents non-eculizumab sequences in each reference. AMG1002, ¶58.

Though different portions of eculizumab's amino acid sequence were taught in different references, the POSA is presumed to be knowledgeable of all the pertinent art—*i.e.*, all the portions of eculizumab and its general structure. *Standard Oil Co. v. American Cyanamid Co.*, 774 F.2d 448, 454 (Fed. Cir. 1985). Based on these teachings, the POSA would have had a reason to combine the references with a reasonable expectation of success as explained below in Sections XIII and XIV.

VI. Person of ordinary skill in art.

A person of ordinary skill in the art (POSA) is a hypothetical person, presumed to be aware of all pertinent art, who thinks along conventional wisdom in the art, and is a person of ordinary creativity. *KSR Int'l Co. v. Teleflex Inc.*, 550 US 398, 421 (2007); *Standard Oil*, 774 F.2d at 454. A POSA in the field of the '149 patent had knowledge of the scientific literature and have skills relating to the design and generation of antibodies, the complement system, and the application of antibodies as therapeutics before March 15, 2007. AMG1002, ¶19. A POSA also had knowledge of laboratory techniques and strategies used in immunology research, including practical applications of the same. *Id.* Typically, a POSA would have had an M.D. and/or a Ph.D. in immunology, biochemistry, cell biology,

molecular biology, pharmaceutics, or a related discipline, with at least two years of experience in the field. *Id.* Also, a POSA may have worked as part of a multidisciplinary team and drawn upon not only his or her own skills, but also taken advantage of certain specialized skills of others on the team, *e.g.*, to solve a given problem; for example, a clinician and a formulation chemist may have been part of a team. *Id.*

VII. Claim construction.

Claims must be given their ordinary and customary meaning in light of the specification—"the meaning that the term would have to a person of ordinary skill in the art in question at the time of the invention." *Phillips v. AWH Corp.*, 415 F.3d 1303, 1312-1313 (Fed. Cir. 2015) (en banc); *see also*, 37 C.F.R. §42.100(b); 83 Fed. Reg. 51340, 51358 (Oct. 11, 2018).

The meaning of all claim terms in the '149 patent are plain on their face and require no further construction. AMG1002, ¶61. Amgen reserves the right to rebut any claim construction arguments Alexion might raise.

VIII. Identification of the challenge (37 C.F.R. §42.104(b)).

Amgen requests IPR based on the grounds summarized below.

Ground	35 U.S.C. Section (pre-AIA)	Claim	References
1	§102(b)	1	Hillmen

2	§102(b)	1	Hill '05
3	§102(b)	1	Bowdish
4	§103(a)	1	Bell, Bowdish, and Evans
5	§103(a)	1	Evans and Mueller

- Hillmen et al., *N. Engl. J. Med.* 350(6):552-559 (2004) ("**Hillmen**") published February 5, 2004. AMG1004, 552.

- Hill et al., *Blood* 106(7):2559-2565 (2005) ("**Hill '05**") published October 1, 2005. AMG1047, 2559.

- US 2003/0232972 A1 ("**Bowdish**"), published December 18, 2003. AMG1006, face.

- US 2005/0191298 A1 ("**Bell**"), published September 1, 2005. AMG1005, face.

- U.S. Patent No. 6,355,245 ("**Evans**"), issued March 12, 2002. AMG1007, face.

- WO 97/11971 ("**Mueller**"), published April 3, 1997. AMG1008.

These references are prior art under 35 U.S.C. §102(b) because each published more than one year before March 15, 2007, the '149 patent's earliest claimed priority date.

IX. The same or substantially the same prior art or arguments were not previously presented to the Office.

The arguments and evidence presented herein were not before the examiner during prosecution and, therefore, do not constitute "the same or substantially the same prior art or arguments" under 35 U.S.C. §325(d).

During prosecution, the examiner rejected Alexion's claims as (i) anticipated by Hillmen in view of Thomas; (ii) anticipated by Appel; and (iii) anticipated by Wang. AMG1015, 598-600. Those rejections rested solely on disclosures in Thomas and Evans for eculizumab sequence information. *Id.* The examiner later allowed the '149 patent claims mistakenly believing—because of Alexion's mischaracterization of the art—that the sequence and structure of eculizumab were not already known.

Though Hillmen was referenced by the examiner during prosecution, this Petition presents it in a different light, along with new references—Bell, Bowdish, and Mueller, which teach the IgG2/IgG4 constant domain missing from the art raised during prosecution.

Bell and a parent application to Bowdish (US 2003/0049683 A1) was cited but not relied upon during prosecution, and Mueller was not cited at all.Thus, this Petition presents important information that the examiner failed to appreciate or consider, including information never even presented to the examiner.

Consequently, this Petition is not the same as, substantially the same as, or cumulative of any previous arguments. Rather, the art combinations here, which were not raised by the examiner during prosecution, provide the complete sequence of eculizumab, thereby teaching the very thing the examiner mistakenly concluded was missing from the prior art.

Because the Grounds set forth herein provide new evidence, new combinations of art, and new arguments to address the erroneous bases through which Alexion obtained the '149 patent, § 325(d) does not preclude instituting this Petition.

X. Ground 1: Hillmen anticipates claim 1.

Hillmen anticipates claim 1. AMG1002, ¶¶70-77. A reference anticipates when it discloses each and every claim limitation "either expressly or inherently." *In re Crish*, 393 F.3d 1253, 1256 (Fed. Cir. 2004). "Under the principles of inherency, if the prior art necessarily … includes[] the claimed limitations, it anticipates." *MEHL/Biophile Int'l Corp. v. Milgraum*, 192 F.3d 1362, 1363 (Fed. Cir. 1999).

A. Alexion admitted that Hillmen's eculizumab necessarily has the claimed sequence.

Dr. Balthasar confirms that Hillmen disclosed all the limitations of claim 1, either expressly or inherently. AMG1002, ¶¶71-73. Hillmen expressly disclosed

"an antibody that binds C5" as claimed because Hillmen disclosed administering *eculizumab* formulations to patients, and eculizumab was a known antibody that binds C5. AMG1004, Abstract. For example, Hillmen disclosed that "patients with PNH received infusions of *eculizumab*…." AMG1004, Abstract. Hillmen further described eculizumab as an "*antibody against terminal complement protein C5*." AMG1004, Abstract. And Hillmen's antibody necessarily "comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4" because Alexion admitted that Hillmen's eculizumab—which was already in the public domain—necessarily possesses those very amino acid sequences.

During prosecution, Alexion submitted a list of eculizumab clinical studies—including Hillmen's Phase 2 Pilot Study (Study number "C02-001")—and affirmatively stated that "the antibody (eculizumab) used in each of the studies … *contained the heavy and light chain sequences of SEQ ID NOs: 2 and 4*." AMG1015, 738(¶6); *see also, id.*, 736 (study number "C02-001"). Alexion is bound by its admissions made during prosecution. *See, e.g., Vitronics Corp. v. Conceptronic, Inc.*, 90 F.3d 1576, 1583 (Fed. Cir. 1996); *Tyler Refrigeration v. Kysar Indus. Corp.*, 777 F.2d 687, 690 (Fed. Cir. 1985).

The FDA Medical Review in Alexion's approval package for Soliris® confirmed that "Study C02-001" was published in "Hillmen, P et al. … NEJM. 2004; 350:552-558" (i.e., Hillmen). AMG1024, 109. Similarly, Australia's

Pharmaceutical Benefits Advisory Committee ("PBAC") produced a public summary document for Soliris® showing that clinical trial "C02-001 (Pilot Study)" was published in "N Engl J Med 2004, 350:552-559." AMG1025, 2.

Because Alexion's admission confirms that the "eculizumab" disclosed in Hillmen necessarily comprised a heavy chain consisting of SEQ ID NO:2 and a light chain consisting of SEQ ID NO:4, as claimed, Hillmen inherently discloses the claimed sequences.

Despite admitting to the Office that the eculizumab disclosed in Hillmen necessarily comprised a heavy chain consisting of SEQ ID NO:2 and a light chain consisting of SEQ ID NO:4, Alexion misleadingly argued during prosecution that its contribution over the art was the specific amino acid sequence of eculizumab. AMG1014, 586-587, 738-744. However, as *Crish* makes clear, "just as the discovery of properties of a known material does not make it novel, *the identification and characterization of a prior art material also does not make it novel*." *Crish*, 393 F.3d at 1258. There is "[a] long line of cases confirm[ing] that one cannot establish novelty by claiming a known material by its properties." *Id.*

In *Crish*, the applicant claimed an hINV promoter region based on its nucleotide sequence. The court stated that the pertinent inquiry for its anticipation analysis is "whether the claimed [invention] was new," and determined that:

> The promoter region of hINV *was not new* … hINV was *known and used years before* … The only arguable contribution to the art that Crish's application makes is the identification of the nucleotide sequence of the promoter region of hINV. However, just as the discovery of properties of a known material does not make it novel, *the identification and characterization of a prior art material also does not make it novel*.

Id.; *see also, Atlas Powder Co. v. IRECO Inc.,* 190 F.3d 1342, 1347 (Fed. Cir. 1999 ("the discovery of a previously unappreciated property of a prior art composition … does not render the old composition patentably new to the discoverer."); *Abbott Labs. v. Baxter Pharms.*, 471 F. 3d 1363, 1368 (Fed. Cir. 2006) (lack of knowledge of a compound's property is "wholly irrelevant to the question of whether the [patent at issue] claims something 'new' over the disclosure of the [prior art].").

Cases such as *Crish* are squarely applicable here and compel finding anticipation. Alexion admitted Hillmen necessarily disclosed the claimed antibody. The '149 patent's mere claim to the amino acid sequence that Alexion admits was a property of a prior art compound (eculizumab) contributes nothing over the prior art.

B. Hillmen's disclosure is enabling.

Hillmen also provided an enabling disclosure of the claimed antibody. An anticipatory publication "must be capable, *when taken in conjunction with the knowledge of those skilled in the art* to which it pertains, of placing that invention in the possession of the public." *In re Donohue*, 632 F.2d 123, 125 (CCPA 1980) ("*Donohue I*"); *see also*, *In re Donohue*, 766 F.2d 531, 533 (Fed. Cir. 1985) ("*Donohue II*") (public possession "is effected if one of ordinary skill in the art could have *combined the publication's description* of the invention *with his own knowledge* to make the claimed invention.") An anticipatory reference must also "enable one skilled in the art to make the anticipating subject matter" without undue experimentation. *Elan Pharms. v. Mayo Found.*, 346 F.3d 1051, 1054 (Fed. Cir. 2003).

To determine whether experimentation would be undue, one must examine "(1) the quantity of experimentation; (2) the amount of direction or guidance present; (3) the presence or absence of working examples; (4) the nature of the invention; (5) the state of the prior art; (6) the relative skill of those in the art; (7) the predictability or unpredictability of the art; and (8) the breadth of the claims." *Impax Labs. v. Aventis Pharms.*, 545 F.3d 1312, 1314-1315 (Fed. Cir. 2008) (citing *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988)). Applying these factors, Dr. Balthasar explains that Hillmen, coupled with the general knowledge in the art,

would have enabled a POSA to possess eculizumab having the claimed sequences. AMG1002, ¶¶74-76.

The alleged invention and single claim are directed to an antibody (eculizumab). This antibody was expressly disclosed in Hillmen; and Alexion admitted that Hillmen's eculizumab had the claimed sequences. AMG1004, Abstract, 553-554; AMG1015, 738(¶6), 736; AMG1024, 109; AMG1025, 2. And the general knowledge in the prior art was replete with disclosures, direction, and guidance about eculizumab's structure and amino acid sequence. AMG1002, ¶¶42-58, 75-76.

Dr. Balthasar's Figure 10 above illustrates the art disclosing eculizumab amino acid sequences. *See* Section IV, *supra*; AMG1002, ¶58. Dr. Balthasar explains two independent ways in which a POSA would have obtained eculizumab's amino acid sequence:

(1) A POSA would have known that **Bowdish** disclosed the entire amino acid sequence of eculizumab with the exception of the heavy chain CDR3 region, and that **Evans** disclosed the eculizumab heavy chain CDR3 region (the missing piece from Bowdish). AMG1006, ¶¶[0191]-[0193], Figure 13A-13B (SEQ ID NOs:67 and 69); AMG1007, 44:4-13 (SEQ ID NO:20); AMG1002, ¶75.

(2) A POSA also would have known that **Evans** disclosed the amino acid

> sequences of eculizumab's heavy and light chain variable domains, and **Mueller** disclosed the hybrid IgG2/IgG4 heavy chain and light chain constant domains of eculizumab. AMG1007, 44:4-13 (SEQ ID NO:20); AMG1008, 52-53, 58-61; AMG1002, ¶75.

And given the high level of skill in the relevant field, the POSA would have readily obtained eculizumab's sequences as claimed from the art using only routine experimentation.

Armed with the general knowledge in the relevant field, a POSA reading Hillmen would not have "needed to experiment unduly to gain possession of the invention." *Impax*, 545 F.3d at 1315-1316; AMG1002, ¶76. Here, just as in *In re Donohue*, "the primary reference named a composition falling within the scope of the claims and indicated that it had previously been made and tested; additional references showed that a method of making this composition would have been within the knowledge of one of ordinary skill in the art." *Donohue I* at 126.

The law compels finding anticipation here. The claimed antibody was not new—Hillmen taught eculizumab. And under *Crish*, simply adding the amino acid sequence of an already known antibody to the claims does not confer novelty. Moreover, Alexion's admission that Hillmen's eculizumab inherently possesses the claimed sequences is binding under *Vitronics.* And under *Donohue I* and *Donohue II*, Hillmen is enabling in view of the multiple publications of eculizumab's

sequence in the art. Accordingly, Hillmen anticipates claim 1.

XI. Ground 2: Hill '05 anticipates claim 1.

Hill '05 also anticipates claim 1 because Hill '05 disclosed "an antibody that binds C5," eculizumab—a known antibody that necessarily possesses the claimed amino acid sequences. AMG1002, ¶¶78-87.

A. Alexion admitted that Hill '05's eculizumab necessarily has the claimed sequence

Hill '05 is an Alexion publication describing results from a one-year extension study involving the same 11 patients enrolled in the Hillmen Phase 2 Pilot Study. AMG1047, 2559-2560. Hill '05 disclosed administering eculizumab formulations to patients over a one-year extension period, concluding that "[r]esults of this 1-year extension study showed that eculizumab therapy continues to be safe and well tolerated in PNH patients." AMG1047, 2565.

Hill '05 disclosed "an antibody that binds C5" as claimed because Hill '05 disclosed administering *eculizumab* formulations to patients, and eculizumab was a known antibody that binds C5. AMG1047, Abstract, 2560. For example, Hill '05 disclosed that patients in the extension study received "a maintenance dose of *eculizumab*." AMG1047, 2560. Hill '05 further described eculizumab as an "a *humanized monoclonal antibody* that specifically targets the *complement protein C5*." AMG1047, 2559. And Hill '05's eculizumab "comprises a heavy chain

consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4" because Alexion admitted that Hill '05's eculizumab necessarily possesses those very amino acid sequences .

In the list of eculizumab clinical studies Alexion submitted to the USPTO during prosecution, Alexion also included Hill '05's extension study (Study number "E02-001") when affirmatively stating that "the antibody (eculizumab) used in each of the studies … *contained the heavy and light chain sequences of SEQ ID NOs: 2 and 4*." AMG1015, 738(¶6); *see also, id.*, 736 (study number "E02-001"). Again, Alexion's admission is binding. *Vitronics* 90 F.3d at 1583; *Tyler Refrigeration*, 777 F.2d at 690.

Hill '05 explicitly confirms that it is an extension study of Hillmen's Phase 2 Pilot Study, stating "[w]e previously reported the outcome of an open-label study of eculizumab in patients with PNH [*citing Hillmen*] … Here we report the results of a 1-year follow-up study designed to assess the long-term efficacy and safety of eculizumab in patients with PNH." AMG1047, 2559. Hill '05 also disclosed that "[t]he acute-phase study was an initial 12-week, open-label trial of eculizumab in 11 patients with PNH and has been previously described in detail [*citing Hillmen*] … The current study was an open-label *extension of that acute-phase study*." AMG1047, 2560; AMG1002, ¶¶79-83. This is consistent with Alexion's admission to the USPTO that study number E02-001 (Hill '05) is entitled "Extension Study in

Patients … who Previously Participated in Study C02-001 [Hillmen]." AMG1015, 736.

Because Alexion's admission confirms that the "eculizumab" disclosed in Hill '05 necessarily comprised a heavy chain consisting of SEQ ID NO:2 and a light chain consisting of SEQ ID NO:4, as claimed, Hill '05 inherently discloses an antibody comprising the claimed sequences.

As already discussed, eculizumab was not new. Alexion serially published on eculizumab well before March 15, 2007. *See, e.g.,* AMG1004, Abstract, 554; AMG1042, Abstract; AMG1047, Abstract, 2560; AMG1011, Abstract; AMG1005, ¶¶[0081]-[0096]; AMG1012, Abstract, 1235; AMG1013, Abstract; AMG1002, ¶¶42-58. And merely claiming an inherent property of a known antibody —as Alexion claims in the '149 patent—does not confer novelty. *Crish*, 393 F.3d at 1258; *Atlas Powder*, 190 F.3d at 1347; *Abbott Labs.*, 471 F.3d at 1368.

B. Hill '05's disclosure is enabling.

Hill '05 is enabling for the same reasons discussed above in Ground 1. The alleged invention and single claim are directed to an antibody (eculizumab) that was expressly disclosed in Hill '05. AMG1047, Abstract, 2559-2560. And as already discussed, the general knowledge in the prior art had ample disclosures, direction, and guidance on eculizumab and its amino acid sequence. AMG1002, ¶¶84-86. *See* Section IV, *supra*.

Again, Dr. Balthasar explains two independent ways in which a POSA would have obtained eculizumab's amino acid sequence: (1) from the "5G1.1" scaffold sequences in **Bowdish** and the HCDR3 sequence in **Evans** (AMG1006, ¶¶[0191]-[0193], Figure 13A-13B (SEQ ID NOs:67 and 69); AMG1007, 44:4-13 (SEQ ID NO:20)); or (2) from the heavy and light chain variable region sequences in **Evans** and the heavy and light chain constant domain sequences in **Mueller** (AMG1007, 44:4-13 (SEQ ID NO:20); AMG1008, 52-53, 58-61). AMG1002, ¶85. Given the high level of skill in the relevant field, the POSA would have readily obtained eculizumab's sequences as claimed from the art using only routine experimentation. *Impax*, 545 F.3d at 1315-1316; AMG1002, ¶86.

As in Ground 1, the law also compels finding anticipation in Ground 2. The claimed antibody was not new because Hill '05 taught eculizumab. Later claiming the amino acid sequence of a known antibody does not make what was already publicly known now novel. *Crish*, 393 F.3d at 1258; *Atlas Powder,* 190 F.3d at 1347; *Abbott Labs.*, 471 F. 3d at 1368. Moreover, Alexion is bound by its admission that Hill '05's eculizumab inherently possesses the claimed sequences. *Vitronics*, 90 F.3d at 1583; *Tyler Refrigeration*, 777 F.2d at 690. And a POSA taking Hill '05's disclosure in conjunction with the knowledge of skill in the art would have been able to possess the claimed antibody without undue experimentation. *Donohue I*, 632 F.2d at 125; *Donohue II*, 766 F.2d at 533.

XII. Ground 3: Bowdish anticipates claim 1.

Bowdish anticipates claim 1 because Bowdish inherently disclosed the claimed "antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO:2 and a light chain consisting of SEQ ID NO:4." AMG1002, ¶¶88-103.

A. Bowdish disclosed the claimed antibody.

Bowdish taught methods of making peptide-antibody recombinant proteins, and described using a 5G1.1 antibody as the starter "scaffold" antibody sequence for creating a recombinant TPO-mimetic+h5G1.1 antibody. AMG1006, ¶[0191]. Bowdish provided the full 5G1.1 antibody amino acid sequence except for the heavy chain CDR3 (HCDR3) sequence, which Bowdish replaced with the TPO-mimetic peptide sequence, LPIEGPTLRQWLAARAPV. AMG1006, ¶¶[0191]-[0193], Figs. 13A-13B, and SEQ ID NOs. 67 and 69; AMG1002, ¶¶89-93. As explained in more detail below, Bowdish's "5G1.1" starter antibody necessarily possessed the claimed sequences.

Bowdish provides the complete sequences of the heavy and light chains of the recombinant TPO-mimetic+h5G1.1 antibody ("5G1.1+TPO") as SEQ ID NO:67 ("5G1.1-TPO Heavy Chain") and SEQ ID NO:69 ("5G1.1 Light Chain") in Figures 13A and 13B, respectively. AMG1006, Figures 13A-13B; AMG1002, ¶¶91-92. Dr. Balthasar's Figure 3 below illustrates the structure of Bowdish's TPO-mimetic+h5G1.1antibody, showing the location of the TPO peptide (blue) in the

HCDR3 region of the polypeptide of SEQ ID NO:67 (heavy chain), and the polypeptide of SEQ ID NO:69 (light chain), where green represents the 5G1.1 "scaffold" sequences that are to SEQ ID NO:2 and SEQ ID NO:4:

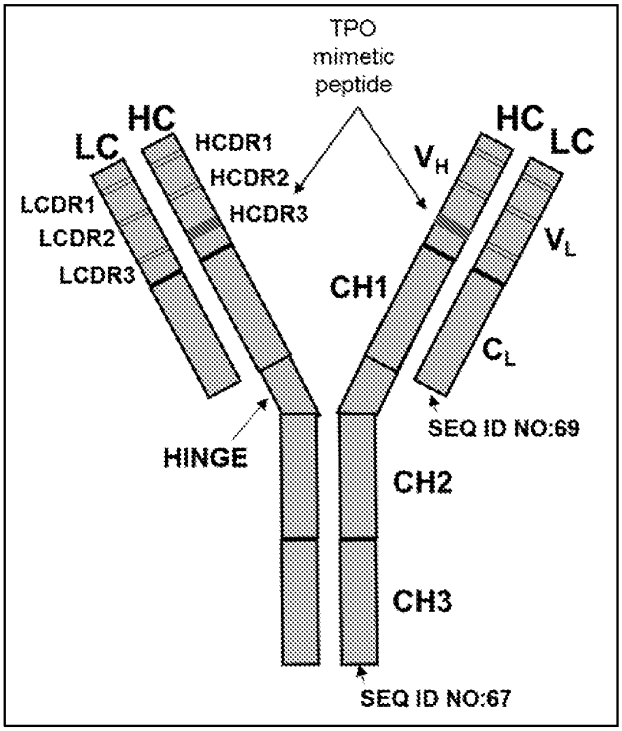


Figure 3.

AMG1002, ¶¶50, 92; AMG1006, Figures 13A-13B.

As Dr. Balthasar explains, the mature portion of Bowdish's SEQ ID NO:69 (i.e., the light chain) is 100% identical to the '149 patent's SEQ ID NO:4 as claimed; and the mature portion of Bowdish's SEQ ID NO:67 (i.e., the heavy chain) is 100% identical to the '149 patent's SEQ ID NO:2 as claimed, with the exception of the HCDR3 sequence. AMG1002, ¶¶96-100.

Bowdish's sequences in SEQ ID NOs: 69 and 67 each contain italicized portions, which Bowdish explicitly denoted as "leader sequence[s]." As Dr. Balthasar explains, a POSA would have known that any leader sequence would be cleaved from the mature antibody sequence. AMG1002, ¶91. Thus, the POSA would have known that the leader sequences in Bowdish's Figures 13A-13B are not part of the mature antibody sequence[13]. AMG1006, Figures 13A-13B; AMG1002, ¶91; AMG1045, 582.

A POSA would have understood that the only portion of the "scaffold" 5G1.1 antibody sequence not expressly disclosed in Bowdish is the HCDR3 sequence because Bowdish taught that "[t]he TPO mimetic peptide graft in Fab clone X4b has been *transplanted into the heavy chain CDR3* of another antibody framework, *5G1.1* … The sequence was *cloned into 5G1.1* in such a fashion as to

[13] Alexion is estopped from arguing to the contrary. During prosecution, Alexion amended original SEQ ID NO:4 to *remove* the leader sequence, arguing that "the mature light chain sequence is an *inherent* portion of the precursor sequence … and could have been readily identified at the relevant filing date using well established rules and art-recognized techniques…." AMG1015, 546; *see Vitronics Corp.*, 90 F.3d at 1583; *Tyler Refrigeration*, 777 F.2d at 690.

replace the native CDR3." AMG1006, ¶[0191]; AMG1002, ¶¶91-93. Dr. Balthasar's Figure 3 above shows this, where green represents portions of 5G1.1 amino acid sequence present in Bowdish's TPO-mimetic+h5G1.1 antibody and blue represents the TPO-mimetic peptide sequence replacing the native HCDR3.

Bowdish's 5G1.1 "scaffold" antibody, therefore, inherently possessed a heavy chain of SEQ ID NO:4 and a light chain of SEQ ID NO:2 as claimed. Bowdish disclosed that the starter scaffold antibody 5G1.1 was produced according to Evans, stating that "[c]onstruction of 5G1.1 is described in [Evans], incorporated herein by reference."[14] AMG1006, ¶[0191].

Evans disclosed preparing different humanized C5-binding antibodies referred to as "5G1.1" antibodies. AMG1007, 19:47-49, 37:35-39:30, 40:31-45:4; AMG1002, ¶¶94-95. As Dr. Balthasar explains, a POSA would have understood that *all* of the 5G1.1 antibody heavy chain variable regions in Evans contain *the same CDR3 sequence*: YFFGSSPNWYFDV. AMG1007, Fig. 19, 43:13-14, 43:26-27, 43:33-34, 43:60-61, 44:2-3, 44:12-13, 44:21-22, 44:30-31, 44:39-40, 44:49-50, 44:59-60, 45:3-4; AMG1002, ¶95. Thus, a POSA would have known that the heavy chain of Bowdish's 5G1.1 starter antibody contained the YFFGSSPNWYFDV CDR3 sequence, regardless of which "version" of Evans'

[14] *See* note 5, *supra*.

humanized 5G1.1 the POSA considered. AMG1002, ¶95.

In view of the above, Bowdish's starter 5G1.1 antibody necessarily contained Evans' YFFGSSPNWYFDV HCDR3 sequence as its native HCDR3. AMG1002, ¶¶96-100. This is shown in Dr. Balthasar's Figure 12 below:

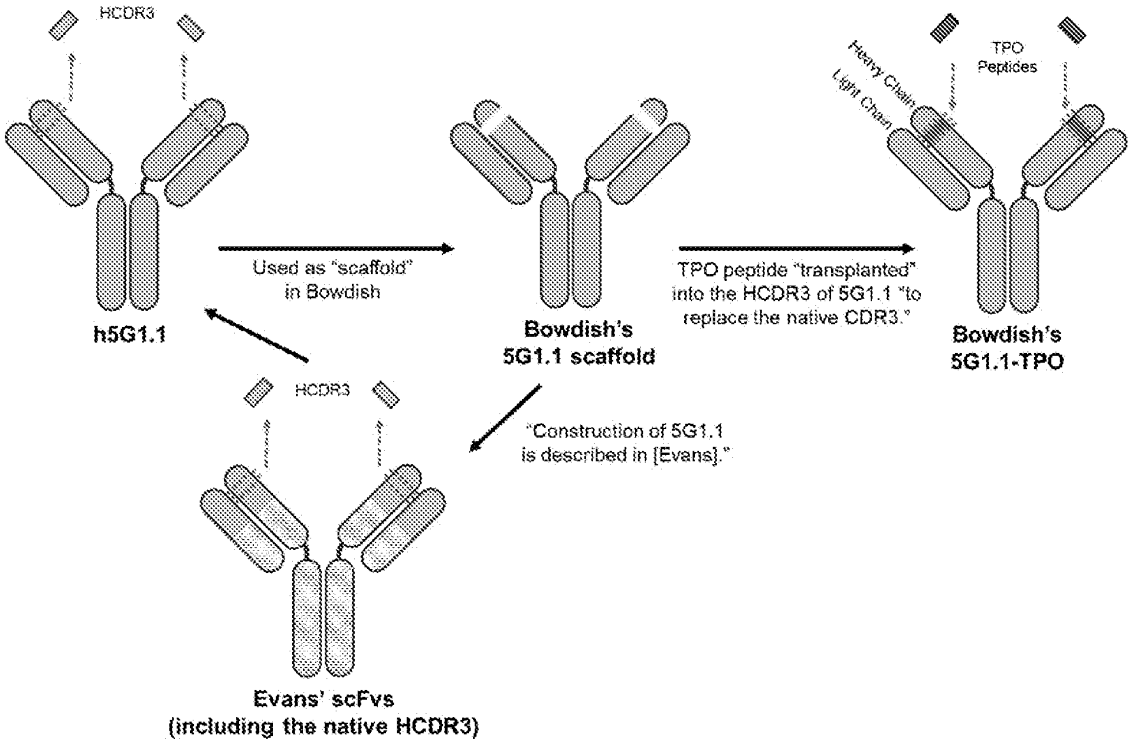


Figure 12.

Dr. Balthasar confirms that inserting Evans' HCDR3 sequence in place of Bowdish's TPO-mimetic peptide sequence in SEQ ID NO:67 creates an antibody heavy chain sequence that is a 100% match with SEQ ID NO: 2 from the '149 patent. AMG1002, ¶¶98-99. As already discussed above, Bowdish's light chain

SEQ ID NO:69 is a 100% match with SEQ ID NO:4 from the '149 patent. AMG1002, ¶100. Accordingly, Bowdish's disclosure of "5G1.1" as the starter scaffold sequence for creating a TPO-mimetic+h5G1.1 antibody is necessarily a disclosure of an anti-C5 antibody that binds C5 and has the claimed sequences. *MEHL/Biophile*, 192 F.3d at 1365.

B. Bowdish disclosure is enabling.

Bowdish is enabling for many of the reasons discussed above in Grounds 1 and 2. AMG1002, ¶¶101-192. As Dr. Balthasar explains, a POSA would have known that Bowdish disclosed the entire amino acid sequence of the 5G1.1 starter antibody with the exception of the HCDR3 region, and that Evans disclosed the native HCDR3 region (the missing piece from Bowdish). AMG1006, ¶¶[0191]-[0193], Figure 13A-13B (SEQ ID NOs:67 and 69); AMG1007, 44:4-13 (SEQ ID NO:20); AMG1002, ¶102; *see also, Donohue I*, 632 F.2d at 125; *Donohue II*, 766 F.2d at 533.

Given the narrow scope of claim 1 and the high level of skill in the relevant field, the POSA would have readily obtained the heavy and light chain sequences as claimed from the art using only routine experimentation. And, armed with the general knowledge in the relevant field, a POSA reading Bowdish would not have needed undue experimentation to gain possession of the claimed anti-C5 antibody. *Impax*, 545 F.3d at 1315-1316; *Wands*, 858 F.2d at 731; AMG1002, ¶102.

Bowdish therefore anticipates claim 1. AMF1002, ¶103.

XIII. Ground 4: claim 1 would have been obvious over Bell, Bowdish, and Evans.

Dr. Balthasar explains that a POSA reading Bell would have constructed an anti-C5 antibody by combining the 5G1.1 sequences taught in Bowdish and Evans. AMG1002, ¶¶104-113. And a POSA would have had reasons to combine these references with a reasonable expectation of success in arriving at the claimed antibody. AMG1002, ¶¶114-119. There are no objective indicia of nonobviousness. *See* Section XII.C.

A. Claim 1 would have been obvious.

1. Bell expressly taught all the limitations of claim 1 except eculizumab's amino acid sequence.

Bell disclosed "an antibody that binds C5" as claimed because Bell disclosed using "an *anti-C5 antibody* selected from the group consisting of *h5G1.1-mAb (eculizumab)*…" and also that the antibody "h5G1.1-mAb" was "undergoing clinical trials under the tradename *eculizumab*." AMG1005, ¶¶[0012], [0052] and [0082]. Bell described an anti-C5 antibody (eculizumab) clinical trial involving a 12-week "pilot study," followed by a 52-week extension study for a total of 64 weeks, followed by a second extension study for a total of two years.[15] AMG1005,

[15] Because the clinical study taught in Bell is the same C02-001 study in Hillmen and E02-001 study in Hill '05, which disclose the eculizumab amino acid

¶[0082]. The only information explicitly not recited in Bell is eculizumab's amino acid sequences (SEQ ID NOs: 2 and 4 as claimed), but these sequences were readily found in Bowdish and Evans. AMG1002, ¶107.

2. Bowdish and Evans taught the claimed amino acid sequences.

As already discussed in Ground 3, Bowdish described using a h5G1.1 antibody as the starter "scaffold" antibody sequence for creating a recombinant TPO-mimetic+h5G1.1 antibody. Bowdish provided the full h5G1.1 amino acid sequence except for the heavy chain CDR3 (HCDR3) sequence, which was replaced with the TPO-mimetic peptide sequence. AMG1006, ¶¶[0191]-[0193], Figs. 13A-13B, and SEQ ID NOs.:67 and 69; AMG1002, ¶¶108-110. And the missing HCDR3 sequence from Bowdish was taught by Evans. AMG1007, 44:12-13; AMG1002, ¶¶111-112. Dr. Balthasar's Figure 14 below depicts this

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sequences of SEQ ID NOs: 2 and 4, Bell, too, therefore anticipates claim 1 for the same reasons discussed above for Hillmen and Hill '05. AMG1015, 738(¶6); *see also, id.*, 736 (study numbers C02-001 and E02-001); AMG1005, ¶[0082]; AMG1002, ¶108. Hill '04 (study number X03-001), Hillmen '06 (study number C04-001), and Young '06 (study number C04-002) also anticipate claim 1 for the same reasons. AMG1015, 738(¶6), 736; *see also*, AMG1011, Abstract; AMG1012, 1235; AMG1013, Abstract; AMG1002, ¶108.

combination of art providing Bowdish's complete "scaffold" antibody sequence:

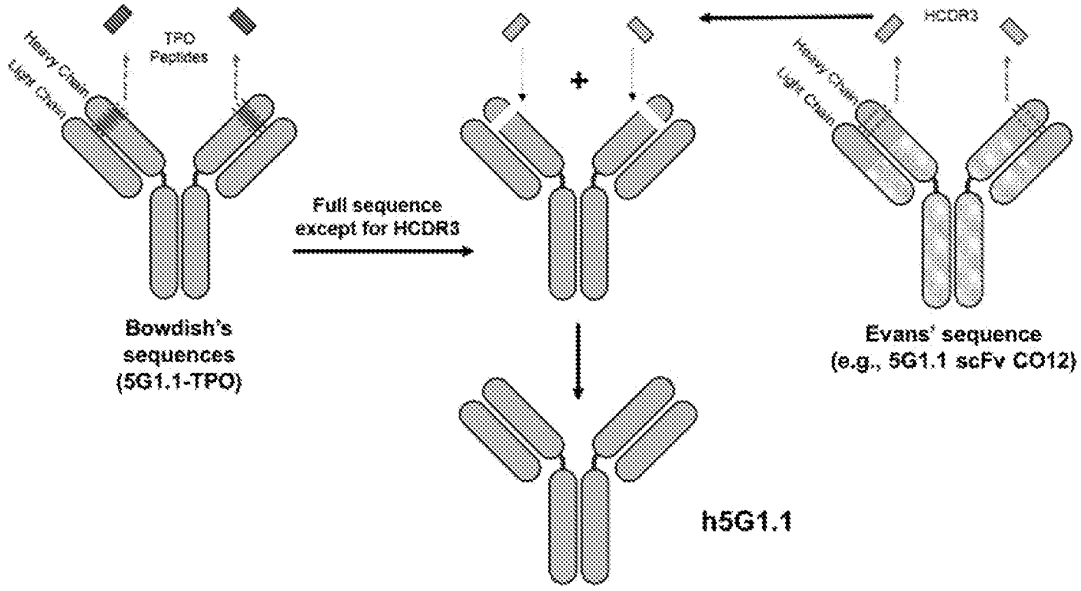


Figure 14.

AMG1002, ¶113.

Dr. Balthasar's Figure 3 (presented above in Ground 2) illustrates the structure of Bowdish's recombinant TPO-mimetic+h5G1.1 chimeric antibody, showing the location of the TPO-mimetic peptide (blue) in the native HCDR3 region of the polypeptide of SEQ ID NOs:67 (i.e., the heavy chain), and the polypeptide of SEQ ID NO:69 (i.e., the light chain), where green represents sequences 100% identical to SEQ ID NOs: 2 and 4 as claimed. AMG1002, ¶50; AMG1006, Figures 13A-13B. The missing HCDR3 piece, however, was taught in Evans, and Bowdish expressly directed a POSA to Evans. AMG1006, ¶[0191].

As already discussed, Evans disclosed a series of humanized 5G1.1 scFvs, and a POSA would have known that all of the heavy chain variable regions in Evans' scFvs contain the same YFFGSSPNWYFDV CDR3 sequence. AMG1007, Fig. 19, 43:13-14, 43:26-27, 43:33-34, 43:60-61, 44:2-3, 44:12-13, 44:21-22, 44:30-31, 44:39-40, 44:49-50, 44:59-60, 45:3-4; AMG1002, ¶111. Dr. Balthasar's Figure 14 above shows this, where Evans' HCDR3 sequence is extracted and inserted into Bowdish's construct. AMG1002, ¶¶112-113.

As Dr. Balthasar explains, a POSA would have expected that inserting Evans' YFFGSSPNWYFDV HCDR3 sequence in place of Bowdish's TPO-mimetic peptide sequence in SEQ ID NO:67 would provide the complete heavy chain sequence of the anti-C5 antibody. Indeed, Dr. Balthasar confirms that inserting Evans' HCDR3 sequence in place of Bowdish's TPO-mimetic peptide sequence[16] in SEQ ID NO:67 creates a heavy chain sequence that is a 100% match with SEQ ID NO: 2 from the '149 patent. AMG1002, ¶112. And as already discussed above, Bowdish's light chain SEQ ID NO:69 is a 100% match with SEQ ID NO:4 from the '149 patent. AMG1002, ¶112.

[16] To be clear, a POSA would have known that this would be achieved by replacing DNA encoding the TPO-mimetic (SEQ ID NO:65) with DNA encoding the HCDR3. AMG1002, ¶97.

3. A POSA would have had a reason to combine the references with a reasonable expectation of success.

Bell taught that targeting complement protein C5 with eculizumab (h5G1.1) is safe and effective for treating PNH patients, providing ample reason for a POSA to make a humanized anti-C5 antibody such as eculizumab. AMG1005, ¶¶[0083]-[0096]. Because Bell does not expressly provide the amino acid sequence of its anti-C5 antibody, a POSA would have looked to other known teachings in the art pertaining to eculizumab (5G1.1), like Bowdish and Evans.

A POSA is "presumed to be aware of all the pertinent prior art." *Standard Oil*, 774 F.2d at 454. A POSA, therefore, would have been well aware that Bell's anti-C5 antibody, eculizumab, was also known in the art as humanized "5G1.1," "h5G1.1," or "h5G1.1-mAb" with a hybrid IgG2/IgG4 constant region. AMG1002, ¶¶114-115; AMG1005, ¶[0052]; AMG1034, 1279; *see also*, Section IV, *supra*. To possess the amino acid sequence of that antibody, a POSA would have consulted Bowdish because it taught using a humanized 5G1.1 antibody (including the hybrid IgG2/IgG4 heavy chain constant domain) as the starter antibody sequence when creating the TPO-mimetic+h5G1.1 antibody and further provided the amino acid sequence of the chimeric antibody. AMG1002, ¶¶115-116. Knowing that the only change made to the h5G1.1 amino acid sequence in Bowdish was replacing the original HCDR3 region in the scaffold h5G1.1 with a TPO-mimetic peptide, a

POSA seeking a humanized C5-binding antibody would have had reason to restore the original HCDR3 region to complete the eculizumab. AMG1002, ¶116.

The POSA would have looked to Evans for the missing HCDR3 sequence because both Bell and Bowdish explicitly direct the artisan to Evans for information on how each's h5G1.1 antibody was originally created. AMG1005, ¶[0052]; AMG1006, ¶[0191]; AMG1002, ¶116. For example, Bell stated: "[m]ethods for the preparation of *h5G1.1-mAb* [eculizumab] … are described in [Evans][17] … the disclosures of which are incorporated herein in their entirety by this reference." AMG1005, ¶[0052]. And Bowdish similarly stated: "[c]onstruction of *5G1.1* is described in [Evans], incorporated herein by reference."[18] AMG1006, ¶[0191].

This combination of prior art would have led a POSA to make a simple substitution of one known element for another—i.e., replace the TPO-mimetic

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[17] Bell also cites Thomas. AMG1005, ¶[0052]. A POSA would not have chosen to combine Bowdish with Thomas' antibody over Evan's because Thomas's antibody is an IgG4 isotype, and not a human hybrid IgG2/IgG4 isotype, which was known to be less immunogenic and thus preferred over an IgG4 isotype. AMG1031, 448, 451; AMG1002, ¶¶46-47; *see also*, Section IV, *supra.*

[18] *See* note 5, *supra.*

peptide sequence in Bowdish's TPO-mimetic+h5G1.1 antibody with the HCDR3 sequence from Evans—to yield predictable results: a complete anti-C5 antibody. *KSR*, 550 US at 416 ("[A] combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results."); AMG1002, ¶117.

And, as Dr. Balthasar confirms by sequence alignment, the anti-C5 antibody obtained by replacing the TPO-mimetic peptide sequence in Bowdish's TPO-mimetic+h5G1.1 antibody with the HCDR3 sequence from Evans comprises a heavy chain consisting of SEQ ID NO:2 and a light chain consisting of SEQ ID NO:4 as claimed. AMG1002, ¶112.

A POSA would have reasonably expected to succeed in making the anti-C5 antibody as claimed because it would have required only basic molecular biology techniques to substitute Evans' HCDR3 sequence in place of Bowdish's TPO-mimetic peptide sequence. AMG1002, ¶118. A POSA also would have had an expectation of success in producing the anti-C5 antibody because antibody production methods were well-known in the art, and the antibody would be expected to bind C5. See, e.g., AMG1006, ¶¶[0130]-[0131]; AMG1002, ¶118.

Accordingly, claim 1 would have been obvious. And there are no objective indicia that support patentability. *See* Section XII.C., *infra*.

XIV. Ground 5: Claim 1 would have been obvious over Evans and Mueller.

Claim 1 also would have been obvious in view of Evans and Mueller. AMG1002, ¶¶120-137. Discussed below, a POSA would have had a reason to combine these references with a reasonable expectation of successfully making the claimed antibody. AMG1002, ¶¶123-128, 136-137. And no objective indicia support patentability. *See* Section XII.C.

A. Claim 1 would have been obvious.

1. Evans and Mueller disclose the claimed amino acid sequences.

As discussed in detail below, Evans disclosed the complete amino acid sequences of the heavy and light chain variable domains of anti-C5 antibodies. AMG1007, 44:4-13, SEQ ID NO:20; AMG1002, ¶¶121, 123-124, 129-131. And Mueller disclosed the amino acid sequence of an anti-C5 antibody's light chain constant domain and the hybrid IgG2/IgG4 heavy chain constant domain. AMG1008, 58-61; AMG1002, ¶¶121, 125, 132. Dr. Balthasar's Figure 16 below depicts this combination of art providing the complete anti-C5 antibody sequence:

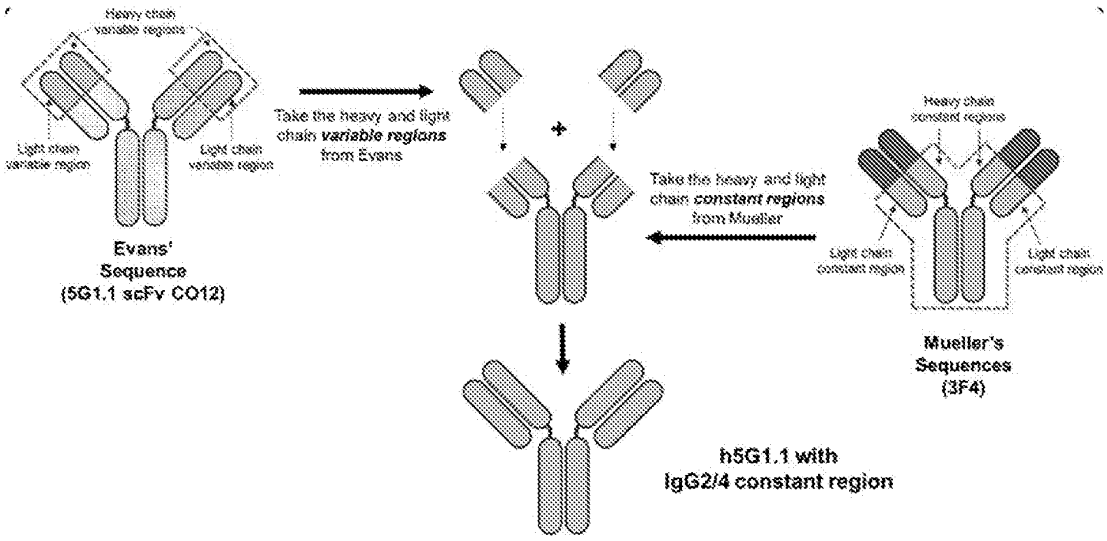


Figure 16.

See AMG1002, ¶133.

Evans—which Alexion previously said claims Soliris® and provides written description and enablement support for claims directed to eculizumab (AMG1009, 4; AMG1049, 838-839)—discloses making the original mouse 5G1.1 monoclonal antibody. AMG1007, 37:36-39:30 (Example 7); AMG1002, ¶124. Evans further described constructing a series of humanized 5G1.1 antibody constructs containing the heavy and light chain CDR sequences from the mouse 5G1.1 antibody inserted into a human framework. AMG1007, 42:58-45:4. In particular, Evans described nine different humanized 5G1.1 scFv constructs[19] along with their amino acid

[19] A POSA would have known that a scFv comprises an antibody's light and heavy chain variable domains connected by a linker. *See, e.g.,* AMG1007, 6:39-41

sequences. *Id.* (construct numbers 2 and 11-18); AMG1002, ¶124. Evans also described combining the antibody variable regions with constant domains—including hybrid IgG constant domains—to make a complete anti-C5 antibody. AMG1007, 45:24-33.

In looking for a constant domain to pair with Evans' variable regions, a POSA would have looked to Mueller because Mueller taught antibody constant regions designed with a lower propensity to activate the immune system (and complement)—a desirable feature for a complement inhibiting antibody. AMG1008, 7:28-31, 8:23-26, 12:27-30; AMG1002, ¶¶125-128. A POSA reading Evans also would have looked to Mueller for "h5G1.1" sequence information because Mueller disclosed a 5G1.1 antibody with a hybrid IgG2/IgG4 constant domain. AMG1002, ¶¶125-128. Mueller taught methods for making "chimeric antibodies containing the C1 and hinge region of human IgG2 and the C2 and C3 regions of human IgG4 … (HuG2/G4 mAb)." AMG1008, 12:27-30; *see also, id.,* 8:23-26. In particular, Mueller described a control antibody "h5G1.1 CO12 HuG2/G4 mAb," which a POSA would have readily identified as a humanized

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("single chain antibodies may include one each of only VH and VL domains, in which case they are *referred to as scFv antibodies*"); *see also,* AMG1040, 45-48; AMG1002, ¶124.

anti-C5 antibody because of the "h5G1.1" nomenclature coupled with the hybrid IgG2/IgG4 constant domain ("HuG2/G4"). AMG1008, 12:37, FIG. 15; AMG1005, ¶[0052]; AMG1034, 1279; AMG1049, 838-839; AMG1002, ¶¶54-55, 125-128.

As Dr. Balthasar explains, Mueller disclosed the amino acid sequence of a hybrid IgG2/IgG4 heavy chain constant domain when Mueller disclosed the sequence of the chimeric anti-VCAM "3F4" antibody. AMG1002, ¶¶129, 132; AMG1008, 58-61. A POSA would have known that a chimeric antibody contains the variable region from a non-human antibody and the constant region from a human antibody, and therefore would have understood that Mueller's chimeric 3F4 HuG2/G4 mAb heavy chain contains the variable regions from murine antibody 3F4 (the blue portions in Dr. Balthasar's Figure 16 above) and the constant regions of human hybrid IgG2/IgG4 (the green portions in Dr. Balthasar's Figure 16). AMG1002, ¶¶132-133; AMG1040, 29-30.

Mueller separately disclosed the amino acid sequences of the mature 3F4 heavy and light chain variable regions (i.e., the blue portion in Dr. Balthasar's Figure 16). AMG1008, Figure 9; AMG1002, ¶132. A POSA aligning the 3F4 heavy and light chain variable region sequences from Figure 9 with the sequences of the 3F4 HuG2/G4 chimeric antibody would have identified the 3F4 variable regions (the regions a POSA would have excluded) as amino acids 20-137 of the 3F4 HuG2/G4 heavy chain and amino acids 20-131 of the 3F4 light chain.

AMG1008, Figure 9, 52-53, 58-61; AMG1002, ¶132.

A POSA therefore would have immediately known that the remainder of the 3F4 HuG2/G4 heavy chain (amino acids 138-463) is the hybrid IgG2/IgG4 constant region of that antibody, and that the remainder of the 3F4 light chain (amino acids 132-238) is the light chain constant region of that antibody (i.e., the green portion in Dr. Balthasar's Figure 15). AMG1008, 52-53, 56-57; AMG1002, ¶132. Given that Mueller used this humanized anti-C5 antibody as an isotype control for 3F4 HuG2/G4, a POSA would have reasonably expected the disclosed heavy and light chain constant regions to be the same as those in the 3F4 HuG2/G4 chimeric antibody. AMG1002, ¶¶55, 125.

As Dr. Balthasar explains, overwhelming evidence in the art would have further confirmed a POSA's belief that the amino acid sequences of the heavy and light chain constant regions of Mueller's 3F4 HuG2/G4 antibody are the same as those in a 5G1.1 antibody, e.g., eculizumab. AMG1002, ¶¶45, 54-55, 125, 132. Publications such as Bowdish, Tacken, Mueller II, and Evans all disclosed portions of eculizumab constant regions that either overlapped with or were exact matches to the heavy and light chain constant regions of Mueller's 3F4 HuG2/G4 antibody. AMG1002, ¶¶45, 54-55, 125, 132; AMG1006, Figs. 13A-13B; AMG1034, 1279; AMG1031, Abstract, 448, Fig. 7; AMG1007, 43:50-55 (SEQ ID NO:15).

With the heavy and light chain constant domain sequences obtained from

Mueller, a POSA would have looked back to Evans to complete the amino acid sequence of the anti-C5 antibody. AMG1002, ¶¶127-128. Knowing that Mueller refers to the control antibody as "h5G1.1 CO12 HuG2/G4 mAb," the POSA would have referred to the series of humanized 5G1.1 scFvs taught in Evans and readily identified construct no. 12 (SEQ ID NO:20) as the scFv of interest because Evans used the same "CO12" nomenclature as Mueller by designating it "5G1.1 scFv CO12." AMG1007, 44:4-13; AMG1002, ¶¶127-128. This is shown in Dr. Balthasar's Figure 16 above, where the green portions depict Evans' heavy and light chain variable regions. AMG1002, ¶133; AMG1007, 44:4-13, SEQ ID NO:20.

That Evans taught additional 5G1.1 scFv constructs is of no moment because, "for an obviousness analysis, even the fact that 'a specific embodiment is taught to be preferred is not controlling, since all disclosures of the prior art, including unpreferred embodiments, must be considered.'" *In re Thomas*, 151 Fed. App'x. 930, 934 (Fed. Cir. 2005) (quoting *Merck & Co., Inc. v. Biocraft Labs., Inc.*, 874 F.2d 804, 807 (Fed. Cir. 1989). Here, any one of the combinations of Mueller's constant regions with Evans' variable regions would have been obvious, and does not make the combination with any one pair of variable regions any less obvious. *See Merck*, 874 F.2d at 807 ("That the [asserted prior art] discloses a multitude of effective combinations does not render any particular formulation less

obvious.")

A POSA would have known that Evans' humanized 5G1.1 scFvs each contains a single polypeptide sequence comprising (1) a humanized heavy chain variable region of 5G1.1, (2) a linker, and (3) a humanized light chain variable region of 5G1.1. AMG1002, ¶¶124, 130; AMG1007, 6:39-41; AMG1040, 45-48. As Dr. Balthasar explains, a POSA would have been able to readily identify the heavy and light chain variable regions within SEQ ID NO: 20 of Evans. AMG1002, ¶131. For example, a POSA would have known that the linker in "5G1.1 scFv CO12" (SEQ ID NO:20) is amino acids 112-126 because this 15-amino acid sequence (GGGGSGGGGSGGGGS) was well known in the art as common a linker sequence in scFv antibodies. AMG1007, SEQ ID NO:20 (Certificate of Correction, 42-44); AMG1037, ¶¶[0021], [0097]; AMG1002, ¶131.

Alexion has also argued during prosecution that "the mature light chain sequence … could have been readily identified at the relevant filing date using well established rules and art-recognized techniques" and provided Adderson (AMG1048) as an example showing "the characteristic mature N-terminus (DIQ) of a light V kappa antibody light chain." AMG1015, 546; AMG1048, Fig. 6. Thus, the first two amino acids of Evans' SEQ ID NO:20 (MA) are a leader sequence, based on Alexion's admission that a mature kappa light chain starts with the sequence "DIQ" on its N terminus. AMG1002, ¶131; AMG1048, 734 (Fig. 6).

Accordingly, a POSA would have understood that the mature light and heavy chain variable regions of Evans' anti-C5 antibody correspond to amino acids 3-111 and 127-248 of SEQ ID NO:20, respectively. AMG1007, 44:4-13; SEQ ID NO:20; AMG1002, ¶131.

Finally, a POSA would have expected that combining Evans' variable region sequences with Mueller's constant domain sequences[20] would provide the complete heavy and light chain sequences of an anti-C5- antibody. *See* Dr. Balthasar's Figure 16 above; AMG1002, ¶¶133-135.

As Dr. Balthasar shows by sequence alignment, the combination of eculizumab's heavy and light chain variable regions in Evans' SEQ ID NO:20 with Mueller's constant regions would make an anti-C5 antibody having SEQ ID NOs:2 and 4, as claimed. AMG1002, ¶¶134-135.

2. A POSA would have had a reason to combine the references with a reasonable expectation of success.

In seeking to make an anti-C5 antibody, a POSA reading Evans would have looked to Mueller because Mueller taught antibodies with constant regions designed with a lower propensity to activate the immune system (and

[20] To be clear, a POSA would have known that this would be achieved by combining DNA encoding Evans' variable region sequences with that encoding Mueller's constant region. AMG1002, ¶97.

complement). AMG1008, 12:27-30; AMG1002, ¶¶125-126. Moreover, Mueller disclosed an h5G1.1 antibody named "h5G1.1 CO12 HuG2/G4 mAb" with a hybrid IgG2/IgG4 heavy chain constant region, which a POSA would have readily understood to be the anti-C5 antibody, *eculizumab*. AMG1002, ¶¶ AMG1002, ¶¶45, 54-55, 125.

Evans and Mueller taught complementary, familiar elements of anti-C5 antibody amino acid sequences. AMG1002, ¶¶129, 136. Thus, by combining familiar elements in the art according to known methods, the artisan would have predictably arrived at an antibody comprising a light chain consisting of SEQ ID NO:2 and a heavy chain consisting of SEQ ID NO:4 as claimed. *KSR*, 550 US at 416 ("a combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results."). A POSA would have easily confirmed this prediction by comparing the constructed sequences with another Alexion publication WO 2005/007809, which taught detailed descriptions of expression vectors designed for placing antibody variable regions in frame with chimeric IgG2/IgG4 heavy chain constant regions. AMG1032, 7, 28-32, FIG. 5; AMG1002, ¶136.

A POSA would have reasonably expected to successfully make an anti-C5 antibody as claimed because it would have required only basic molecular biology techniques to combine Evans' scFv variable regions with Mueller's constant

domains. AMG1002, ¶136. A POSA also would have had an expectation of success in producing an anti-C5 antibody as claimed because antibody production methods already were well-known. See, e.g., AMG1006, ¶¶[0130]-[0131]; AMG1002, ¶136.

B. Objective indicia do not support patentability.

"To be afforded substantial weight, the objective indicia of non-obviousness must be tied to the *novel elements* of the claim at issue." *Univ. Pierre et Marie Curie v. Focarino*, 738 F.3d 1337, 1347 (Fed. Cir. 2013). Objective evidence that is not "both claimed and *novel in the claim*" lacks nexus to the invention. *In re Kao*, 639 F.3d 1057, 1068 (Fed. Cir. 2011).

Alexion argued during prosecution of a related patent that "the non-natural, protein-engineered, heavy chain of eculizumab" (i.e., the hybrid IgG2/IgG4 constant domain) provided "surprising and unpredictable" results such as decreased effector function, reduced immunogenicity and increased half-life. AMG1014, 588, 593(¶8). Eculizumab's hybrid IgG2/IgG4 constant domain was well known in the art (e.g., AMG1034, 1279), however, and cannot be a "novel element." *Marie Curie*, 738 F.3d at 1347; *Kao*, 639 F.3d at 1068. Accordingly, the alleged "surprising and unpredictable" features of eculizumab have no nexus with the challenged claim and do not support non-obviousness. *Id.*

Moreover, Alexion's alleged results would not have been unexpected to a

POSA. AMG1002, ¶¶138-140. Mueller II taught in 1997 that antibodies with a hybrid IgG2/IgG4 heavy chain "[do] not contain the antibody sequences necessary for FcR binding," and would not contain "any new epitopes that would likely be immunogenic." AMG1031, 448, 451. It was also well known that a hybrid IgG2/IgG4 heavy chain would "have increased half-life." *See, e.g.,* AMG1032, 5, 19; AMG1002, ¶140. There is nothing unexpected here.

Petitioner is not aware of any other alleged objective indicia relevant to the '149 patent claim, and reserves the right to rebut any evidence Alexion asserts in this proceeding. *Amneal Pharms., LLC v. Supernus Pharms., Inc.*, IPR2013-00368, Paper 8, at 12-13 (Dec. 17, 2013); AMG1002, ¶141.

XV. Certification that the Patent May Be Contested via *Inter Partes* Review by the Petitioner and Standing (37 C.F.R. §42.104(a)).

Amgen certifies that (1) the '149 patent is available for IPR and (2) Amgen is not barred or estopped from requesting IPR of the '149 patent's single claim.

XVI. Mandatory Notices (37 C.F.R. §42.8(a)(1).

Real party-in-interest 37 C.F.R. §42.8(b)(1): Amgen Inc.

Related Matters (37 C.F.R. §42.8(b)(2)): Amgen has concurrently filed petitions for IPR of U.S. Patent Nos. 9,718,880 (IPR2019-00740) and 9,725,504 (IPR2019-00739), which are related to the '149 patent and also owned by Alexion.

Lead and back-up counsel (37 C.F.R. §42.8(b)(3)) for Amgen Inc. are

Lead Counsel	Back-Up Counsel
Deborah Sterling (Reg. No. 62,732) STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C. 1100 New York Avenue, NW Washington, DC 20005 202.772.8508 (telephone) 202.371.2540 (facsimile) dsterling-PTAB@skgf.com	David H. Holman (Reg. No. 61,205) Scott A. Schaller (Reg. No. 60,167) David W. Roadcap (Reg. No. 68,956) STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C. 1100 New York Avenue, NW Washington, DC 20005 202.371-2600 (telephone) 202.371.2540 (facsimile) dholman-PTAB@sternekessler.com sschalle-PTAB@ sternekessler.com droadcap-PTAB@sternekessler.com

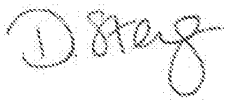
Notice of Service Information (37 C.F.R. §42.8(b)(4)): Please direct all correspondence regarding this Petition to counsel at the above addresses. Amgen consents to service by email at the addresses above.

Procedural Statements: This Petition is filed in accordance with 37 C.F.R. §42.106(a). Concurrently filed herewith are a Power of Attorney and Exhibit List under 37 C.F.R. §42.10(b) and §42.63(e), respectively. The required fee is paid through Deposit Acct. No. 19-0036 (Customer ID No. 45324). The Office is authorized to charge any fee deficiency, or credit any overpayment, to Deposit Acct. No. 19-0036 (Customer ID No. 45324).

XVII. Conclusion.

The challenged claim is unpatentable as anticipated or obvious and IPR is warranted.

Respectfully submitted,
STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C



Date: February 28, 2019
1100 New York Avenue, N.W.
Washington, D.C. 20005-3934
(202) 371-2600

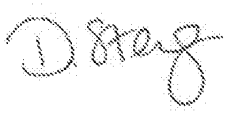
Deborah A. Sterling, Ph.D.
Registration No. 62,732
Lead Attorney for Petitioner

10498316.1

CERTIFICATE OF WORD COUNT (37 C.F.R. § 42.24(d))

I certify that Amgen Inc.'s Petition for *Inter Partes* Review for U.S. Patent No. 9,732,149 contains 11,154 words as counted by the word-processing program used to generate this response. This total does not include the table of contents, certificate of service, or this certificate of word count.

Respectfully submitted,
STERNE, KESSLER, GOLDSTEIN & FOX L.L.C.



Date: February 28, 2019
1100 New York Avenue, N.W.
Washington, D.C. 20005-3934
(202) 371-2600

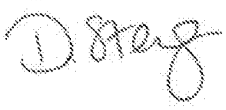
Deborah A. Sterling, Ph.D.
Registration No. 62,732
Lead Attorney for Petitioner

CERTIFICATE OF SERVICE (37 C.F.R. § 42.6(e)), §42.105(a))

I certify that the above-captioned "Petition for *Inter Partes* Review for U.S. Patent No. 9,732,149" was served in its entirety upon the Patent Owner on February 28, 2019, via FedEx, at the correspondence address of record indicated in the Patent Office's public PAIR system for U.S. Patent No. 9,732,149:

Nelson Mullins Riley & Scarborough LLP/Alexion
One Post Office Square
30th Floor
Boston MA 02109-2127

STERNE, KESSLER, GOLDSTEIN & FOX L.L.C.



Date: February 28, 2019
1100 New York Avenue, N.W.
Washington, D.C. 20005-3934
(202) 371-2600

Deborah A. Sterling, Ph.D.
Registration No. 62,732
Lead Attorney for Petitioner

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mation about drugs and patient-specific drug information to other pharmacists, other health-care professionals, and patients as appropriate.

15. Initiation of and participation in drug and drug-related (e.g., medication administration devices) research, including formal clinical drug investigations.

ASHP believes that pharmacists have a responsibility to communicate advances in the development and delivery of these and other clinical pharmacy services to the health-care community and the public through appropriate publications, presentations, and programs.

ASHP statement on unit dose drug distribution

Am J Hosp Pharm. 1989; 46:2346

The unit dose system of medication distribution is a pharmacy-coordinated method of dispensing and controlling medications in organized health-care settings. The unit dose system may differ in form, depending on the specific needs of the organization. However, the following distinctive elements are basic to all unit dose systems: medications are contained in single unit packages; they are dispensed in a form that is as ready-to-administer as possible; for most medications, not more than a 24-hour supply[a] of doses is delivered to or available at the patient-care area at any time.[1,2]

Numerous studies concerning unit dose drug distribution systems have been published over the past several decades. These studies indicate categorically that unit dose systems, with respect to other drug distribution methods, are (1) safer for the patient, (2) more efficient and economical for the organization, and (3) a more effective method of utilizing professional resources.

More specifically, the inherent advantages of unit dose systems over alternative distribution procedures are

1. A reduction in the incidence of medication errors.
2. A decrease in the total cost of medication-related activities.
3. A more efficient usage of pharmacy and nursing personnel, allowing for more direct patient-care involvement by pharmacists and nurses.
4. Improved overall drug control and drug-use monitoring.
5. More accurate patient billing for drugs.
6. The elimination or minimization of drug credits.
7. Greater control by the pharmacist over pharmacy workload patterns and staff scheduling.
8. A reduction in the size of drug inventories located in patient-care areas.
9. Greater adaptability to computerized and automated procedures.

In view of these demonstrated benefits, ASHP considers the unit dose system to be an essential part of drug distribution and control in organized health-care settings in which drug therapy is an integral component of health-care delivery.

[a] In long-term-care facilities, a larger supply of medication (e.g., 48 or 72 hours) may be acceptable.

References

1. Summerfield MR. Unit dose primer. Bethesda, MD: American Society of Hospital Pharmacists; 1983.
2. ASHP technical assistance bulletin on hospital drug distribution and control. *Am J Hosp Pharm.* 1980; 37: 1097–103.

Approved by the ASHP House of Delegates, June 5, 1989. Approved by the ASHP Board of Directors, November 16, 1988. Supersedes earlier versions approved by the ASHP House of Delegates on June 8, 1981, and by the Board of Directors on April 19, 1975, and November 13–14, 1980.

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Reports

2286 **Robotic system for i.v. antineoplastic drug preparation: Description and preliminary evaluation under simulated conditions**
Dennis D. Cote and Mark G. Torchia

2294 **Factors influencing pharmacists' selection of their first practice setting**
Edmund A. Carter and Richard Segal

2300 **System for voluntary reporting of adverse drug reactions in a university hospital**
Thomas E. Vorce-West, Lynda Barstow, and Bill Butcher

2304 **Accuracy and time requirements of a bar-code inventory system for controlled substances**
Kim Crawford Dinklage, Sara J. White, Julie C. Lenhart, and Harold N. Godwin

2308 **National survey of critical-care pharmaceutical services**
Joseph F. Dasta, Richard Segal, and Anthony Cunningham

2313 **Evaluation of five procedures for measuring nonstandard doses of nifedipine liquid**
Wendy J. Rosen and Cary E. Johnson

2318 **Effect of primary fluids and dilutional volumes on the delivery of cefazolin sodium by a membrane infusion device**
Milap C. Nahata, Diane E. Durrell, and Marcia A. Miller

2321 **Stability of famotidine 20 and 40 mg/L and amino acids in total parenteral nutrient solutions**
Linda Bullock, Joseph F. Fitzgerald, Melvin R. Glick, Robert B. Parks, Joseph G. Schnabel, and Bruce G. Hancock

2326 **Stability of famotidine 20 and 50 mg/L in total nutrient admixtures**
Linda Bullock, Joseph F. Fitzgerald, and Melvin R. Glick

2329 **Stability of famotidine 20 and 40 mg/L in total nutrient admixtures**
Jose B. Montoro, Leonor Pou, Pilar Salvador, Carmen Pastor, and Santiago M. Cano

Note

2333 **Long-term stability of famotidine 20 mg/L in a total parenteral nutrient solution**
Joseph E. DiStefano, Francis P. Mitrano, Richard J. Baptista, Mary M. Der, Anthony P. Silvestri, John D. Palombo, and Bruce R. Bistrian

Primer

2336 **Situational theory of leadership**
David J. Waller, Steven R. Smith, and John T. Warnock

ASHP Reports

2342 **ASHP statement on the pharmacist's role with respect to drug delivery systems and administration devices**

2344 **ASHP statement on the pharmacist's role in clinical pharmacokinetic services**

2345 **ASHP statement on the pharmacist's clinical role in organized health-care settings**

2346 **ASHP statement on unit dose drug distribution**

2347 **ASHP guidelines on selecting pharmaceutical manufacturers and suppliers**

AIDS Facts

2349 AS-101 . . . AIDS hotlines for the deaf . . . Preventing pathogen transmission via respiratory resuscitation devices . . . Efficacy of low-dose zidovudine therapy

2351 Classified Advertising

2388 CP Digest

2398 Current Literature

Journal References . . . Book Reviews: *Training Manual for Intravenous Admixture Personnel, Fourth Edition (Hunt)*, Clyde Buchanan . . . *USP DI, Ninth Edition (United States Pharmacopeial Convention, Inc.)*, Lucinda G. Miller . . . Books Received

2404 Advertising Index

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Samsung Bioepis Exhibit No. 1002
Page 2660

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VOLUME 46 NUMBER 11 NOVEMBER 1989

NO 7 8

clinical career ladders

robotic system for i.v. drug preparation

factors influencing selection of first practice setting

voluntary reporting of adverse drug reactions

bar-code inventory system for controlled substances

national survey of critical-care pharmaceutical services

situational leadership

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Samsung Bioepis Exhibit No. 1002
Page 2661

PUBLIC SUMMARY DOCUMENT
Product: Eculizumab, solution concentrate for I.V. infusion, 300 mg in 30 mL, Soliris®
Sponsor: Alexion Pharmaceuticals Australasia Pty Ltd
Date of PBAC Consideration: March 2009

1. Purpose of Application

The submission sought a Section 100 (Highly Specialised Drugs Program) PBS listing, or a recommendation for inclusion on the Life Saving Drugs Program (LSDP) for treatment of paroxysmal nocturnal haemoglobinuria (PNH).

Highly Specialised Drugs are medicines for the treatment of chronic conditions, which, because of their clinical use or other special features, are restricted to supply to public and private hospitals having access to appropriate specialist facilities.

Life Saving Drugs Program
The Commonwealth Government provides funds under an appropriation item established for the specific purpose of assisting access to expensive and lifesaving drugs accepted by the PBAC as clinically effective, but not available as pharmaceutical benefits because of a failure to meet cost effectiveness criteria. Financial assistance for such drugs is approved in accordance with specified eligibility criteria and subject to certain conditions as agreed by the Ministers for Health and Finance.

2. Background

At the July 2008 meeting, the PBAC rejected the application for Section 100 listing of eculizumab for the treatment of patients with PNH on the basis of an unacceptably high and highly uncertain estimated cost per additional death avoided over a 2-year period.

The PBAC also rejected this application for consideration for the LSDP. The Committee agreed that eculizumab may meet the criteria for the LSDP for an as yet unidentified subgroup of patients with PNH, but that it was not possible to identify this subgroup at the present time. The Committee noted the sponsor' s indication that it was working on a set of eligibility criteria to identify a population of patients that would benefit most from treatment with eculizumab.

The Public Summary Document for eculizumab from the July 2008 meeting can be accessed from
http://www.health.gov.au/internet/main/publishing.nsf/Content/211923B8041DA9D2CA2574EF00060D0C/$File/Eculizumab%20Final%20PSD%20Alexion%20Pharmaceuticals%20Inc.pdf

3. Registration Status

Eculizumab was TGA registered on 20 March 2009 for the treatment of patients with paroxysmal nocturnal haemoglobinuria (PNH) to reduce haemolysis.

4. Listing Requested and PBAC's View

Section 100 (Highly Specialised Drug)
Private hospital authority required
Treatment of patients with paroxysmal nocturnal haemoglobinuria to reduce haemolysis.
or

Samsung Bioepis Exhibit No. 1002
Page 2662

Inclusion on the Life Saving Drugs Program for treatment of paroxysmal nocturnal haemoglobinuria to reduce haemolysis.

For PBAC's view see Recommendation and Reasons.

5. Clinical Place for the Proposed Therapy

Paroxysmal nocturnal haemoglobinuria is a clonal haemopoietic stem cell disorder that is extremely rare, progressive and life-threatening. Complement mediated intravascular haemolysis is the central mechanism responsible for morbidities and mortality in PNH including thromboembolism, renal dysfunction, pulmonary hypertension, severe anaemia and disabling fatigue.

Currently, therapeutic management of PNH is supportive and mainly addresses the treatment of anaemia and prevention of thrombotic events.

Eculizumab would provide a treatment option for patients with PNH to reduce haemolysis, however it is not curative for the underlying disease.

6. Comparator

The submission nominated supportive care as the main comparator. The comparator was accepted, as previously, by the PBAC as appropriate.

7. Clinical Trials

The submission presented the same clinical trial evidence as in the submission to the July 2008 PBAC meeting. One 26 week randomised placebo controlled trial (TRIUMPH), two uncontrolled single arm eculizumab studies (52 week SHEPHERD and 12 week PILOT) and the E05-001 extension study (median duration 22 months) were presented.

The trials published at the time of submission are as follows:

Trial/First author	Protocol title/ Publication title	Publication citation
Randomised trial		
C04-001 (TRIUMPH) Hillmen P, et al.	The Complement Inhibitor Eculizumab in Paroxysmal Nocturnal Haemoglobinuria.	N Engl J Med 2006; 355 (12):1233-1243.
Supportive non-randomised trials		
C04-002 (SHEPHERD) Brodsky R, et al.	Multicentre Phase 3 Study of the Complement Inhibitor Eculizumab for the Treatment of Patients with Paroxysmal Nocturnal Haemoglobinuria.	Blood 2008; 111 (4):1840-1847.
C02-001 (Pilot Study) & 2 extension studies Hillmen P ,et al	Effect of Eculizumab on Hemolysis and Transfusion Requirements in Patients with Paroxysmal Nocturnal Hemoglobinuria.	N Engl J Med 2004, 350:552-559.
Hill A, et al.	Sustained response and long-term safety of eculizumab in paroxysmal nocturnal haemoglobinuria	Blood 2005; 106:2559-65

Samsung Bioepis Exhibit No. 1002
Page 2663

Trial/First author	Protocol title/ Publication title	Publication citation
E05-001 (Phase 3 Common Extension Trial) Hillmen P , et al.	Effect of the Complement Inhibitor Eculizumab on Thromboembolism in Patients with Paroxysmal Nocturnal Haemoglobinuria.	Blood 2007; 110 (12):4123-8.

8. Results of Trials

At the July 2008 meeting the PBAC considered that the direct evidence from the TRIUMPH trial to establish the superiority of eculizumab in clinical effectiveness was limited to transfusions avoided and Hb stabilisation, and claimed benefits in terms of fewer thromboembolic events, fewer deaths and improved kidney function were not adequately demonstrated.

The re-submission presented new data regarding the effect of eculizumab on kidney function and damage of other end-organs. The key additional *post hoc* analyses of severe morbidity events of individual end-organs before and during eculizumab treatment in extension study E05-001 are summarised below:

End-organ	Pre-treatment (1683 pt-years)		During treatment (382 pt-years)		P-value	Relative rate reduction	*Absolute difference (events/100 pt-yrs*
	No. of events	Events/ 100 pt-yrs	No. of events	Events/ 100 pt-yrs			
Brain	29	1.72	4	1.05	NS	39%	*0.67*
Liver	52	3.09	8	2.10	NS	32%	*0.99*
GI	66	3.92	3	0.79	Sig	80%	*3.13*
Kidney	71	4.22	8	2.10	Sig	50%	*2.12*
TE	124	7.37	5	1.31	Sig	82%	*6.06*
Compo-site*	271	16.10	28	7.34	Sig	54%	*8.76*

* Composite Severe Morbidity Event included non-overlapping events in the 5 most common systems - brain, liver, GI, renal and thrombosis

The proportions of patients with events were not reported, except for pre-treatment thromboembolic events, 31.8% (62/195). The claim of long-term improvement in end-organ function and therefore survival with eculizumab treatment is based on post-hoc before and after comparisons in the uncontrolled E05-001 study with median treatment duration of 22 months.

At the July 2008 meeting, the PBAC was not satisfied that the comparative toxicity of eculizumab and BSC was adequately elucidated, as two non-fatal cases of meningococcal infection occurred despite immunisation in the clinical trial/studies and longer term safety data were very limited.

No new toxicity data were presented in the re-submission except for one additional death reported in study E05-001 and the inclusion of PSUR 2 (10 reported deaths and 1 fatal meningococcal infection case during the 6-month reporting period).

9. Clinical Claim

The re-submission claimed that eculizumab is therapeutically superior to best supportive care for the treatment of PNH to reduce morbidity and mortality with similar safety issues.

Samsung Bioepis Exhibit No. 1002
Page 2664

For PBAC's view see Recommendation and Reasons.

10. Economic Analysis

The economic evaluation was updated, using the proposed new effective price and 3-year mortality rates for patients receiving eculizumab (from the included eculizumab trial/studies) and BSC (derived from de Latour 2008 study). In the previous submission the incremental cost effectiveness ratio (ICER) was estimated to be greater than $200,000 per additional death avoided over a two year period, whilst in the re-submission the incremental cost effectiveness ratio (ICER) was also estimated to be greater than $200,000 per additional death avoided over a three year period

In the previous submission, neither costs nor outcomes were discounted. In the current re-submission, only costs but not outcomes were discounted.

11. Estimated PBS Usage and Financial Implications

The financial cost per year to the PBS was estimated to be $10-$30 million in Year 5
The likely number of patients per year was estimated to be less than 10,000 in Year 5.

The PBAC considered the utilisation of eculizumab for PNH was also uncertain as the extent of under diagnosis of PNH in Australia is unclear and there was uncertainty in the applicability of the UK incidence and prevalence to the Australian population

12. Recommendation and Reasons

The comparator of best supportive care (BSC) was accepted, as previously, by the PBAC as appropriate. The submission presented the same clinical trial evidence as in the submission to the July 2008 PBAC meeting. One 26 week randomised placebo controlled trial (TRIUMPH), two uncontrolled single arm eculizumab studies (52 week SHEPHERD and 12 week PILOT) and the E05-001 extension study (median duration 22 months) were presented. The Committee considered that uncertainty remained in the difference in mortality of patients receiving eculizumab and those receiving best supportive care and in the magnitude of the absolute benefit of eculizumab over best supportive care in reduction of thromboembolic events. The effectiveness of eculizumab in preserving end-organ function was also not clear as improvements were based on post-hoc before and after comparisons in the uncontrolled E05-001 extension study and there was still concern about the lack of long-term clinical effectiveness data.

The PBAC considered that the evidence presented was not sufficient to support the claim of similar safety of eculizumab to best supportive care due to the lack of long term placebo controlled trials. The increased risk of meningococcal infection with eculizumab treatment remained a concern, especially the increased risk of contracting meningococcal strain B infection for which there is no vaccine available. Although the submission claimed that eculizumab reduced PNH related fatigue, the potential for patients to experience fatigue as an adverse effect of treatment with eculizumab was noted, as fatigue was acknowledged in the submission as a moderate to severe symptom of PNH which causes significant patient distress.

The Committee considered that, with an annual drug cost approaching $500,000 per year, the incremental cost per additional death avoided was unacceptably high and uncertain. The utilisation of eculizumab for PNH was also uncertain as the extent of under diagnosis of PNH

Samsung Bioepis Exhibit No. 1002
Page 2665

in Australia is unclear and there was uncertainty in the applicability of the UK incidence and prevalence to the Australian population.

The PBAC noted the advice from the Highly Specialised Drugs Working Party that again supported listing of eculizumab under the Highly Specialised Drugs Program subject to meeting clinical and cost effectiveness criteria.

However, the PBAC rejected listing eculizumab on the PBS on the basis of an unacceptably high and highly uncertain cost-effectiveness.

In relation to the criteria for inclusion on the Life Saving Drugs Program (LSDP), the Committee accepted that PNH may shorten the lifespan of some patients suffering from the disease and that the submission's proposed link between treatment with eculizumab resulting in the reduction of thromboembolic events and an improved lifespan of some patients with PNH was not unreasonable (Criterion 2). The PBAC accepted eculizumab is safe to use in patients with bone marrow dysfunction and chronic kidney disease and hence that Criterion 4 for the LSDP was met. The Committee considered that eculizumab may be clinically effective for some patients with PNH, and hence that the drug meets Criterion 5 of the LSDP. With regard to Criterion 10 of the LSDP, the PBAC considered that the proposed funding algorithm did not clearly identify those patients with PNH who would most benefit from treatment with eculizumab, however that criteria for initiation and continuation of treatment with eculizumab could be developed to identify those patients with PNH who would benefit most from treatment with eculizumab.

The PBAC therefore considered that eculizumab meets the criteria for inclusion on the LSDP but that specific requirements for initiation and continuation of treatment would need to be developed by independent experts to identify those patients with paroxysmal nocturnal haemoglobinaemia (PNH) who would benefit most from treatment with eculizumab.

The PBAC noted that although eculizumab meets the criteria for the LSDP, the inclusion of eculizumab on LSDP would mean a new direction for that program, as the disease being treated is not a rare inherited enzyme deficiency, for which the LSDP was established. The PBAC also noted the steep escalation of costs associated with recent applications for consideration of drugs under the LSDP.

The PBAC noted that the submission meets the criteria for an Independent Review.

13. Context for Decision

The PBAC helps decide whether and, if so, how medicines should be subsidised in Australia. It considers submissions in this context. A PBAC decision not to recommend listing or not to recommend changing a listing does not represent a final PBAC view about the merits of the medicine. A company can resubmit to the PBAC or seek independent review of the PBAC decision.

14. Sponsor's Comment

The Sponsor agrees that eculizumab is an appropriate drug to be listed and funded under the Life Savings Drug Program. It is Alexion's intent to work closely with the LSDP and the Health Ministry to find rapid solutions for access to funded eculizumab therapy for patients at highest risk from this ultra-rare, destructive, progressive and life threatening disease of PNH.

We look forward to creating a unique and sustainable funding model for the LSDP and eculizumab in PNH, for all stakeholders; from Government to patients.

Samsung Bioepis Exhibit No. 1002
Page 2667

The EMBO Journal vol.13 no.1 pp.110-117, 1994

Paroxysmal nocturnal haemoglobinuria (PNH) is caused by somatic mutations in the PIG-A gene

Monica Bessler[1], Philip J.Mason[1], Peter Hillmen[1], Toshio Miyata[2], Norio Yamada[3], Junji Takeda[2], Lucio Luzzatto[1,4] and Taroh Kinoshita[2]

[1]Department of Haematology, Hammersmith Hospital, Royal Postgraduate Medical School, Du Cane Road, London W12 0NN, UK, [2]Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565 and [3]Department of Internal Medicine, The Branch Hospital, Nagoya University School of Medicine, Nagoya 461, Japan
[4]Corresponding author

Communicated by L.Luzzatto

Paroxysmal nocturnal haemoglobinuria (PNH), an acquired clonal blood disorder, is caused by the absence of glycosyl phosphatidylinositol (GPI)-anchored surface proteins due to a defect in a specific step of GPI-anchor synthesis. The cDNA of the X-linked gene, PIG-A, which encodes a protein required for this step has recently been isolated. We have carried out a molecular and functional analysis of the PIG-A gene in four cell lines deficient in GPI-linked proteins, obtained by Epstein-Barr virus (EBV) transformation of affected B-lymphocytes from PNH patients. In all four cell lines transfection with PIG-A cDNA restored normal expression of GPI-linked proteins. In three of the four cell lines the primary lesion is a frameshift mutation. In two of these there is a reduction in the amount of full-length mRNA. The fourth cell line contains a missense mutation in PIG-A. In each case the mutation was present in the affected granulocytes from peripheral blood of the patients, but not in normal sister cell lines from the same patient. These data prove that PNH is caused in most patients by a single mutation in the PIG-A gene. The nature of the mutation can vary and most likely occurs on the active X-chromosome in an early haematopoietic stem cell.

Key words: paroxysmal nocturnal haemoglobinuria/PIG-A/mRNA stability/somatic mutation

Introduction

Paroxysmal nocturnal haemoglobinuria (PNH) is an acquired haemolytic blood disorder often associated with cytopenia and recurrent venous thrombosis (Dacie and Lewis, 1972; Rotoli and Luzzatto, 1989). Previous evidence indicates that PNH results from the expansion of an abnormal clone which arises by a somatic mutation occurring in an early haematopoietic stem cell (Oni *et al.*, 1970). Cells of the abnormal clone are deficient in all cell surface proteins which are normally anchored to the membrane by a glycosyl phosphatidylinositol (GPI) molecule (De Sandre *et al.*, 1956; Nicholson-Weller *et al.*, 1983; Pangburn *et al.*, 1983; Hansch *et al.*, 1987; Selvaraj *et al.*, 1988; Rosse *et al.*, 1990). At least two of these missing proteins protect blood cells against complement attack. The deficiency in GPI-linked proteins is due to a defect in the GPI-anchor assembly in the endoplasmic reticulum. The defect has been pinpointed to the step catalysed by the α-1,6-*N*-acetylglucosaminyl-transferase (Armstrong *et al.*, 1992; Hillmen *et al.*, 1993c; Takahashi *et al.*, 1993). Recently, the cDNA corresponding to the gene responsible for this particular step in the anchor biosynthesis, named PIG-A (for phosphatidylinositol glycan complementation class A), has been isolated by expression cloning, sequenced and mapped to the X-chromosome (Miyata *et al.*, 1993; Takeda *et al.*, 1993). The PIG-A cDNA contains a 1452 bp open reading frame that encodes a putative membrane protein of 484 amino acid residues (Miyata *et al.*, 1993). Whether the PIG-A gene actually encodes the α-1,6-*N*-acetylglucosaminyltransferase is not yet certain, but in two Japanese patients with PNH it has been shown that an abnormality in the PIG-A gene is responsible for the disease. In one patient a single base pair deletion at a 5′ splice site caused abnormal splicing and a truncated mRNA. In the other patient a reduced amount of normal sized RNA was found (Takeda *et al.*, 1993).

In the present study we have analysed four cell lines deficient in GPI-anchored proteins which we have obtained by Epstein-Barr (EBV) transformation of the affected B cells from four British patients with PNH (Hillmen *et al.*, 1993b). Each one of the four cell lines has an abnormality in the PIG-A gene. In three cell lines we found frameshift mutations and two of these are associated with a severely reduced level of normal sized RNA. The fourth cell line had a point mutation in the coding region of the PIG-A gene. We have verified that these lesions are due to somatic mutations because they are not present in normal cell lines isolated from the same patients. These data confirm that mutations of the PIG-A gene are responsible for PNH in at least the majority of patients, and they show that a whole array of different mutations in the same gene can cause the PNH phenotype.

Results

Correction of the PNH phenotype by transfection of the PIG-A cDNA

We have previously obtained from patients with PNH EBV-transformed lymphoblastoid cell lines which have been fully characterized and shown to be deficient in GPI-linked proteins (Hillmen *et al.*, 1993b). Four such PNH cell lines from four different patients were transfected by electroporation with a plasmid competent for expression of PIG-A cDNA and then tested for the presence of surface CD59. Expression of this molecule was promptly restored in each case (Figure 1). When cells were grown in a hygromycin-containing medium after transfection, there was gradual enrichment for CD59-positive cells. After 20 days practically all cells had an almost normal amount of CD59 (Figure 1). The transfected cells expressing CD59 also expressed CD55

Samsung Bioepis Exhibit No. 1002
Page 2668

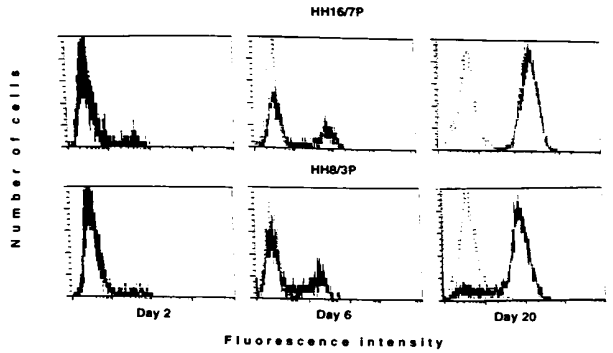


Fig. 1. CD59 expression on the surface of PNH cells after transfection with PIG-A cDNA. The two PNH cell lines HH16/7P and HH8/3P are derived from two different PNH patients. The cells were transfected with the PIG-A cDNA (continuous line) or an irrelevant control cDNA (CD8) (dotted line) on day 0. Selection for the transfectants in hygromycin-containing medium was started on day 2. CD59 expression was determined by flow cytometry using an anti-CD59 monoclonal antibody.

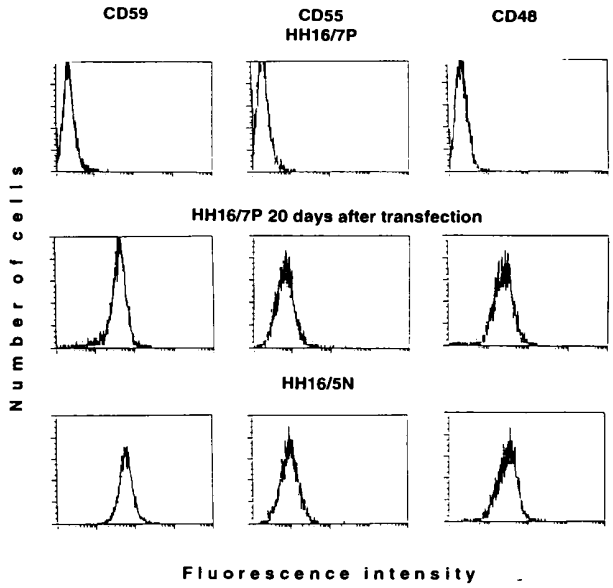


Fig. 2. Expression of several GPI-linked proteins on the surface of PNH cells before and after transfection with PIG-A cDNA and selection in hygromycin. CD59, CD55 (DAF) and CD48 on the surface of the PNH cells were determined 20 days after transfection using the appropriate monoclonal antibody and flow cytometry. Top row, untransfected cell line HH16/7P; middle row, PNH cell line HH16/7P 20 days after transfection; bottom row, the phenotypically normal sister cell line HH16/5N.

and CD48 at a level comparable with that of normal lymphoblastoid cell lines (see Figure 2).

Analysis of PIG-A transcripts in PNH cell lines

Since PIG-A fully corrects the PNH phenotype, we were satisfied that it must have been the defective molecule in these cell lines. Specifically, it seemed likely that a mutation in the PIG-A gene is responsible for the PNH phenotype. Poly(A)-enriched mRNA from each of the four cell lines was analysed by Northern blotting. For comparison we used HeLa cell RNA, RNA from a normal lymphoblastoid cell line obtained from a normal individual, as well as RNA from

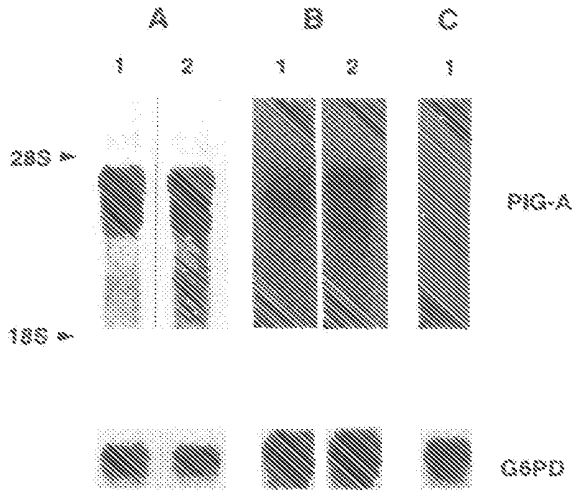


Fig. 3. Northern blot analysis of PIG-A mRNA in normal and PNH lymphoblastoid cell lines. Ten micrograms of poly(A)-enriched RNA were applied to each lane. Filters were hybridized with the PIG-A cDNA probe. The films were exposed at −80°C for 14 days. The same filters were subsequently hybridized to a G6PD cDNA probe (Persico *et al.*, 1986) and exposed overnight. Lanes 1A, 1B and 1C: mRNA from the PNH cell lines HH16/7P, HH8/3P and HH5/7P. Lanes 2A and 2B: RNA from the corresponding phenotypically normal sister cell lines. (No phenotypically normal cell line was available from patient HH5.) The position of 18S and 28S rRNA bands is shown on the left.

normal lymphoblastoid cell lines obtained from three of the four patients from whom the PNH cell lines were derived.

In normal cells we found three different mRNA species. Based on the known sizes of 28S (Conzalez *et al.*, 1985) and 18S RNAs (McCallum *et al.*, 1985), we estimate their sizes to be 3.8, 3.3 and 2.8 kb, respectively (see Figure 3). In the PNH cell lines HH20/9P and HH16/7P, the mRNA patterns were indistinguishable from those of their normal sister cell lines HH20/17N and HH16/5N or of lymphoblastoid cells from a normal person and of HeLa cells. On the other hand, the PNH cell line HH8/3P showed a sharply different pattern from that of its normal sister cell line HH8/4N. Although all three mRNA species were present, their relative abundance was markedly altered. Specifically, the medium-size (3.3 kb) mRNA, hardly visible in the normal cell line, was the major mRNA species in the PNH cell line; by contrast, the largest (3.8 kb) was barely visible (Figure 3). The PNH cell line HH5/7P (for which we do not have a normal sister cell line) has yet a different pattern: here the 3.8 kb mRNA is missing altogether, whereas the two smaller mRNA species are present in apparently normal amounts (Figure 3).

In order to further define the abnormalities of PIG-A mRNA, this was reverse transcribed and the coding region was amplified by polymerase chain reaction (PCR). From RNA of two normal lymphoblastoid cell lines obtained from normal persons and from HeLa cells, as well as from all three phenotypically normal lymphoblastoid cell lines obtained from three of our PNH patients, three major amplification products were detected using primers 11 and 12. The largest amplification product has the predicted size of the coding region (1500 bp); two additional products were ~1200 and 800 bp in size, respectively (Figure 4, lane 3). Sequence analysis showed that the largest band was the

Samsung Bioepis Exhibit No. 1002
Page 2669

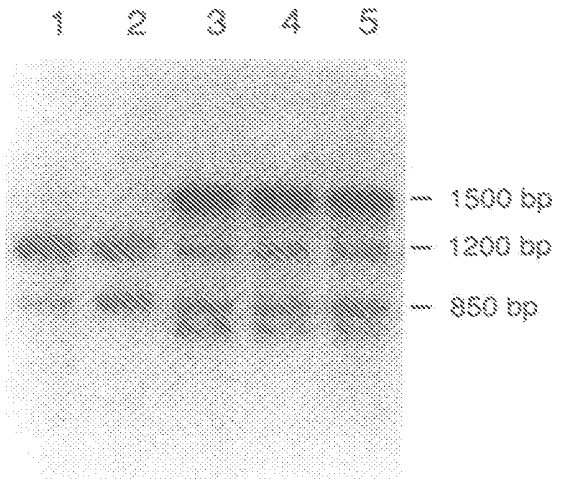


Fig. 4. Autoradiograph of RT−PCR analysis of PIG-A mRNA from PNH cell lines. The entire coding region of the PIG-A cDNA was amplified using primers 11 and 12 (Table I). The radiolabelled RT−PCR products were analysed on a 2% agarose gel. Lane 1, HH8/3P; lane 2, HH5/7P; lane 3, JY cell; lane 4, HH20/9P; lane 5, HH16/7P.

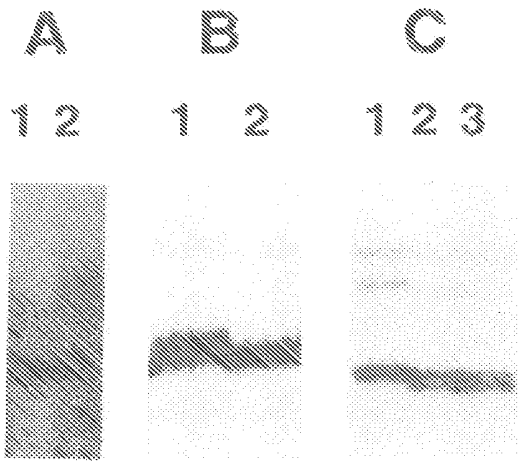


Fig. 5. SSCP analysis used to localize mutations found in PNH cell lines. (**A**) Heat-denatured 180 bp *Fok*I restriction fragment from the 3′ amplification product of the PIG-A cDNA (primers c and f, Table I) from cell lines of patient HH20. Lane 1, HH20/9P; lane 2, HH20/17N. (**B**) Non-denatured 270 bp *Hin*dIII−*Taq*I fragment of exon 2 amplified from genomic DNA (primers −I1 and I2, Table I) from lymphoblastoid cell lines of patient HH8. Lane 1, HH8/3P; lane 2, HH8/4N. (**C**) Non-denatured 340 bp *Hin*dIII−*Taq*I fragment of exon 2 amplified from genomic DNA of PNH cell line HH5/7P (lane 1), and from the patient's granulocytes (lane 2) and lymphocytes (lane 3). For patient HH20 (A), the mutation was localized by the band shift present in the denatured fragments after digestion with *Fok*I (A) and *Bst*NI (not shown). In patient HH8, who is a female PNH patient and who turned out to be heterozygous for the mutation, the two bands for the non-denatured 270 bp *Hin*dIII−*Taq*I fragment are due to different electrophoretic mobility of the heteroduplex. Heteroduplex formation is also the cause of the two bands in the non-denatured 340 bp *Hin*dIII−*Taq*I fragment from granulocytes and lymphocytes (lanes 2C and 3C) of patient HH5 (C), who is a male patient and has a mixture of normal and PNH cells in his peripheral blood.

product to be expected from the primers used, i.e. it had the sequence of PIG-A cDNA from nucleotide −36 to 1476 (numbers refer to cDNA unless stated otherwise). The second largest band had the same sequence, except for a 374 bp excision from nucleotide 342 to 715. The third band had a 658 bp excision from nucleotide 58 to 715. Analysis of the genomic structure revealed that exon 2 of the PIG-A gene extends from nucleotide −62 to nucleotide 715 (data not shown). At each of the two positions 58 and 342 we note a GT dinucleotide, which is a potential 5′ splicing site. We presume, therefore, that the three reverse transcription (RT)−PCR products reflect mRNA species arising by several alternative splicing pathways, in which portions of exon 2 are spliced out of the primary transcript. Thus, it seems likely that only the 1500 bp RT−PCR product corresponds to functional mRNA. It appears that normal cells also produce smaller, presumably non-functional mRNA species.

When RNA from our PNH cell lines was similarly subjected to RT−PCR analysis, HH20/9P and HH16/7P yielded patterns which were indistinguishable in size and relative amounts from the products of normal lymphoid cells (Figure 4, lanes 4 and 5). In contrast, RNA from cell lines HH8/3P and HH5/7P yielded only a major band of ~1200 bp and a minor band of ~800 bp, but no band of 1500 bp, corresponding to the full size of the mRNA coding region (Figure 4, lanes 1 and 2).

In order to assess the functional competence of the PIG-A mRNA from PNH cell lines, the largest cDNA products obtained by RT−PCR were cloned into the pEB expression vector and transfected into the GPI-anchor-deficient mutant cell line JY5 (originally used for expression cloning of the PIG-A cDNA; Miyata *et al.*, 1993). Transfection of the 1500 bp RT−PCR product from the JY cell line (the phenotypically normal parent cell line of JY5) restored expression of the surface antigen CD59 on JY5. By contrast, transfection of the 1500 bp product of HH20/9P and HH16/7P failed to restore CD59 expression, indicating that the apparently normal-sized mRNAs from these two cell lines are non-functional, and are therefore likely to have a mutation in the coding region. The 1200 bp cDNA products of HH8/3P and HH5/7P also failed, not surprisingly, to restore CD59 expression after transfection into the JY5 cell line.

Analysis of the molecular lesions in PNH cell lines at the DNA level

For the two cell lines HH20/9P and 16/7P, the mRNA patterns combined with the results of the transfection experiments suggested point mutations within the coding region. SSCP analysis of HH20/9P cDNA and comparison with the analysis of HH20/17N showed a band shift close to the 3′ end of the coding region. *Fok*I and *Bst*NI digests of the 700 bp sized RT−PCR amplification products localized the mutation between nucleotides 1331 and 1388. By sequence analysis we found that indeed in HH20/9P an AT doublet at position 1348−1349 is replaced by a single C.

The mutation in HH16/7P could not be located by SSCP analysis, but direct sequencing of the PCR product of exon 2 revealed a C to T replacement at position 464.

For cell lines HH8/3P and HH5/7P, which had altered mRNA patterns, SSCP analysis of *Hin*dIII/*Taq*I digests and of *Bst*XI digests of exon 2 amplified from genomic DNA showed band shifts which localized the mutation between nucleotides 228 and 425 for cell line HH8/3P, and between

Samsung Bioepis Exhibit No. 1002
Page 2670

nucleotides 505 and the 3′ end of the 996 bp amplification product for cell line HH5/7P (see Figure 5). By sequencing the entire exon 2 we found indeed that in HH8/3P a GC doublet at positions 336–337 was replaced by a single T (Figure 6A). In HH5/7P we found instead a 5 bp insertion, TAGAT, at position 634. Since the primers used (−I1 and I2; Table I) are located 54 bp 5′ and 115 bp 3′, respectively, of the intron–exon boundaries of exon 2 we were able to verify that these flanking regions, including the splice sites, had an entirely normal sequence.

The PNH phenotype results from somatic mutations

In order to confirm that the mutations observed are not constitutional, we took advantage of the normal sister cell lines and of fresh patient material when available. In all three patients for whom we did obtain a PNH cell line and a phenotypically normal cell line, the mutation present in the PNH cell line was not present in the normal sister cell line. For patient HH5 for whom no phenotypically normal cell lines were available, both normal and mutated genes were shown to be present in DNA from the patient's mononuclear cells by sequencing M13 clones. HH8 was a female patient, and the cell line HH8/3P was found by sequence analysis to be heterozygous for the mutation described. This mutation predicts loss of a *Dde*I restriction site, and this was shown to be missing in the PNH cell line HH8/3P (thus also ruling out a possible PCR artefact), whereas it was retained in the patient's normal cell line HH8/4N (see Figure 7). By using the same approach, we have been able to show that all of the cDNA from the PNH cells lacks the *Dde*I site (see Figure 7), indicating that there is no detectable transcript from the normal allele.

We next confirmed the presence of the mutation in the patient's peripheral blood granulocytes. When the amplification product of exon 2 from granulocyte genomic DNA was cloned into M13, two out of 14 clones sequenced showed the nucleotide change at position 336, whereas the remaining 12 were normal. Considering that the PNH granulocytes are also heterozygous, the proportion of M13 clones with the mutation (~14%) fits reasonably well with

Table I. Oligonucleotides used in reverse transcription and PCR analysis of the PIG-A gene

Purpose	Primer sequence (5′→3′)	Designation	Position (5′→3′)	Size of the amplified product (bp)
Primer for cDNA synthesis	AATGATATAGAGGTAGCATAAC	13	1586–1565	
Amplification of portions of cDNA	ACTGGCGGCCATGGAACTCAC[a]	a	−85 to −65	641
	AAGACACACAAATGATGTGG	d	556–537	
	TTACAAACAAGCTTCTAACCGT[a]	b	497–518	518
	CTCAGGAATTCCACCAACTCT	e	1014–994	
	TCATTTTGGAAGAAGTTCG[a]	c	806–824	700
	CTTTcTAGAACTATTACAAATGTT	f	1505–1482	
	accagagctcGGTTGCTCTAAGAACTGATGTC	11	−36 to −14	1532
	accaggtaccTCTTACAATCTAGGCTTCCTTC	12	1476–1455	
Amplification of genomic exon 2	AATATCTAgATCTGGATTTGTA[a]	−I1	I1 (−75 to −54)[b]	991
	gggaaTTCTTTGCTATAATTTATAG	I2	I2 (115–134)[c]	

Lower case indicates non-PIG-A sequences designed to introduce restriction enzyme sites.
[a]5′ biotinylated.
[b]Number of bp 5′ of the intron 1–exon 2 boundary.
[c]Number of bp 3′ of the exon 2–intron 2 boundary.

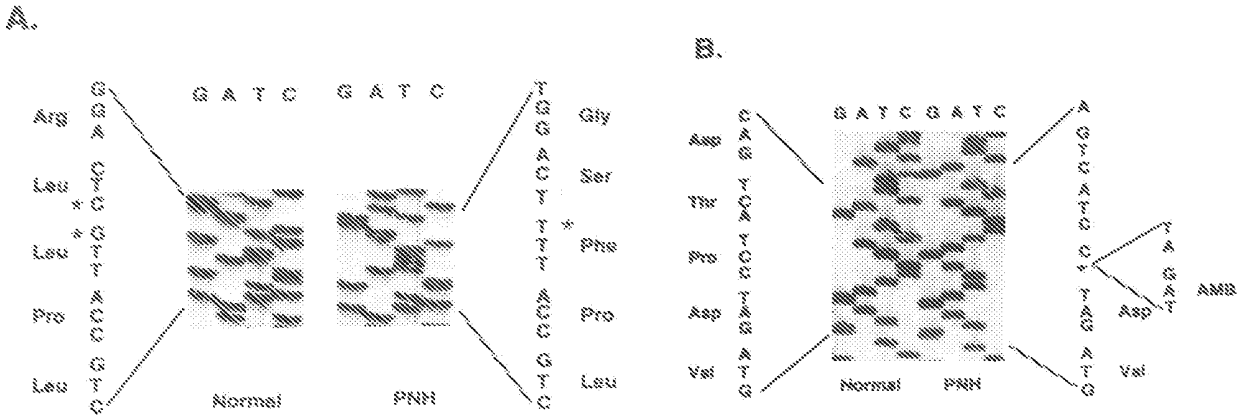


Fig. 6. Nucleotide sequence of genomic DNA encompassing the mutation found in the two PNH cell lines. (**A**) Nucleotide sequence obtained from M13 clones carrying the normal and the mutant allele in HH8/3P. (**B**) Nucleotide sequence of M13 clones obtained from HH5/7P and from the patient's normal lymphocytes.

Samsung Bioepis Exhibit No. 1002
Page 2671

the proportion of granulocytes deficient in GPI-anchored membrane proteins, previously determined to be 40% in this patient (Bessler *et al.*, 1992). A similar estimate was also obtained by visual inspection of an ethidium bromide-stained agarose gel after electrophoresis of the PCR product digested with *Dde*I (see Figure 7).

In the male patient HH5, the mutation detected in the cell line HH5/7P was also demonstrated in the patient's granulocytes (95% of which belong to the PNH clone) (Figure 6B), and in three out of six M13 clones obtained from the patient's mononuclear cells, again in good agreement with the previous finding that 55% of mononuclear cells were deficient in GPI-anchored membrane proteins.

Discussion

Expression of PIG-A regularly corrects the PNH phenotype

Transfection of our four PNH cell lines with the PIG-A cDNA restored the expression of three different GPI-linked surface proteins which were missing from these cells (Hillmen *et al.*, 1993b). It seems safe to assume that the same applies to all GPI-linked proteins, and that we have thus achieved a full correction of the PNH phenotype. We had previously shown that the absence of GPI-linked surface proteins in all of these cell lines was due to a specific block in GPI-anchor biosynthesis (Hillmen *et al.*, 1993c). The restoration of normal expression of all GPI-linked proteins we have tested thus proves that the product of the PIG-A cDNA is indeed able to relieve the block in GPI-anchor biosynthesis. Along with the data recently reported on other cell lines from two Japanese patients (Takeda *et al.*, 1993), this brings to six the total number of cases in which it has been formally proved that the absence of a normal PIG-A gene product produces the PNH phenotype.

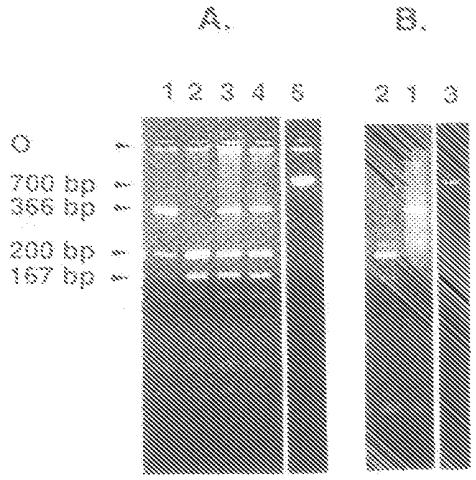


Fig. 7. Analysis of the PNH mutation in genomic DNA and in cDNA of patient material. All samples were from patient HH8. Genomic DNA was amplified using the primers $-I_1$ and d (Table I), which results in a 700 bp product (panel A, lane 5). cDNA was amplified with primers a and d (Table I), yielding a 622 bp product (panel B, lane 3). *Dde*I digestion of these products yields several fragments, the two largest being 200 and 167 bp, respectively (lane 2 in both panels A and B). Since in this patient the PNH mutation abolishes a *Dde*I site, these two fragments are replaced by a single 366 bp fragment. (**A**) Genomic DNA digested with *Dde*I, run on a 3% agarose gel and stained with ethidium bromide. Lane 1, HH8/3P (PNH cell line); lane 2, HH8/4N (normal cell line); lane 3, granulocytes; lane 4, lymphocytes; lane 5, undigested PCR product of HH8/4N. (**B**) RT–PCR products digested with *Dde*I. Lane 1, HH8/3P; lane 2, HH8/4N; lane 3, undigested RT–PCR product of HH8/4N. Analysis of the genomic DNA shows that in HH8/3P undigested and digested fragments are present (panel A, lane 1), in keeping with the cell line being heterozygous for the mutation. In granulocytes (40% PNH cells: panel A, lane 3) and lymphocytes (20% PNH cells: panel A, lane 4) the relative amounts of the restriction fragments are in agreement with the notion that the patient's PNH blood cells are heterozygous for the same mutation. Analysis of the cDNA from HH8/3P with *Dde*I shows the presence of only the undigested 366 bp fragment (panel B, lane 1), demonstrating that only the mutant allele is expressed in the PNH cell line, which is in agreement with the mutation having occurred on the active X chromosome.

Multiple mRNA species from the PIG-A gene

In order to characterize the genetic changes underlying the PNH phenotype, we first chose to analyse the PIG-A mRNA in normal cells. Northern blots of poly(A)-enriched RNA from both phenotypically normal lymphoblastoid cells and HeLa cells revealed three different mRNA species. The most prominent band was estimated to be 3.8 kb in size based on ribosomal markers (Conzalez *et al.*, 1985; McCallum *et al.*, 1985). In addition, we observed consistently a less intense band at 2.8 kb and a very faint band at 3.3 kb. [The smaller mRNA species had not been detected on Northern blots of total RNA (Miyata *et al.*, 1993; Takeda *et al.*, 1993), presumably because of their low abundance.] From comparison with the signal obtained with a housekeeping gene probe (G6PD; see Figure 3), we infer that the PIG-A mRNA has very low abundance in lymphoblastoid cells.

Although we have not formally established the correspondence between these mRNA species and the RT–PCR products, the latter technique confirms the presence of multiple mRNA species. The two smaller PCR products indicate the presence of mRNA molecules in which portions of exon 2 have been eliminated: these must arise through the use of two alternative splice sites within exon 2. From sequence analysis, we find that each of these two mRNA species has a frameshift, with the open reading frame being terminated by a stop codon after only four and seven codons, respectively.

Alternative splicing is often used to produce multiple distinct mRNAs from a single gene, and this may introduce functional diversity amongst the products of a single gene (Smith *et al.*, 1989; Green, 1991). This process may be regulated in a developmentally specific manner or in response to specific physiological conditions to produce stage- or condition-specific mRNAs. In some cases, when the product is not functional, alternative splicing can act as an on/off switch of a gene at a post-transcriptional level: for instance, this has been well documented for a number of *Drosophila* genes (Bingham *et al.*, 1988). In the case of the PIG-A gene, alternative splicing gives rise to several non-functional products, but whether this phenomenon has a regulatory role remains to be determined.

Heterogeneity of PIG-A mutations in PNH

Analysis of the RNA and DNA from our four PNH cell lines has revealed four different mutations, all in the PIG-A gene, causing severely diminished expression of GPI-anchored membrane proteins on the cell surface. Although in two cell lines (HH20/9P and HH16/7P) the RNA pattern was normal, cDNA from all four, inserted in an expression vector, failed to restore the synthesis of GPI-anchored protein in the test

Samsung Bioepis Exhibit No. 1002
Page 2672

cell line JY5. SSCP analysis of the amplification products obtained from cDNA and genomic DNA, followed by digestion with appropriately selected restriction enzymes, showed a band shift in three cell lines and, therefore, was a useful tool in localizing the site of the mutation in each.

In patient HH20, once the position of the mutation was localized by SSCP analysis, sequence analysis showed that an AT doublet at position 1348–1349 was replaced by a single C. The mutation results in a frameshift which causes a termination of the open reading frame after four codons. The resulting protein would be truncated at residue 454, thus lacking the last 30 C-terminal amino acids. The fact that the PNH cell line is completely deficient in GPI-anchored membrane proteins suggests that this putative protein is not functional. This assumption is supported by the transfection experiment which failed to restore the expression of GPI-anchored membrane proteins in JY5 after transfection of the PIG-A cDNA obtained from this patient's cell line. The loss of function might be due to changes in the active site. An alternative possibility is that the truncated protein is not retained in the membrane of the endoplasmic reticulum and, therefore, is not available where it is needed for GPI-anchor synthesis.

In patient HH16, direct sequencing of exon 2 revealed a C to T change at position 464, resulting in the substitution of phenylalanine for serine at amino acid position 155. The fact that the PNH phenotype does indeed result from this amino acid replacement was proven by its absence in the normal sister cell line and by the functional transfection assay. Presumably, this single amino acid replacement causes functional inactivation of PIG-A.

The PNH cell lines of patients HH8 and HH5 showed abnormal mRNA patterns by Northern blot and RT–PCR analysis. In HH8/3P, we found that a GC doublet was replaced by a single T at position 336. Although the mutation is only 6 bp 5′ to the alternative splice at position 342, it does not affect the 5′ consensus (Oshima and Gotoh, 1987). It does, however, produce a frameshift and termination of the open reading frame after 11 codons in the full-length PIG-A mRNA. On the other hand, the mutation produces an open reading frame of 1080 bp in the second largest transcript. Since the PNH cell line lacked the expression of GPI-anchored proteins and no expression of CD59 was achieved after transfection of the 1200 bp RT–PCR product into the JY5 cells, the putative protein of 360 amino acids cannot have any activity with respect to GPI-anchor synthesis.

In the PNH cell line of patient HH5, a 5 bp insertion (TAGAT) at position 634 also produces a frameshift and inserts an in-frame stop codon. The insertion is 81 nucleotides upstream of the 3′ end of exon 2, and therefore is unlikely to affect splicing. The 1200 and 800 bp RT–PCR products, the only ones found in this cell line, are unaffected by the mutation since the 3′ end of exon 2, which includes position 634, is spliced out in both of the smaller products.

In patient HH8, the mutation we have identified might alter splicing because it is very close to the splicing site, and altered efficiency of splicing by a mutation not very close to a splice site (as in patient HH5) has been reported (Smith *et al.*, 1989). However, an alternative and perhaps more attractive explanation of the abnormal RNA patterns observed in these two cell lines is that premature termination of translation decreases the stability of the PIG-A mRNA (Urlaub *et al.*, 1989). This would explain why the two smaller transcripts of normal lymphoid cell lines, which have stop codons, have low abundance; at the same time, it would explain the reduced amount or complete lack of the full-length transcript in HH8/3P and HH5/7P. Low abundance of a mutated RNA due to an early stop codon has been well documented for β-globin mRNA in certain cases of thalassaemia (Baserga and Benz, 1988). By the same mechanism, restoration of the reading frame in the mRNA corresponding to the second RT–PCR product in HH8/3P would explain the prominent middle-sized mRNA species seen in the Northern blot (Figure 3). It is interesting that, by contrast, the premature termination of transcription did not markedly alter the relative abundance of the largest sized RNA species in patient HH20, presumably because here the abnormal stop codon is near the normal stop codon. Thus, analysis of the molecular defect in these four PNH patients has revealed in each a different mutation within the coding region of the PIG-A gene, and all of these mutations are different from the previously reported mutation found in a Japanese patient, which was in a 5′ splice site and prevented production of full-length mRNA.

Somatic mutations in the PIG-A gene are responsible for PNH in all patients tested thus far

With the single exception of the patient suffering from a similar condition as a result of an inherited CD59 deficiency, in all patients with PNH the mutation is always in the PIG-A gene. The fact that the genetic lesions found in PNH are heterogeneous is not surprising, since we are dealing with somatic rather than inherited mutations. The most likely explanation for the mutation being always in the PIG-A gene is that, as previously suggested by somatic cell hybridization experiments (Hillmen *et al.*, 1993a), and proven by direct mapping (Takeda *et al.*, 1993), the PIG-A gene is on the X chromosome. Thus, deficiency of other enzymes involved in the GPI-anchor biosynthetic pathway, which are probably encoded in autosomal genes, would require inactivating mutations of both alleles, whereas with PIG-A just one inactivating mutation is sufficient. Whereas with inherited mutations X-linkage is classically associated with a much higher frequency of disease in males than in females, this is not true in the case of PNH. The explanation is clearly that at the somatic cell level, as a result of X chromosome inactivation, there is only one active X in each cell in both males and females. The PIG-A gene maps on the short arm of the X, but not within the pseudo-autosomal region, and the data reported in two cases (Takeda *et al.*, 1993 and patient HH8, see Figure 7) indicate that PIG-A is subject to inactivation in female somatic cells. Thus, the PNH mutation must have taken place on the active X chromosome after X chromosome inactivation, and therefore it is responsible for the disease in these patients.

Previous evidence suggested that PNH is due to the expansion of an abnormal cell clone thought to arise by a somatic mutation in an early haematopoietic stem cell (Oni *et al.*, 1970; Dacie and Lewis, 1972; Rotoli *et al.*, 1984). More recent biochemical studies suggested that a single metabolic block might be responsible for PNH in the majority of patients (Armstrong *et al.*, 1992; Hillmen *et al.*, 1993c; Takahashi *et al.*, 1993), consistent with complementation analysis using somatic cell fusion between GPI-anchor-deficient mutant mouse thymoma cell lines (Thy-1

Samsung Bioepis Exhibit No. 1002
Page 2673

mutants) and lymphoblastoid cell lines obtained from PNH patients (Armstrong *et al.*, 1992; Takahashi *et al.*, 1993). Somatic cell hybridization between normal and PNH lymphoblastoid cell lines showed that the mutant allele was recessive (Hillmen *et al.*, 1993a). This work, together with the previous work by Takeda *et al.* (1993), now proves directly the somatic mutation theory of the pathogenesis of PNH, because the mutations in the PIG-A gene found in PNH cell lines are not present in normal sister cell lines from the same patients or in the normal cells from the patient's peripheral blood.

Materials and methods

Cell lines

The production of the B-lymphoblastoid cell lines from the PNH patients (normal: HH20/17N, HH16/5N, and HH8/4N; PNH HH20/9P, HH16/7P, HH5/7P and HH8/3P) has been described previously (Hillmen *et al.*, 1993b). The lymphoblastoid cell line JY and its GPI-deficient mutant JY5 (Holander *et al.*, 1988) were also used. The cell lines were cultured in RPMI 1640 medium containing 10% fetal calf serum.

Transfection with PIG-A cDNA

Full-length cDNA was cloned into the EBV-based mammalian expression vector pEB (Miyata *et al.*, 1993). The PIG-A cDNAs prepared by RT–PCR (see below) were also cloned into the pEB vector. Ten micrograms of the pEB containing the PIG-A cDNA were transfected into $1-3 \times 10^7$ lymphoblastoid cells by electroporation. The electroporation was carried out with a Gene-PulserTM₁ (Bio-Rad) at 220–250 V and 960 μFD in 0.8 ml culture medium. To select for transfectants, 50–200 μg/ml hygromycin B (Calbiochem) were added to the culture medium 2 days after transfection. Dead cells were removed repetitively by centrifugation over a Ficoll gradient. Transfection conditions and hygromycin concentration varied for each cell line and were optimized previously. A cDNA encoding CD8, an antigen usually not present on B-cells, in a similar vector [EBO-pcD-Leu2 (Margolskee *et al.*, 1988) generously provided by R.F.Margolskee, Roche Institute of Molecular Biology, Nutley, NJ] was used as a control plasmid.

Analysis of GPI-anchored membrane proteins

Quantitation of the expression of GPI-linked membrane proteins was performed by flow cytometry (FACscan, Becton Dickinson) using monoclonal antibodies towards CD59 (Bric229, Bioproducts Laboratories), CD55 (IA10, a gift from Prof. V.Nussenzwig, Department of Pathology, NYU Medical Center, New York, NY), CD48 (LoMN25, a gift from Dr B.De La Parra, University of Louvain, Louvain, Belgium) as described previously (Hillmen *et al.*, 1993b).

Analysis of PIG-A mRNA

Total RNA was isolated using the guanidine isothiocyanate method (Chirgwin *et al.*, 1979). Poly(A)$^+$ RNA was obtained by oligo dT-cellulose column chromatography (Collaborative Research Incorporated). Northern blots were prepared using formaldehyde-containing gels (Mason *et al.*, 1993). The blots were probed with the PIG-A cDNA probe. This probe corresponds to nucleotides −76 to 2606 of the PIG-A cDNA (Takeda *et al.*, 1993).

Generation of cDNA-PCR products for functional analysis and gel analysis

Total RNA (4 μg) was reverse transcribed using 50 pmol of primer 13 (Table I) with 200 U of RNase H-free reverse transcriptase (SuperscriptTM₁ Gibco BRL) in 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 100 μg/ml bovine serum albumin (BSA) in a total volume of 20 μl. The coding region of PIG-A was amplified from the cDNA with 50 pmol of each primer 11 and 12 (Table I) in 50 μl of 10 mM Tris–Cl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatine and 25 μM dNTP by 25 cycles of a reaction consisting of incubation for 1 min at 93°C, 1 min at 55°C and 3 min at 72°C; 10 μCi [α-^{32}P]dCTP (Amersham) were used to radiolabel PCR products. The PCR products were analysed by electrophoresis in 2% agarose gel, followed by autoradiography.

Generation of cDNA-PCR products for SSCP analysis and sequencing

Total RNA (2 μg) was used in the reverse transcriptase reaction. Reverse transcription was performed for 2 h at 37°C in 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DDT, 1 mM each of dATP, dCTP, dTTP and dGTP, and 105 μg/ml random hexamer primer (pd(N)$_6$, Pharmacia) in a total volume of 40 μl using 300 U of reverse transcriptase M-MLV RT (Gibco BRL) and 30 U of ribonuclease inhibitor RNasin (Promega). The reaction was terminated by incubation at 65°C for 10 min and centrifugation for 2 min at 4°C. Three microlitres of cDNA were used for each 100 μl PCR reaction.

DNA amplification

DNA was prepared by the SDS/Proteinase K extraction procedure (Sambrook *et al.*, 1989). For the amplification of exon 2 from genomic DNA, two intronic primers were chosen (Table I). The primers used to amplify the 1452 bp coding region of the PIG-A cDNA are shown in Table I. The PCR was performed in a total volume of 100 μl containing 0.5 mM dNTPs, 100 ng of each primer, 2 U of Taq DNA polymerase (Boehringer Mannheim), 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris–Cl (pH 8.8), 6.7 mM $MgCl_2$, 10 μg gelatine and 10 mM β-mercaptoethanol. The amplification reaction was carried out using a Perkin Elmer Cetus thermal cycler (Perkin Elmer Cetus Instruments). A 30 cycle protocol was used with denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 3 min. Reactions were electrophoresed in a 1–1.5% agarose gel, stained in ethidium bromide and visualized on a UV transluminator.

Analysis by single-strand conformation polymorphism

The entire exon 2 amplified from genomic DNA, including the flanking intron–exon boundaries and the entire coding region of the PIG-A cDNA amplified in three portions using the appropriate cDNA as template (Table I), were screened for mutation by SSCP analysis. Fragments were labelled during the amplification reaction by adding 0.1 μl of [α-^{32}P]dCTP (50 pmol, 2000 Ci/mmol) (Amersham) to each 25 μl reaction mixture. Then 5 μl of each amplified sample were digested with the combination of restriction enzymes so that the entire coding region was resolved into several overlapping fragments of a size between 100 and 400 bp. After digestion, 5 μl of the digest were withdrawn and mixed with an equal volume of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.5% xylene cyanol. The mixture was heat-denatured at 95°C and snap-frozen on dry ice. Four microlitres of each sample per lane before and after heat denaturation were applied to a 6% polyacrylamide gel (32 × 40 × 0.03 cm; 0.5 cm/lane) containing 90 mM Tris–borate buffer (pH 8.3) and 10% glycerol. Electrophoresis was performed at 3–6 W for 10–16 h at 25°C. The gel was dried on filter paper and exposed to Fuji RX 100 X-ray films at −80°C for 12–48 h with an intensifying screen. Whenever abnormal mobility was detected, the corresponding PCR product was further investigated by nucleic acid sequencing.

Nucleotide sequencing

When biotinylated primers were used in the PCR, the nucleotide sequence was determined by direct solid phase sequencing after obtaining single-stranded DNA by denaturation in 0.1 M sodium hydroxide and separation of the strands by the use of streptavidin-coated magnetic beads (Dynabeads® M-280) and a magnetic particle concentrator (Dyndal MPC® -E) according to the instructions of the distributor. Sequencing was performed by dideoxy chain termination using T7 DNA polymerase (Sequenase™, Biochemical Corp.). When direct sequencing was not informative, the PCR fragments were cloned into phage M13 and sequenced from single-stranded M13 DNA. In all cases exon 2 was sequenced from genomic DNA. Other portions corresponding to abnormal fragments detected by SSCP analysis were sequenced from cDNA.

Analysis of the genomic PIG-A gene

A total of 6×10^5 plaques of a human genomic library in λ2001 (prepared from the Jurkat T-cell line) were screened with the same probe used in the Northern analysis. Two different phages hybridizing to the PIG-A probe were isolated and mapped by restriction enzyme digestion. A 1.2 kb *Hin*dIII fragment and a 2.3 kb *Hin*dIII–*Eco*RI fragment that contained nucleotides −62 to 715 of the cDNA without interruption by any intron sequences were subcloned into pUC12 and 13, and partially sequenced in order to obtain the intron–exon boundaries of exon 2. Exon 1, containing the first 23 nucleotides of the 5′ end of the published cDNA sequence, was located in a 3.2 kb *Hin*dIII fragment 5′ to exon 2.

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Samsung Bioepis Exhibit No. 1002
Page 2674

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Samsung Bioepis Exhibit No. 1002
Page 2675

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Samsung Bioepis Exhibit No. 1002
Page 2676

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TYSABRI® (natalizumab)

DESCRIPTION

TYSABRI® (natalizumab) is a recombinant humanized IgG4κ monoclonal antibody produced in murine myeloma cells. Natalizumab contains human framework regions and the complementarity-determining regions of a murine antibody that binds to α4-integrin. The molecular weight of natalizumab is 149 kilodaltons. TYSABRI® is supplied as a sterile, colorless, and clear to slightly opalescent concentrate for intravenous (IV) infusion.

Each 15 mL dose contains 300 mg natalizumab; 123 mg sodium chloride, USP; 17.0 mg sodium phosphate, monobasic, monohydrate, USP; 7.24 mg sodium phosphate, dibasic, heptahydrate, USP; 3.0 mg polysorbate 80, USP/NF, in water for injection, USP at pH 6.1.

CLINICAL PHARMACOLOGY

General

TYSABRI® binds to the α4-subunit of α4β1 and α4β7 integrins expressed on the surface of all leukocytes except neutrophils, and inhibits the α4-mediated adhesion of leukocytes to their counter-receptor(s). The receptors for the α4 family of integrins include vascular cell adhesion molecule-1 (VCAM-1), which is expressed on activated vascular endothelium, and mucosal addressin cell adhesion molecule-1 (MadCAM-1) present on vascular endothelial cells of the gastrointestinal tract. Disruption of these molecular interactions prevents transmigration of leukocytes across the endothelium into inflamed parenchymal tissue. *In vitro*, anti-α4-integrin antibodies also block α4-mediated cell binding to ligands such as osteopontin and an alternatively spliced domain of fibronectin, connecting segment-1 (CS-1). *In vivo*, TYSABRI® may further act to inhibit the interaction of α4-expressing leukocytes with their ligand(s) in the extracellular matrix and on parenchymal cells, thereby inhibiting further recruitment and inflammatory activity of activated immune cells.

The specific mechanism(s) by which TYSABRI® exerts its effects in multiple sclerosis have not been fully defined. In multiple sclerosis, lesions are believed to occur when activated inflammatory cells, including T-lymphocytes, cross the blood-brain barrier (BBB). Leukocyte migration across the BBB involves interaction between adhesion molecules on inflammatory cells, and their counter-receptors present on endothelial cells of the vessel wall. The clinical effect of natalizumab in multiple sclerosis may be secondary to blockade of the molecular interaction of α4β1-integrin expressed by inflammatory cells with VCAM-1 on vascular endothelial cells, and with CS-1 and/or osteopontin expressed by parenchymal cells in the brain. Data from an experimental autoimmune encephalitis animal model of multiple sclerosis demonstrate reduction of leukocyte migration into brain parenchyma and reduction of plaque formation detected by magnetic resonance imaging (MRI) following repeated administration of natalizumab. The clinical significance of these animal data is unknown.

Pharmacokinetics

Following the repeat intravenous administration of a 300 mg dose of natalizumab to multiple sclerosis

Samsung Bioepis Exhibit No. 1002
Page 2678

patients, the mean maximum observed serum concentration was 98 ± 34 mcg/mL. Mean average steady-state natalizumab concentrations over the dosing period were approximately 30 mcg/mL. The mean half-life of 11 ± 4 days was observed with a clearance of 16 ± 5 mL/hour. The distribution volume of 5.7 ± 1.9 L was consistent with plasma volume.

Pharmacokinetics of TYSABRI® in pediatric multiple sclerosis patients or patients with renal or hepatic insufficiency have not been studied.

Pharmacodynamics

TYSABRI® administration increases the number of circulating leukocytes, (including lymphocytes, monocytes, basophils, and eosinophils) due to inhibition of transmigration out of the vascular space. TYSABRI® does not affect the number of circulating neutrophils **(see PRECAUTIONS, Laboratory Tests)**.

CLINICAL STUDIES

TYSABRI® was evaluated in two ongoing randomized, double-blind, placebo-controlled trials in patients with multiple sclerosis. Both studies enrolled patients who experienced at least one clinical relapse during the prior year and had a Kurtzke Expanded Disability Status Scale (EDSS) score between 0 and 5.0.

In both studies, neurological evaluations were performed every 12 weeks and at times of suspected relapse. Magnetic resonance imaging evaluations for T1-weighted gadolinium (Gd)-enhancing lesions and T2-hyperintense lesions were performed annually.

Study 1 enrolled patients who had not received any interferon-beta or glatiramer acetate for at least the previous 6 months; approximately 94% had never been treated with these agents. Median age was 37, with a median disease duration of 5 years. Patients were randomized in a 2:1 ratio to receive TYSABRI® 300 mg IV infusion (n=627) or placebo (n=315) every 4 weeks for up to 28 months.

Study 2 enrolled patients who had experienced one or more relapses while on treatment with AVONEX® (Interferon beta-1a) 30 mcg intramuscularly (IM) once weekly during the year prior to study entry. Median age was 39, with a median disease duration of 7 years. Patients were evenly randomized to receive TYSABRI® 300 mg (n=589) or placebo (n=582) every 4 weeks for up to 28 months. All patients continued to receive AVONEX® 30 mcg IM once weekly.

Results for each study were analyzed at a pre-specified time and are shown in Tables 1 and 2. Median patient time on study was 13 months in both studies. Safety and efficacy of treatment with TYSABRI® beyond one year are not known.

The exact relationship between MRI findings and the clinical status of patients is unknown. Changes in lesion area often do not correlate with changes in disability progression. The prognostic significance of the MRI findings in these studies has not been evaluated.

Samsung Bioepis Exhibit No. 1002
Page 2679

Table 1. 13-Month Clinical and 1-Year MRI Endpoints in Study 1 (Monotherapy Study)

	TYSABRI® n=627	**Placebo** n=315
Clinical Endpoints		
Annualized relapse rate	0.25	0.74
Relative reduction (percentage)	66%	
Percentage of patients remaining relapse-free	76%	53%
MRI Endpoints		
New or newly enlarging T2-hyperintense lesions		
Median	0.0	3.0
Percentage of patients with:		
0 lesions	60%	22%
1 lesion	18%	13%
2 lesions	6%	7%
3 or more lesions	16%	58%
Gd-enhancing lesions		
Median	0.0	0.0
Percentage of patients with:		
0 lesions	96%	68%
1 lesion	3%	13%
2 or more lesions	1%	19%

All analyses were intent-to-treat. For each endpoint, $p<0.001$. Determination of p-values: relapse rate by Poisson regression adjusting for baseline relapse rate, EDSS, presence of Gd-enhancing lesions, age; percentage relapse-free by logistic regression adjusting for baseline relapse rate; and MRI endpoints by ordinal logistic regression adjusting for baseline lesion number.

Table 2. 13-Month Clinical and 1-Year MRI Endpoints in Study 2 (Add-On Study)

	TYSABRI® plus AVONEX® n=589	**Placebo plus AVONEX®** n=582
Clinical Endpoints		
Annualized relapse rate	0.36	0.78
Relative reduction (percentage)	54%	
Percentage of patients remaining relapse-free	67%	46%

Samsung Bioepis Exhibit No. 1002
Page 2680

	TYSABRI® plus AVONEX® n=589	Placebo plus AVONEX® n=582
MRI Endpoints		
New or newly enlarging T2-hyperintense lesions		
Median	0.0	1.0
Percentage of patients with:		
0 lesions	67%	40%
1 lesion	26%	29%
2 lesions	4%	10%
3 or more lesions	3%	21%
Gd-enhancing lesions		
Median	0.0	0.0
Percentage of patients with:		
0 lesions	96%	76%
1 lesion	3%	12%
2 or more lesions	1%	12%

All analyses were intent-to-treat. For each endpoint, $p<0.001$. Determination of p-values: relapse rate by Poisson regression adjusting for baseline relapse rate, EDSS, presence of Gd-enhancing lesions, age; percentage relapse-free by logistic regression adjusting for baseline relapse rate; and MRI endpoints by ordinal logistic regression adjusting for baseline lesion number.

INDICATIONS AND USAGE

TYSABRI® is indicated for the treatment of patients with relapsing forms of multiple sclerosis to reduce the frequency of clinical exacerbations. This indication is based on results achieved after approximately one year of treatment in ongoing controlled trials of two years in duration. The safety and efficacy of TYSABRI® beyond one year are unknown.

Safety and efficacy in patients with chronic progressive multiple sclerosis have not been established.

CONTRAINDICATIONS

TYSABRI® should not be administered to patients with known hypersensitivity to TYSABRI® or any of its components.

WARNINGS

Hypersensitivity

TYSABRI® has been associated with hypersensitivity reactions, including serious systemic reactions (e.g., anaphylaxis) which occurred at an incidence of <1%. These reactions usually occur within 2 hours of the start of the infusion. Symptoms associated with these reactions can include urticaria, dizziness, fever, rash, rigors, pruritus, nausea, flushing, hypotension, dyspnea, and chest pain. Generally, these reactions are associated with antibodies to TYSABRI®.

Samsung Bioepis Exhibit No. 1002
Page 2681

If a hypersensitivity reaction occurs, discontinue administration of TYSABRI® and initiate appropriate therapy **(see ADVERSE REACTIONS, Infusion-related Reactions)**. Patients who have experienced a hypersensitivity reaction should not be re-treated with TYSABRI®. The possibility of antibodies to TYSABRI® should be considered in patients who have hypersensitivity reactions **(see ADVERSE REACTIONS, Immunogenicity)**.

PRECAUTIONS

Immunosuppression

In Studies 1 and 2, concomitant treatment of relapses with a short course of corticosteroids was not associated with an increased rate of infection. The safety and efficacy of TYSABRI® in combination with other immunosuppressive agents have not been evaluated. Patients receiving these agents should not receive concurrent therapy with TYSABRI® because of the possibility of increased risk of infections.

Information to Patients

If patients experience symptoms consistent with a hypersensitivity reaction (e.g., urticaria with or without associated symptoms) during or following an infusion of TYSABRI®, they should report these symptoms to their physician immediately **(see WARNINGS, Hypersensitivity)**.

Laboratory Tests

TYSABRI® induces increases in circulating lymphocytes, monocytes, eosinophils, basophils, and nucleated red blood cells. Observed increases persist during TYSABRI® exposure, but are reversible, returning to baseline levels usually within 16 weeks after the last dose. Elevations of neutrophils are not observed.

Drug Interactions

After multiple dosing, interferon beta-1a (AVONEX® 30 mcg IM once weekly) reduced TYSABRI® clearance by approximately 30%. The similarity of the TYSABRI®-associated adverse event profile between Study 1 (without co-administered AVONEX®) and Study 2 (with co-administered AVONEX®) indicates that this alteration in clearance does not necessitate reduction of the TYSABRI® dose to maintain safety **(see ADVERSE REACTIONS, General)**.

Results of studies in multiple sclerosis patients taking TYSABRI® and concomitant interferon beta-1a (AVONEX® 30 mcg IM once weekly) or glatiramer acetate were inconclusive with regard to the need for dose adjustment of the beta-interferon or glatiramer acetate.

Carcinogenesis, Mutagenesis, and Impairment of Fertility

No clastogenic or mutagenic effects of natalizumab were observed in the Ames or human chromosomal aberration assays. Natalizumab showed no effects on *in vitro* assays of α4-integrin positive tumor line proliferation/cytotoxicity. Xenograft transplantation models in SCID and nude

Samsung Bioepis Exhibit No. 1002
Page 2682

mice with two α4-integrin positive tumor lines (leukemia, melanoma) demonstrated no increase in tumor growth rates or metastasis resulting from natalizumab treatment.

Reductions in female guinea pig fertility were observed in one study at dose levels of 30 mg/kg, but not at the 10 mg/kg dose level (2.3-fold the clinical dose). A 47% reduction in pregnancy rate was observed in guinea pigs receiving 30 mg/kg relative to control. Implantations were seen in only 36% of animals having corpora lutea in the 30 mg/kg group versus 66-72% in the other groups. Natalizumab did not affect male fertility at doses up to 7-fold the clinical dose.

Pregnancy (Category C)

In reproductive studies in monkeys and guinea pigs, there was no evidence of teratogenic effects at doses up to 30 mg/kg (7 times the human clinical dose based on a body weight comparison). In one study where female guinea pigs were exposed to natalizumab during the second half of pregnancy, a small reduction in pup survival was noted at post-natal day 14 with respect to control (3 pups/litter for the group treated with 30 mg/kg natalizumab and 4.3 pups/litter for the control group). In one of five studies that exposed monkeys or guinea pigs during pregnancy, the number of abortions in treated (30 mg/kg) monkeys was 33% versus 17% in controls. No effects on abortion rates were noted in any other study. TYSABRI® underwent trans-placental transfer and produced *in utero* exposure in developing guinea pigs and cynomolgus monkeys. When pregnant dams were exposed to natalizumab at approximately 7-fold the clinical dose, serum levels in fetal animals at delivery were approximately 35% of maternal serum natalizumab levels. A study in pregnant cynomolgus monkeys treated at 2.3-fold the clinical dose demonstrated natalizumab-related changes in the fetus. These changes included mild anemia, reduced platelet count, increased spleen weights, and reduced liver and thymus weights associated with increased splenic extramedullary hematopoiesis, thymic atrophy, and decreased hepatic hematopoiesis. In offspring born to mothers treated with natalizumab at 7-fold the clinical dose, platelet counts were also reduced. This effect was reversed upon clearance of natalizumab. There was no evidence of anemia in these offspring.

There are no adequate and well-controlled studies of TYSABRI® therapy in pregnant women. Because animal reproduction studies are not always predictive of human response, this drug should be used during pregnancy only if clearly needed. If a woman becomes pregnant while taking TYSABRI®, discontinuation of TYSABRI® should be considered.

Nursing Mothers

It is not known whether TYSABRI® is excreted in human milk. Because many drugs and immunoglobulins are excreted in human milk, and because the potential for serious adverse reactions is unknown, a decision should be made whether to discontinue nursing or TYSABRI® taking into account the importance of therapy to the mother.

Geriatric Use

Clinical studies of TYSABRI® did not include sufficient numbers of patients aged 65 years and over to determine whether they respond differently than younger patients.

Samsung Bioepis Exhibit No. 1002
Page 2683

Pediatric Use

Safety and effectiveness of TYSABRI® in pediatric multiple sclerosis patients below the age of 18 have not been studied. TYSABRI® is not indicated for use in pediatric patients.

Immunizations

No data are available on the effects of vaccination in patients receiving TYSABRI®. No data are available on the secondary transmission of infection by live vaccines in patients receiving TYSABRI®.

ADVERSE REACTIONS

General

The most frequently reported serious adverse reactions with TYSABRI® were infections (2.1% versus 1.3% in placebo, including pneumonia [0.6%]), hypersensitivity reactions (1.3%, including anaphylaxis/anaphylactoid reaction [0.8%]), depression (0.8%, including suicidal ideation [0.5%]), and cholelithiasis (0.8%) **(see WARNINGS, Hypersensitivity and ADVERSE REACTIONS, Infections)**.

The most frequently reported adverse reactions resulting in clinical intervention (i.e., discontinuation of TYSABRI®), were urticaria (1%) and other hypersensitivity reactions (1%) **(see WARNINGS, Hypersensitivity)**.

Because clinical trials are conducted under widely varying and controlled conditions, adverse reaction rates observed in clinical trials of TYSABRI® cannot be directly compared to rates in the clinical trials of other drugs and may not reflect the rates observed in practice. The adverse reaction information does, however, provide a basis for identifying the adverse events that appear to be related to drug use and a basis for approximating rates.

A total of 1,617 multiple sclerosis patients, in both controlled and uncontrolled studies, have been exposed to TYSABRI® with a median duration of exposure of 20 months.

Table 3 enumerates adverse events and selected laboratory abnormalities that occurred in Study 1 at an incidence of at least 1 percentage point higher in TYSABRI®-treated patients than was observed in the placebo group. The adverse event profile in Study 2 was similar.

Samsung Bioepis Exhibit No. 1002
Page 2684

Table 3. Adverse Reactions in Study 1

Adverse Events (Preferred Term)	TYSABRI® n=627 Percentage	Control n=312 Percentage
General		
Headache	35%	30%
Fatigue	24%	18%
Arthralgia	15%	11%
Allergic reaction	7%	3%
Urinary urgency/frequency	7%	5%
Chest discomfort	4%	2%
Local bleeding	3%	1%
Rigors	3%	1%
Syncope	2%	1%
Infection		
Urinary tract infection	18%	15%
Lower respiratory tract infection	15%	14%
Gastroenteritis	9%	5%
Vaginitis*	8%	5%
Tonsillitis	5%	3%
Psychiatric		
Depression	17%	14%
Gastrointestinal		
Abdominal discomfort	10%	9%
Abnormal liver function test	5%	3%
Skin		
Rash	9%	7%
Dermatitis	5%	4%
Pruritus	4%	2%
Menstrual disorders*		
Irregular menstruation/dysmenorrhea	7%	2%
Amenorrhea	2%	0%
Neurologic		
Tremor	3%	2%

*percentage based on female n

Samsung Bioepis Exhibit No. 1002
Page 2685

Infections

In Studies 1 and 2, the rate of infection was approximately 1 per patient-year in both TYSABRI®-treated patients and placebo-treated patients. The infections were predominately upper respiratory tract infections, influenza, and urinary tract infections. Most patients did not interrupt treatment with TYSABRI® during the infection. In Study 1, the incidence of serious infection was 2.1% in TYSABRI®-treated patients versus 1.3% in placebo-treated patients. No difference was seen between treatment groups in Study 2.

Infusion-related Reactions (see WARNINGS, Hypersensitivity)

An infusion-related reaction was defined in clinical trials as any adverse event occurring within 2 hours of the start of an infusion. Approximately 22% of TYSABRI®-treated multiple sclerosis patients experienced an infusion-related reaction, compared to 17% of placebo-treated patients. Events more common in the TYSABRI®-treated patients included headache, dizziness, fatigue, hypersensitivity reactions, urticaria, pruritus, and rigors. Acute urticaria was observed in approximately 2% of patients. Other hypersensitivity reactions were observed in 1% of patients receiving TYSABRI®. Serious systemic hypersensitivity infusion reactions occurred in <1% of patients. All patients recovered with treatment and/or discontinuation of the infusion.

Patients who became persistently positive for antibodies to TYSABRI® were more likely to have an infusion-related reaction than those who were antibody-negative **(see ADVERSE REACTIONS, Immunogenicity)**.

Immunogenicity

Patients in Study 1 and Study 2 were tested for antibodies to natalizumab every 12 weeks. The assays used in these studies were unable to detect low to moderate levels of antibodies to natalizumab. Antibodies were detected in approximately 10% of multiple sclerosis patients receiving TYSABRI® at least once during treatment with persistent antibody-positivity in 6% of patients. Approximately 90% of patients who became persistently antibody-positive by this assay had developed detectable antibodies by 12 weeks. Anti-natalizumab antibodies were neutralizing *in vitro.*

The presence of anti-natalizumab antibodies was correlated with a reduction in serum natalizumab levels. Across studies, the week 12 pre-infusion mean natalizumab serum concentrations in antibody-negative patients were approximately 17 mcg/mL compared to <1 mcg/mL in antibody-positive patients. Persistent antibody-positivity to natalizumab was associated with a substantial decrease in the effectiveness of TYSABRI®. In Study 1, the annualized relapse rate of persistently antibody-positive TYSABRI®-treated patients (0.75) was similar to the annualized relapse rate in subjects who received placebo (0.74). A similar phenomenon was also observed in Study 2.

Infusion-related reactions most often associated with persistent antibody-positivity included hypersensitivity reactions, urticaria, rigors, nausea, vomiting, and flushing. Additional adverse events more common in persistently antibody-positive patients included myalgia, hypertension,

Samsung Bioepis Exhibit No. 1002
Page 2686

dyspnea, anxiety, and tachycardia.

The long-term immunogenicity of TYSABRI® and the effects of low to moderate levels of antibody to natalizumab are unknown **(see ADVERSE REACTIONS, Infusion-related Reactions)**.

Immunogenicity data are highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of antibody-positivity in an assay may be influenced by several factors, including sample handling, timing of sample collection, concomitant medications, and underlying disease. For these reasons, comparison of the incidence of antibodies to TYSABRI® with the incidence of antibodies to other products may be misleading.

OVERDOSAGE

Safety of doses higher than 300 mg has not been adequately evaluated. The maximum amount of TYSABRI® that can be safely administered has not been determined.

DOSAGE AND ADMINISTRATION

The recommended dose of TYSABRI® is 300 mg IV infusion every four weeks. Dilute TYSABRI® concentrate 300 mg/15 mL in 100 mL 0.9% Sodium Chloride Injection, USP, and infuse over approximately one hour. Do not administer TYSABRI® as an IV push or bolus injection **(see Preparation Instructions)**.

Observe patients during the infusion and for 1 hour after the infusion is complete. Promptly discontinue the infusion upon the first observation of any signs or symptoms consistent with a hypersensitivity-type reaction **(see WARNINGS, Hypersensitivity)**.

Preparation Instructions

Use aseptic technique when preparing TYSABRI® solution for IV infusion. Each vial is intended for single use only.

TYSABRI® is a colorless, clear to slightly opalescent concentrate. Inspect the TYSABRI® vial for particulate material prior to dilution and administration. If visible particulates are observed and/or the liquid in the vial is discolored, the vial must not be used. Do not use TYSABRI® beyond the expiration date stamped on the carton or vial.

To prepare the solution, withdraw 15 mL of TYSABRI® concentrate from the vial using a sterile needle and syringe. Inject the concentrate into 100 mL 0.9% Sodium Chloride Injection, USP. No other IV diluents may be used to prepare the TYSABRI® solution.

Gently invert the TYSABRI® solution to mix completely. Do not shake. Inspect the solution visually for particulate material prior to administration.

Following dilution, infuse TYSABRI® solution immediately, or refrigerate solution at 2-8°C, and use

Samsung Bioepis Exhibit No. 1002
Page 2687

within 8 hours. If stored at 2-8°C, allow the solution to warm to room temperature prior to infusion. DO NOT FREEZE.

Administration Instructions

Infuse TYSABRI® 300 mg in 100 mL 0.9% Sodium Chloride Injection, USP over approximately one hour. After the infusion is complete, flush with 0.9% Sodium Chloride Injection, USP.

Use of filtration devices during administration has not been evaluated. Other medications should not be injected into infusion set side ports or mixed with TYSABRI®.

HOW SUPPLIED

TYSABRI® concentrate is supplied as 300 mg natalizumab in a sterile, single-use vial free of preservatives. Each package contains a single-use vial. NDC 59075-730-15

Storage

TYSABRI® single-use vials must be refrigerated between 2-8°C (36°-46°F). Do not use beyond the expiration date stamped on the carton and vial label. DO NOT SHAKE OR FREEZE. Protect from light.

If not used immediately, store the TYSABRI® solution for infusion at 2-8°C (36°-46°F). TYSABRI® solution for infusion must be administered within 8 hours of preparation.

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TYSABRI® (natalizumab)

Manufactured by:
Biogen Idec Inc.
14 Cambridge Center
Cambridge, MA 02142 USA
1-888-489-7227

Distributed by:
Elan Pharmaceuticals, Inc.
San Diego, CA 92121

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U.S. Patent Numbers: 5,840,299, 6,033,665, 6,602,503, 5,168,062, 5,385,839, 5,730,978

Samsung Bioepis Exhibit No. 1002
Page 2688

39181/USA/3 August 1996

Verluma™

Kit for the preparation of Technetium Tc 99m Nofetumomab Merpentan

For Intravenous Use Only

DESCRIPTION

Verluma™ is a kit for the preparation of Technetium Tc 99m Nofetumomab Merpentan, a diagnostic imaging agent for use by intravenous injection. Each kit contains one each of the non-radioactive components listed below, necessary to prepare one patient dose.

Container	Contents*
1	Isopropyl Alcohol, 99%, 1.5mL
2	Phenthioate Ligand (2,3,5,6-tetrafluorophenyl-4,5-bis-S-(1-ethoxyethyl)-thioacetoamidopentanoate), 0.28mg (dried)
3	Glacial Acetic Acid: 0.2 N Hydrochloric Acid 1:7 (v/v), contains 0.16mL
4	Stannous Gluconate Complex containing Sodium Gluconate, 45mg, and Stannous Chloride, Dihydrate, 0.79mg (lyophilized)
5	Reaction Vial - Empty, 10mL
6	1 M Sodium Bicarbonate Buffer, pH 10, 1mL
7	Nofetumomab (Fab fragment of murine monoclonal antibody NR-LU-10) in Phosphate Buffered Saline, 10mg in 1mL
8	Anion Exchange Column, 5mL with 0.2 µm Sterile Filter
9	Elution Vial - Empty, 10mL

* All components are sterile, pyrogen-free and do not contain preservative.

The active ingredient, Nofetumomab (Container 7), is the Fab fragment of an IgG2b murine monoclonal antibody NR-LU-10. The NR-LU-10 antibody is directed against an approximately 40kD glycoprotein antigen, that is expressed in a variety of cancers and some normal tissues. The NR-LU-10 antibody is produced by the serum-free, in-vitro fermentation of mammalian cells. Nofetumomab is derived from the enzymatic digestion of purified NR-LU-10 antibody with papain and is purified by chromatographic separation including viral inactivation and removal procedures.

The imaging agent is formed by combining the kit components with sodium pertechnetate Tc 99m (Technetium Tc 99m; see Dosage and Administration). Sterile and non-pyrogenic sodium pertechnetate Tc 99m from any generator source in 0.9% sodium chloride can be used.

Samsung Bioepis Exhibit No. 1002
Page 2689

As indicated in the directions provided, the isopropyl alcohol (Container 1) is used to dissolve the freeze-dried phenthioate ligand (Container 2). The phenthioate ligand (2,3,5,6-tetra-fluorophenyl-4,5-bis-S-(1-ethoxyethyl)-thioacetoamido-pentanoate) is involved with two reactions: complexation of the Technetium Tc 99m metal with the ligand and conjugation to Nofetumomab.

The stannous gluconate complex (Container 4) performs two functions. The stannous ion reduces the oxidation state of Technetium Tc 99m. The gluconate acts as an intermediate transfer agent to stabilize the reduced Technetium Tc 99m prior to chelation to the phenthioate ligand.

The glacial acetic acid: 0.2 N hydrochloric acid (Container 3) is used to adjust the pH to induce the transchelation reaction (transfer of reduced Technetium Tc 99m from gluconate to phenthioate).

The sodium bicarbonate buffer (Container 6) is used to adjust the Technetium Tc 99m-phenthioate ligand (active ester) reaction mixture to pH 10. The basic pH deprotonates the epsilon amino groups of the available lysine residues on Nofetumomab. The neutral amino group can then react with the active ester to form the covalent amide bond, resulting in the formation of Technetium Tc 99m Nofetumomab Merpentan.

The anion exchange column with sterile filter (Container 8) is used to retain non-protein bound radioactive species and acts to neutralize the pH. The purified diagnostic imaging agent Technetium Tc 99m Nofetumomab Merpentan is eluted from the column into the elution vial (Container 9).

The final compounded product contains 5 - 10mg of Technetium Tc 99m Nofetumomab Merpentan radiolabeled with 555-1110 MBq (15 - 30mCi) of Technetium Tc 99m diluted with 0.9% Sodium Chloride Injection, in a final volume of 15 - 20mL. The final pH for administration is 7.25 ± 0.75. The radiochemical purity must be determined as described in the instructions and must be ≥85% for patient administration.

Physical Characteristics

Technetium Tc 99m decays by isomeric transition with a physical half-life of 6.02 hours. The principal photon that is useful for detection and imaging studies is listed in Table 1.[1]

TABLE 1. PRINCIPAL RADIATION EMISSION DATA

Radiation	Mean % Disintegration	Mean Energy (KeV)
Gamma-2	89.07	140.5

External Radiation

The specific gamma ray constant for Technetium Tc 99m is 5.4 microcoulombs/kg-MBq-hr(0.78R/mCi-hr) at 1 cm. The first half value thickness of lead for Technetium Tc 99m is 0.017cm. A

Samsung Bioepis Exhibit No. 1002
Page 2690

range of values for the relative attenuation of the radiation emitted by this radionuclide that results from the interposition of various thicknesses of lead is shown in Table 2. To facilitate control of the radiation exposure from Megabequerel/millicurie amounts of this radionuclide, the use of a 0.25cm thickness of lead will attenuate the radiation emitted by a factor of about 1,000.

Table 2. RADIATION ATTENUATION BY LEAD SHIELDING

Lead Shield Thickness (cm)	Coefficient of Attenuation
0.017	0.5
0.08	10^{-1}
0.16	10^{-2}
0.25	10^{-3}
0.33	10^{-4}

To allow correction for physical decay of Technetium Tc 99m, the fractions that remain at selected intervals after the time of calibration are shown in Table 3.

TABLE 3. PHYSICAL DECAY CHART FOR TECHNETIUM Tc 99m, HALF-LIFE 6.02 HOURS

Hours	Fraction Remaining	Hours	Fraction Remaining
0*	1.00	8	0.40
1	0.89	9	0.36
2	0.79	10	0.32
3	0.71	11	0.28
4	0.63	12	0.25
5	0.56	18	0.13
6	0.50	24	0.06
7	0.45		

*Calibration Time

CLINICAL PHARMACOLOGY

General

The product contains Nofetumomab, a Fab fragment of a murine monoclonal antibody NR-LU-10, that recognizes an approximately 40 kD glycoprotein antigen (carcinoma associated antigen) expressed on a variety of cancers and some normal tissues[2]. Due either to lack of antigen expression or other unknown factors, neither all patients with small cell lung cancer, nor all malignant lesions will be imaged by this agent. While the overall percentage of antigen positive tumors is not known, immunohistological evaluation of the NR-LU-10 antibody on cryopreserved fresh human tumors confirms strong reactivity with three of three small cell lung cancer tumors, and three of three tumor cell lines tested. *In vitro* studies demonstrate the immunoreactivity of the Fab fragment against the 40 kD glycoprotein carcinoma associated antigen on two of two tumor cell lines tested.

Samsung Bioepis Exhibit No. 1002
Page 2691

Pharmacokinetics

Following intravenous injection, clearance of Technetium Tc 99m Nofetumomab Merpentan is biphasic. It has a rapid distribution phase with a mean serum half-life of 1.5 hours, and a slower elimination phase with a mean half-life of 10.5 hours. Renal clearance is the primary route of elimination, with 64% of the injected dose of Technetium Tc 99m Nofetumomab Merpentan eliminated within the first 22 hours after administration. The secondary route of elimination is through the hepatobiliary system, leading to accumulation of radioactivity in the kidney, urinary bladder, gall bladder, and intestines. *In vitro* data reveal no evidence of Technetium Tc 99m transfer to serum proteins. Based on calculations of photon flux, clearance rates for the imaging agent, and subjective clinical assessment, optimal time for imaging is 14 -17 hours after injection.

Pharmacodynamics

In the Phase 1/2 study, Technetium Tc 99m Nofetumomab Merpentan localized to primary and/or metastatic small cell[3-7] and non-small cell lung cancer[6-9], and adenocarcinomas of the breast, ovary, colorectum, and prostate. Adenocarcinomas of the kidney and pancreas were not detected. Imaging of patients revealed accumulation of radioactivity in some normal sites due to excretion (gall bladder, intestine, kidneys, urinary bladder), nonspecific vascular localization (testes, midline nasal area, liver, spleen), or specific reactivity (pituitary gland, salivary gland, thyroid) of Technetium Tc 99m Nofetumomab Merpentan. Radioactivity also may appear to accumulate in other non-tumor areas such as regions of inflammation, increased vascular pool, or recent surgical areas. The usefulness of Technetium Tc 99m Nofetumomab Merpentan for detecting metastatic disease (particularly small lesions) may be reduced in these areas, or adjacent areas of non-tumor accumulation.

Clinical Study

A multicenter, single arm, open-label, phase 3 study was conducted in patients with previously untreated, biopsy-confirmed small cell lung cancer. Eighty-nine of 96 patients enrolled in the study were considered evaluable. The 7 inevaluable patients either did not meet one or more of the eligibility criteria, or were not imaged. The primary endpoint was the accuracy of Technetium Tc 99m Nofetumomab Merpentan in staging patients with small cell lung cancer as extensive or limited stage disease. Differentiation of limited from extensive stage disease has both prognostic and therapeutic implications in this disease. The results of the masked interpretation (off-site interpretation without access to clinical or other diagnostic information) with Technetium Tc 99m Nofetumomab Merpentan images were compared with the results of an unmasked battery of other standard diagnostic evaluations (physical exam, bone scan, chest x-ray, CT examinations of head, chest, abdomen, and bone marrow aspirate and/or biopsy). Technetium Tc 99m Nofetumomab Merpentan imaging correctly staged 73 of the 89

Samsung Bioepis Exhibit No. 1002
Page 2692

evaluable patients for an overall accuracy of 82% (see Table 4a). The positive predictive value of Technetium Tc 99m Nofetumomab Merpentan imaging when it demonstrated extensive disease was 94% (44/47). There were 3 patients in whom Technetium Tc 99m Nofetumomab Merpentan imaging identified false positive extensive disease; the false positive sites included uptake in axillary folds in 2 patients and uptake at the site of a recent surgical procedure in one patient.

The sensitivity for detection of extensive disease with Technetium Tc 99m Nofetumomab Merpentan imaging was 77% (44/57), compared with 88% (50/57) for the battery of all standard diagnostic tests. The remaining thirteen patients were under staged by Technetium Tc 99m Nofetumomab Merpentan imaging, with 20 false negative areas for tumor involvement: CNS (n=5), liver (n=5), bone (n=4), adrenal (n=2), pancreas, pericardium, pleural mass, and bone marrow (n=1, each).

Since Technetium Tc 99m Nofetumomab Merpentan imaging is to be performed after a histologic diagnosis is established, many or most patients may have undergone preliminary staging studies, including physical examination, chest x-ray, and CT of the chest; those found to have extensive stage disease may not require further evaluation. In the phase 3 study, 75 of the 89 patients were still considered to have limited stage disease after these three staging procedures. Results of these patients are shown in Table 4b.

As an initial test, Technetium Tc 99m Nofetumomab Merpentan imaging had the highest accuracy 82% (73/89) for clinical staging of any single diagnostic test, and it had the highest sensitivity for extensive disease identifying 44 of 57 cases (see Table 5). However, Technetium Tc 99m Nofetumomab Merpentan was always the second best test for imaging of an individual organ or area. Technetium Tc 99m Nofetumomab Merpentan was relatively insensitive for detection of metastatic disease involving the brain and adrenals (see Table 6). In some patients with extensive staged disease, not all involved organs were imaged.

TABLE 4a. DIAGNOSIS BY VERLUMA™ KIT IMAGING vs. A BATTERY OF STANDARD DIAGNOSTIC EVALUATIONS IN ALL EVALUABLE PATIENTS

		Verluma™ Kit Imaging		Initial Assessment Standard Battery of Tests	
		Extensive	Limited	Extensive	Limited
Final Diagnosis	Extensive	44	13	50	7
	Limited	3	29	2	30

Samsung Bioepis Exhibit No. 1002
Page 2693

TABLE 4b. DIAGNOSIS BY VERLUMA™ KIT IMAGING vs. STANDARD TESTS IN PATIENTS WITHOUT EVIDENCE OF EXTENSIVE DISEASE BY PHYSICAL EXAM, CHEST X-RAY, OR CHEST CT

		Verluma™ Kit Imaging	
		Extensive	Limited
Final Diagnosis	Extensive	31	13
	Limited	3	28

TABLE 5. COMPARATIVE ACCURACY OF VERLUMA™ KIT IMAGING WITH OTHER SINGLE DIAGNOSTIC EVALUATIONS FOR STAGING

Diagnostic Evaluation	Extensive	Limited	Overall Accuracy
Verluma™ Kit Imaging	44/57	29/32	73/89 (82%)
CT Abdomen	33/57	30/32	63/89 (71%)
Bone Scan	24/57	32/32	56/89 (63%)
Bone Marrow (asp/bx)	16/57	32/32	48/89 (54%)
CT Head	12/57	32/32	44/89 (49%)
Physical Exam	8/57	31/32	39/89 (44%)
CT Chest	5/57	32/32	37/89 (42%)
Chest X-Ray	2/57	32/32	34/89 (38%)

TABLE 6. DETECTION RATE FOR VERLUMA™ KIT IMAGING BY ORGAN

Organ	Detection Rate (%)	95% CI
Lung	69/79 (87%)	78 - 94%
Neck/Axilla	20/24 (86%)	67 - 96%
Mediastinum	41/50 (82%)	69 - 91%
Bone	21/26 (81%)	61 - 93%
Bone Marrow	13/17 (76%)	50 - 93%
Liver	17/27 (63%)	42 - 81%
Pleura	8/15 (53%)	27 - 79%
Brain	3/12 (25%)	5 - 57%
Adrenal	0/9	0 - 47%
Abdominal Nodes	0/6	0 - 27%
Head	0/1	0 - 95%
Other	2/7 (29%)	4 - 71%
Total	194/274 (71%)	

INDICATIONS AND USAGE

Technetium Tc 99m Nofetumomab Merpentan is indicated for the detection of extensive stage disease in patients with biopsy-confirmed, previously untreated small cell lung cancer. Where Technetium Tc 99m Nofetumomab Merpentan imaging is interpreted as limited stage disease (limited to one hemithorax,

Samsung Bioepis Exhibit No. 1002
Page 2694

mediastinum, bilateral hilar lymph nodes, ipsilateral supraclavicular nodes), additional diagnostic tests should be performed to exclude extensive stage disease. Bone scan, CT examinations of head, chest, abdomen, chest x-ray, and/or bone marrow aspirate/biopsy have been shown to demonstrate additional sites of involvement in some patients.

Technetium Tc 99m Nofetumomab Merpentan imaging is not indicated for differential diagnosis of suspected lung cancer or suspected metastases. Technetium Tc 99m Nofetumomab Merpentan is intended for single use prior to treatment: it is not intended for assessment of response or evaluation following chemotherapy or radiotherapy (see Precautions).

CONTRAINDICATIONS

Technetium Tc 99m Nofetumomab Merpentan should not be used in patients who are hypersensitive to this or any other product of murine origin.

WARNINGS

Allergic reactions, including anaphylaxis, can occur in patients who receive murine antibodies. Serious allergic reactions have not been observed in clinical trials after Technetium Tc 99m Nofetumomab Merpentan administration. Appropriate medications and equipment for the treatment of hypersensitivity reactions should be available during administration of this agent.

PRECAUTIONS

General

Technetium Tc 99m Nofetumomab Merpentan may not visualize some sites of involvement by small cell lung cancer. A pattern of localization that suggests limited disease does not exclude the possibility of extensive stage disease. For those patients with evidence of limited disease by Technetium Tc 99m Nofetumomab Merpentan imaging, additional tests should be performed to confirm the absence of additional sites of disease (see Phase 3 Trial, Indications and Usage).

A negative Technetium Tc 99m Nofetumomab Merpentan scan (no tumor localization) should not alter patient management, or be used to rule out a diagnosis of small cell lung cancer since some cases of small cell lung cancer may not bear the 40kD antigen (see Phase 3 Trial).

Technetium Tc 99m Nofetumomab Merpentan may accumulate in nontumor sites such as the organs of excretion, certain normal tissues, areas of inflammation, increased blood flow, or recent surgery, and in a variety of other histologic types of cancer. Antibody images should be interpreted in the context of the known sites of inflammation or surgery by a physician experienced in the evaluation of nuclear medicine images. When clinically important, the physician should consider confirmation of areas of increased activity with use of another diagnostic procedure.

Samsung Bioepis Exhibit No. 1002
Page 2695

Clinical studies did not include those patients who had received prior chemotherapy or radiation therapy. Thus, safety and efficacy of Technetium Tc 99m Nofetumomab Merpentan has not been established in such patients.

There are no data to support the safety or efficacy of Technetium Tc 99m Nofetumomab Merpentan readministration. Technetium Tc 99m Nofetumomab Merpentan should be used only once in each patient.

To minimize the radiation dose to the bladder, the patient should be encouraged to drink fluids and to void as often as possible for 12-14 hours after receiving Technetium Tc 99m Nofetumomab Merpentan. Since the hepatobiliary system and gastrointestinal tract are secondary routes of excretion, use of cathartics may reduce radiation exposure. Care should be taken in patients with impaired renal or hepatobiliary function. This product has not been used in such patients.

This product should be prepared according to the product radiolabeling instructions. All kit components, containers and supplies used in the preparation of the radiolabeled Nofetumomab Merpentan are single use and must be disposed of properly. Under no circumstances is any component to be reused. **The contents of the kit are intended only for use in the preparation of Technetium Tc 99m Nofetumomab Merpentan, and individual components are NOT to be administered directly to the patient.**

At least 555 MBq (15mCi) of the final preparation should be administered to ensure that a sufficient dose is used to acquire adequate images. **Reducing the dose of Technetium Tc 99m Nofetumomab Merpentan may adversely impact imaging results, and is not recommended.**

The contents of the Verluma™ kit are not radioactive, however, after sodium pertechnetate Tc 99m is added, adequate shielding of the intermediate formulations and final preparation must be maintained. In the use of any radioactive material, care should be taken to ensure minimum radiation exposure to the patient and personnel consistent with proper patient management.

Radiopharmaceuticals should be used only under the supervision of physicians who are qualified by training and experi-ence in the safe use and handling of radionuclides and whose experience and training have been approved by the appropriate governmental agency authorized to license the use of radionuclides.

Information for Patients

Murine monoclonal antibodies are foreign proteins, and their administration can induce human anti-mouse antibodies (HAMA). While limited data exist concerning the clinical significance of HAMA, the presence of HAMA may interfere with murine antibody-based immunoassays, could compromise the efficacy of in vitro or in vivo diagnostic or therapeutic murine antibody-based agents, and may increase the risk of adverse reactions to such agents. For these reasons, patients should be

Samsung Bioepis Exhibit No. 1002
Page 2696

informed that the use of this product could affect the future use of other murine-based products, including Technetium Tc 99m Nofetumomab Merpentan, and should be advised to discuss prior use of murine antibody-based products with their physicians (see Heterologous Protein Administration).

Heterologous Protein Administration

Murine monoclonal antibodies are heterologous proteins, and their administration can induce HAMA. When considering the administration of Technetium Tc 99m Nofetumomab Merpentan to patients who have previously received murine antibody-based products, physicians should be aware of the potential for HAMA to increase the risk of allergic reactions and to alter clearance and biodistribution of this agent. The quality or sensitivity of the imaging study may be compromised.

Mild transient allergic reactions have occurred rarely in patients receiving this product, and mild serum sickness has been observed in patients receiving other murine monoclonal antibody products.

Drug/Laboratory Test Interactions

The presence of HAMA in serum may interfere with murine antibody-based immunoassays, such as assays for carcinoembryonic antigen and CA 125. If HAMA is known or suspected to be present, the clinical laboratory should be notified that interference may occur.

No data are available on possible drug interactions. Do not mix or administer Technetium Tc 99m Nofetumomab Merpentan with other products. Sufficient time should be allowed for clearance and radioactive decay before and after the use of this product and other products using radionuclides (see Table 3).

Carcinogenesis, Mutagenesis, Impairment of Fertility

Long-term animal studies have not been performed to evaluate the carcinogenic or mutagenic potential of Technetium Tc 99m Nofetumomab Merpentan or to evaluate its effect on fertility in males or females.

Pregnancy Category C

Animal reproduction studies have not been conducted with Technetium Tc 99m Nofetumomab Merpentan. It is also not known whether Technetium Tc 99m Nofetumomab Merpentan can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Technetium Tc 99m Nofetumomab Merpentan should not be administered to a pregnant woman unless, in the opinion of the physician, the information to be gained outweighs the potential risks.

Nursing Mothers and/or Lactating Women

It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Technetium Tc 99m Nofetumomab Merpentan is administered to a nursing woman. Formula feedings should be substituted for breast feedings for at least 60 - 72 hours following administration.

Samsung Bioepis Exhibit No. 1002
Page 2697

Pediatric Use

Safety and effectiveness of Technetium Tc 99m Nofetumomab Merpentan in pediatric patients have not been established.

ADVERSE REACTIONS

Among 515 patients with a variety of carcinomas who have received intravenous doses of Technetium Tc 99m Nofetumomab Merpentan, the following adverse reactions were noted: self-limited elevations in temperature (2 patients), urticaria or mild allergic reactions (3 patients).

In 53 of 89 subjects in the phase 3 trial, with both pre- and postinfusion samples, only 6% (n=3) developed elevated HAMA levels when assayed by an anti-nofetumomab ELISA. Compared to pretreatment HAMA levels, the increases in these 3 patients were 2-fold, 6-fold, and 40-fold, respectively. Analysis of subsequent serum samples in 2 of 3 patients revealed a return of HAMA levels to the normal range within 3 to 4 months. The HAMA level in the third patient (with the 40-fold increase) was declining but did not return to baseline within 4 months. In the patients with elevated HAMA, the antibodies reacted primarily with Nofetumomab and weakly with other mouse immunoglobulins. No toxicity was associated with the HAMA elevations.

Immunohistochemistry analysis demonstrated binding of NR-LU-10, the parent antibody for Nofetumomab, to normal pancreas and salivary glands. Transient changes in serum lipase or amylase have occurred in 20 patients and were not associated with any clinical symptoms. Seven patients exhibited mild elevations in amylase, ten patients experienced mild elevations in lipase levels and 3 patients experienced elevations in both enzymes.

OVERDOSAGE

The maximum amount of Technetium Tc 99m Nofetumomab Merpentan that can be safely administered has not been determined. The largest single dose administered in the clinical studies is 19.9mg of Technetium Tc 99m Nofetumomab Merpentan.

DOSAGE AND ADMINISTRATION

The recommended dose for intravenous administration in a single dose is 5 - 10mg Nofetumomab Merpentan labeled with 1,110 MBq (30mCi) Technetium Tc 99m in 15 - 20mL Sodium Chloride Injection. The dose should be administered over 3 - 5 minutes intravenously by injection from a 20mL shielded syringe. At least 555 MBq (15mCi) of the final preparation should be administered to ensure an adequate dose of radiolabeled antibody is used to obtain satisfactory images 14 - 17 hours after injection. The radiochemical yield may be variable based upon experience and training. **Reducing the dose of Technetium Tc 99m Nofetumomab Merpentan may adversely impact imaging results and is not recommended.**

Samsung Bioepis Exhibit No. 1002
Page 2698

The radiolabeled product may be held for up to six hours at room temperature prior to patient infusion but, at least 555MBq (15mCi) should be administered. The components contained in this kit contain no preservatives.

The patient dose should be measured by a suitable, calibrated radioactivity measurement system immediately prior to administration. Radiochemical purity must be ≥ 85% prior to patient administration as determined by the procedure described in the instructions.

A cathartic may be administered orally after administration of the radiolabeled Nofetumomab Merpentan at an appropriate time to be effective before initiation of imaging; this may reduce background radioactivity in the intestine. Patients should be well hydrated and should void as much as possible prior to image acquisition.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration whenever solution and container permit. If particulate matter and/or discoloration are detected in any kit component or final product, Technetium Tc 99m Nofetumomab Merpentan should not be administered and the distributor should be notified immediately.

Radiation Dosimetry

The estimated radiation dose absorbed by an average 70 kg adult after an intravenous injection of 1,110 MBq (30mCi) of Technetium Tc 99m Nofetumomab Merpentan is shown in Table 7.

TABLE 7. ESTIMATED ABSORBED RADIATION DOSE FOR A 70 kg PATIENT RECEIVING 1,110 MBq (30 mCi) TECHNETIUM Tc 99m NOFETUMOMAB MERPENTAN

	Absorbed Dose	
Tissue	rad/30 mCi	mGy/1,110MBq
Gall Bladder Wall	5.6	56
Intestine Wall (Small)	1.6	16
Intestine Wall (Lower Large)	2.0	19
Intestine Wall (Upper Large)	2.7	27
Kidney	3.9	38
Liver	1.3	13
Lungs	0.74	7.3
Ovaries	0.93	9.3
Red Marrow	0.47	4.7
Spleen	0.56	5.6
Testes	0.45	4.4
Thyroid	0.91	9.0
Uterus	0.87	8.6
Total Body	0.47	4.7

Image Acquisition and Interpretation

Data from clinical trials indicate that optimal diagnostic images are routinely obtained between 14-17 hours after injection. To obtain adequate counting statistics, a planar image of the chest should be acquired in the anterior view for 500,000 counts or

Samsung Bioepis Exhibit No. 1002
Page 2699

10 minutes, whichever is shorter. All subsequent images should be acquired for the same time.

Imaging with Technetium Tc 99m Nofetumomab Merpentan may show artifacts of apparent increased activity at skin folds in the area of the axilla, breast and lower abdomen, particularly in obese patients. Artifacts in the axillae may be eliminated by imaging with the arms extended overhead.

Instructions for the Preparation of Technetium Tc 99m Nofetumomab Merpentan

Note: Read complete directions thoroughly before starting preparation.

General and Procedural Precautions

1) The components should be inspected before use. Should any particulate matter, discoloration, breakage or leakage be noted, contact the distributor.
2) The components of the Verluma™ kit are sterile, non-pyrogenic, and do not contain preservatives. It is important that the user adhere to strict aseptic procedure during the preparation, withdrawal and administration of the Technetium Tc 99m Nofetumomab Merpentan. Use germicide swabs to wipe each vial prior to entry into vial.
3) All components, vials, and supplies used in the preparation of the kit must be disposed of properly. Under no circumstances is any component to be reused. Substitution of components for use with a kit of a different lot number is not recommended.
4) Proper techniques and precautions for handling radioactive materials should be employed. Waterproof gloves should be worn during the labeling procedure. Shielded syringes should be used when adding the sodium pertechnetate eluant to the reaction vial, for withdrawal of intermediate preparations, and administration of the final dose.
5) The Technetium Tc 99m labeling reactions[10] depend on maintaining the stannous ion in a reduced state. Therefore, sodium pertechnetate Tc 99m solution containing oxidants should not be used. If generator-produced Technetium Tc 99m is used, the generator must be eluted at a time that insures that 2,775 - 3,700MBq (75 - 100mCi) be obtained in a volume of 1.0mL.
6) In order to assure appropriate incubation temperatures, provide a shielded water bath at a temperature of 75°C ± 5°C and an ice-chilled shielded container before beginning the preparation of Technetium Tc 99m Nofetumomab Merpentan.
7) The radiochemical purity of Technetium Tc 99m Nofetumomab Merpentan must be checked prior to administration to the patient, according to the instructions below. If the radiochemical purity is not ≥ 85%, the product must not be administered.

Samsung Bioepis Exhibit No. 1002
Page 2700

Verluma 27.09.1996 8:36 Uhr Seite 13

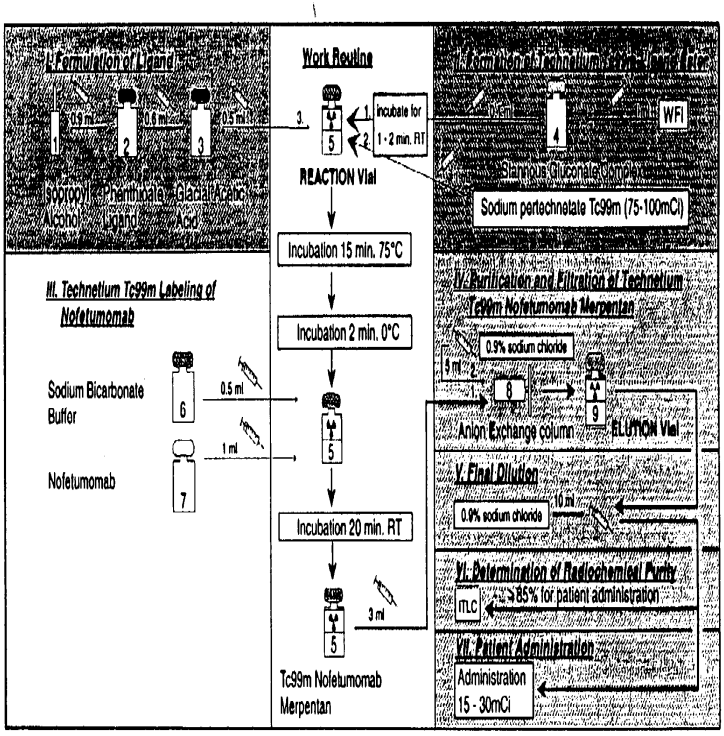
Procedure for the Preparation of Technetium Tc 99m Nofetumomab Merpentan

Note: The directions outlined below must be carefully followed for optimum preparation of the Technetium Tc 99m Nofetumomab Merpentan. The radiochemical yield may be variable based upon training and experience. A training program for preparation of Technertium Tc 99m Nofetumomab Merpentan is available from the distributor.

Required materials not supplied in the kit

1. Any commercial source of oxidant-free sodium pertechnetate Tc 99m in 0.9% sodium chloride, sterile and non-pyrogenic. Labeling requires 2,775 - 3,700 MBq (75 - 100mCi) in 1.0mL volume.
2. Sterile Water for Injection, USP, 1mL.
3. 0.9% Sodium Chloride Injection, USP, 20mL.
4. 1mL insulin syringes, sterile with permanently attached 27 - 28 gauge needles (6 each).
5. 5mL luer-lok syringe, disposable, sterile (2 each).
6. 20mL syringe, disposable, sterile (1 each).
7. 1 x 22 gauge needle, disposable, sterile (5 each).
8. 0.2 µm sterile filter.
9. Germicide to swab vial closures.
10. Lead shielded container to accommodate 10mL vial (2 each).
11. Lead shielded container to accommodate 10mL vial pre-chilled on ice (1 each).
12. Lead shielded container to accommodate 10mL vial for incubation at 75°C (1 each).
13. Timer.
14. Water bath maintained at 75°C ± 5°C.
15. Dose Calibrator.
16. Gamma counter or multichannel analyzer.
17. Ring stand to support column.
18. Adequate lead shielded work station.
19. Silica Gel Impregnated Glass Fiber Sheets as ITLC™ SG, 20 x 20 sheets, Product No. 61886 (available from Gelman Sciences, Inc., Ann Arbor, Michigan).
20. 12% (w/v) aqueous trichloroacetic acid.
21. Thin layer chromatography developing chamber.
22. Oven to activate ITLC for quality control on Technetium Tc 99m Nofetumomab Merpentan.
23. Lead shield for a 20cc syringe (available from nuclear imaging accessory suppliers).
24. Ice Bath.

Samsung Bioepis Exhibit No. 1002
Page 2701



Technetium Tc 99m Nofetumomab Merpentan Preparation Flow Chart

Samsung Bioepis Exhibit No. 1002
Page 2702

The preparation of Technetium Tc 99m Nofetumomab Merpentan consists of six process steps:

I. Formulation of Ligand
II. Formation of Technetium Tc 99m Ligand Ester
III. Technetium Tc 99m Labeling of Nofetumomab
IV. Purification and Filtration of Tc 99m Nofetumomab Merpentan
V. Final Dilution
VI. Determination of Radiochemical Purity
VII. Patient Administration

I. Formulation of Ligand

A. Swab rubber closure of the vial containing the ligand (Container 2) with germicide.
B. Using a sterile, disposable 1mL syringe, aseptically withdraw 0.9 mL of isopropyl alcohol from the freshly opened ampule (Container 1) and aseptically inject the isopropyl alcohol into the ligand vial (Container 2); keep the syringe connected to the vial.
C. Shake the ligand vial (Container 2) until the ligand is thoroughly dissolved. NOTE: This process takes approximately two minutes; complete dissolution is indicated by the disappearance of the thin film of ligand from the bottom surface of the vial (Container 2).
D. Using the attached syringe, aseptically withdraw 0.6mL of the ligand solution (Container 2).
E. Swab the rubber closure of the vial containing the glacial acetic acid: 0.2 N hydrochloric acid solution (Container 3) with germicide.
F. Aseptically inject the 0.6mL of ligand solution into the vial containing the glacial acetic acid: 0.2 N hydrochloric acid solution (Container 3); keep the syringe connected to the vial (Container 3). Gently agitate the solution.
G. Using the attached syringe, aseptically withdraw 0.5mL of the acidified ligand solution (Container 3). Cap the syringe and set it aside.

II. Formation of Technetium Tc 99m Ligand Ester

A. Swab the rubber closure of the stannous gluconate complex (Container 4) and the Sterile Water for Injection vial with germicide. Aseptically inject 1mL of Sterile Water for Injection into the stannous gluconate complex (Container 4); keep the syringe connected to the vial (Container 4). Gently agitate the vial (Container 4) until the lyophilized contents are dissolved.
B. Using the attached syringe, aseptically withdraw 0.1mL of the stannous gluconate solution (Container 4).
C. Obtain the REACTION vial (Container 5) and swab the rubber closure with germicide. Aseptically inject the 0.1mL of stannous gluconate solution into the REACTION vial (Container 5). Affix the "Caution Radioactive Material" label to the REACTION vial (Container 5) and place the vial (Container 5) into a lead shielded container.

Samsung Bioepis Exhibit No. 1002
Page 2703

D. Using a shielded syringe aseptically inject 75 to 100 mCi sodium pertechnetate Tc 99m solution in a volume of 1.0mL into the shielded REACTION vial (Container 5) containing 0.1mL stannous gluconate solution.

E. Gently agitate the REACTION vial (Container 5) to mix the contents.

F. Note the time and store the shielded REACTION vial (Container 5) at room temperature for at least one minute, but no longer than two minutes.

G. Immediately inject the 0.5mL acidified ligand solution (Prepared in Step I.G.) into the REACTION vial (Container 5).

H. Gently agitate the solution to ensure mixing. Place the REACTION vial (Container 5) into a shielded water bath at 75°C ± 5°C for 15 minutes.

I. Immediately transfer the heated REACTION vial (Container 5) into an ice-cold chilled lead container, or shielded ice bath for at least two minutes.

III. Technetium Tc 99m Labeling of Nofetumomab

A. Swab the rubber closure of the vial containing sodium bicarbonate buffer (Container 6) and the vial containing Nofetumomab (Container 7) with germicide.

B. Using a 1mL syringe, aseptically withdraw 0.5mL sodium bicarbonate buffer (Container 6). Replace the needle cap and set the syringe aside.

C. Using a 1mL syringe, aseptically remove 1.0mL from the vial containing Nofetumomab (Container 7). Cap the syringe and set it aside.

D. Remove the REACTION vial (Container 5) containing Technetium Tc 99m ligand ester solution prepared in Step II from the ice bath or chilled lead container and place the vial (Container 5) into another shielded container at room temperature.

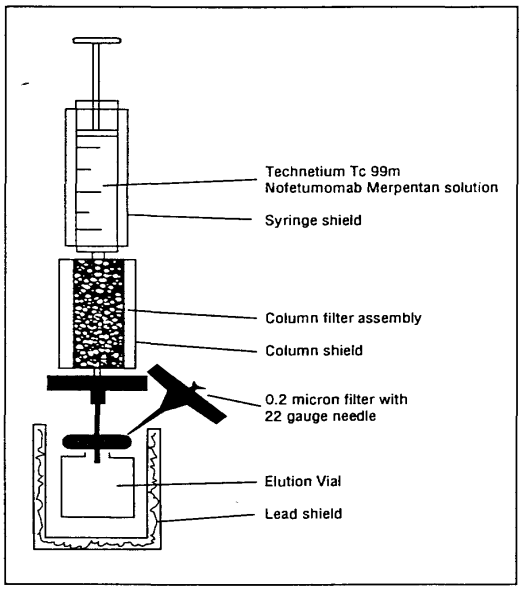
E. Swab the rubber closure of the REACTION vial containing the Technetium Tc 99m ligand ester prepared in Step II (Container 5) with germicide. Aseptically inject the 0.5mL of bicarbonate buffer (prepared in Step III B) into the REACTION vial (Container 5). Gently agitate the shielded vial (Container 5) to ensure mixing.

F. Without delay, gently inject the Nofetumomab in the syringe prepared from Step III C into the REACTION vial (Container 5) containing the buffered Technetium Tc 99m ligand ester (avoid foaming). Gently agitate the shielded vial (Container 5) to mix the contents.

G. Incubate the radioactive reaction mixture (Container 5) in a shielded container at room temperature for 20 minutes.

Samsung Bioepis Exhibit No. 1002
Page 2704

IV. Purification and Filtration of Technetium Tc 99m Nofetumomab Merpentan



A. During the incubation of the Technetium Tc 99m Nofetumomab Merpentan solution (Container 5) (Step III G), aseptically prepare the following components:

1. Affix "Caution Radioactive Material" label to the ELUTION vial (Container 9).
2. Swab the rubber closure of the empty ELUTION vial (Container 9). Aseptically add a sterile disposable needle with an attached 0.2 micron filter to vent the vial (Container 9).
3. Remove the bottom cap from the anion exchange column 0.2 micron filter assembly (Container 8). Attach a sterile disposable needle with needle cap to the 0.2 micron filter assembly.
4. Remove the needle guard from the needle attached to the 0.2 micron filter of the anion exchange column and insert the needle into the ELUTION vial (Container 9).

Samsung Bioepis Exhibit No. 1002
Page 2705

5. Using a 5mL luer-lok syringe with a needle, aseptically withdraw 5mL of Sodium Chloride for Injection, 0.9% USP. Carefully remove any air bubbles from the syringe containing the sodium chloride solution. Replace needle guard and set the syringe aside.

B. After incubation of the Technetium Tc 99m Nofetumomab Merpentan reaction mixture (Container 5) (Step III G), measure total radioactivity of the REACTION vial (Container 5) in a dose calibrator. Subsequently aseptically withdraw the radioactive solution into a shielded 5mL luer-lok syringe. Assure that more than 90% of the radioactivity in the REACTION vial (Container 5) has been transferred to the syringe by measurement of the residual radioactivity in the empty REACTION vial (Container 5). Carefully, remove all air bubbles from the solution while bringing the solution to the top of the syringe barrel.

C. Carefully remove the needle from the syringe containing Technetium Tc 99m Nofetumomab Merpentan and the top cap from the anion exchange column (Container 8) (on the end opposite the 0.2 micron filter). Connect the shielded 5mL syringe containing Technetium Tc 99m Nofetumomab Merpentan to the shielded anion exchange column (Container 8).

D. Slowly inject the Technetium Tc 99m Nofetumomab Merpentan into the anion exchange column (Container 8) collecting the eluate into the ELUTION vial (Container 9).

E. Remove the shielded 5 mL syringe from the top of the anion exchange column (Container 8). Attach the 5mL syringe containing the 5mL of Sodium Chloride for Injection, 0.9% USP.

F. Slowly, over 2 minutes, inject the sodium chloride solution into the anion exchange column (Container 8) collecting the eluate into the same ELUTION vial (Container 9).

G. Measure total reactivity in the ELUTION vial (Container 9) using a dose calibrator.

V. Final Dilution

A. Withdraw 10mL of 0.9% Sodium Chloride for Injection into a shielded sterile, 20mL syringe and then into this syringe withdraw the entire contents of the Technetium Tc 99m Nofetumomab Merpentan vial (ELUTION vial) (Container 9). Mix the solution in the syringe. Drop an aliquot of about 50µl into an eppendorf cup for subsequent quality control by ITLC.

B. Determine the Radiochemical Purity (described below). The preparation must have a purity of 85% or greater. If the result of this test indicates a purity of less than 85% do not administer the product and contact the distributor.

VI. Determination of Radiochemical Purity

A. In order to assess Radiochemical Purity of Technetium Tc 99m Nofetumomab Merpentan, the following must be prepared and assembled in advance:

Samsung Bioepis Exhibit No. 1002
Page 2706

1. Thin layer chromatography developing chamber;
2. Gamma counter calibrated for Technetium Tc 99m;
3. Chromatographic Solvent: Prepare a 12% (w/v) trichloroacetic acid (TCA) in water solution. The solvent can be prepared as a stock reagent and is stable for at least 30 days when stored refrigerated.
4. Silica Gel Impregnated Glass Fiber Sheets: Pre-cut the strips to a final dimension of 2 by 10 cm. NOTE: The strips are fragile, use caution during handling. Activate the pre-cut strips according to manufacturer's instruction. Store the activated strips after activation according to manufacturer's instructions.

B. Test Procedure

1. Carefully remove an activated ITLC chromatographic strip from a storage container using forceps. Using a pencil, carefully mark the origin at approximately 1.2cm from one end of the strip.
2. Spot a small drop (2 to 5µl) of product at the origin. Allow the spot to dry prior to beginning chromatographic development.
3. Place the chromatographic strip into the developing chamber. Do not immerse the origin into the solvent.
4. Develop the chromatographic strip, allowing the solvent to ascend to about 1cm from the strip top. Remove the strip from the developing chamber and allow it to dry.
5. Cut the developed chromatographic strip into three sections. Identify the sections as origin, middle, and solvent front. Using the developing system described above, Technetium Tc 99m Nofetumomab Merpentan remains at the origin and most non-protein bound Technetium Tc 99m labeled material travels with the solvent front. The middle section of the chromatographic strip may be used to verify complete separation between product and impurities (less than 5% of total Technetium Tc 99m activity should be assayed on this section of the strip).
6. Using a suitable radioactivity counter (e.g., gamma counter), count each section of the strip. Count long enough to determine a statistically significant count for each strip section (30 sec).

Calculate the radiochemical purity (percent Technetium Tc 99m Nofetumomab Merpentan) using the following formula: (Counts Origin Area/Counts Solvent ± Counts Middle Section + Counts Origin Area) x 100%.

If the Radiochemical Purity is not ≥ 85%, do not administer the product.

Samsung Bioepis Exhibit No. 1002
Page 2707

VII. Patient Administration

A. Determine the amount of radioactivity in the syringe using a dose calibrator.

B. Record the amount of radioactivity in the syringe, patient i.d., volume and time and date prepared on the lead shield label. Affix the label on the lead shield and deliver product for administration.

How Supplied

Verluma™, Kit for the preparation of Technetium Tc 99m Nofetumomab Merpentan is supplied as a single use kit that contains one each of the following components in sterile, non-pyrogenic form:

Container	Contents
1	Isopropyl Alcohol, 99%, 1.5mL
2	Phenthioate Ligand (2,3,5,6-tetrafluorophenyl-4,5-bis-S-(1-ethoxyethyl)-thioacetoamidopentanoate), 0.28mg (dried)
3	Glacial Acetic Acid: 0.2 N Hydrochloric Acid 1:7 (v/v), 0.16mL
4	Stannous Gluconate Complex containing Sodium Gluconate, 45mg, and Stannous Chloride, Dihydrate, 0.79mg (lyophilized)
5	Reaction Vial - Empty, 10mL
6	1 M Sodium Bicarbonate Buffer, pH 10, 1mL
7	Nofetumomab (Fab fragment of murine monoclonal antibody NR-LU-10) in Phosphate Buffered Saline, contains 10mg in 1mL
8	Anion Exchange Column, 5mL with 0.2 µm Sterile Filter
9	Elution Vial - Empty, 10mL

In addition, the kit also contains one (1) package insert, six (6) vial lead shield labels, and six (6) radiation warning labels.

Storage

The Verluma™ kit should be stored at 2°C to 8°C (36°F to 46°F). Do not freeze or shake. After preparation of Technetium Tc 99m Nofetumomab Merpentan, store at controlled room temperature (20°C to 25°C/68°F - 77°F). Technetium Tc 99m Nofetumomab Merpentan contains no preservatives and must be used within six hours of preparation.

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Samsung Bioepis Exhibit No. 1002
Page 2708

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Samsung Bioepis Exhibit No. 1002
Page 2709

Additional Information

Verluma™ is manufactured by Dr. Karl Thomae GmbH, an affiliated company of Boehringer Ingelheim International GmbH. The NR-LU-10 imaging product was developed by NeoRx Corporation, Seattle, WA.

Distribution of the kit is by DuPont Radiopharmaceutical Division of The DuPont Merck Pharmaceutical Company. Any adverse reaction or product defect found with this product should be reported to DuPont Radiopharmaceutical Division, 1-800-362-2668.

U.S. Patent 4,897,255
U.S. Patent 5,037,630
U.S. Patent 5,120,526
U.S. Patent 5,175,343
U.S. Patent 5,242,679

Made in Germany

Thomae

Manufactured by:

Dr. Karl Thomae GmbH
an affiliated company of
Boehringer Ingelheim International GmbH
55216 Ingelheim/Germany
U.S. License No. 1199

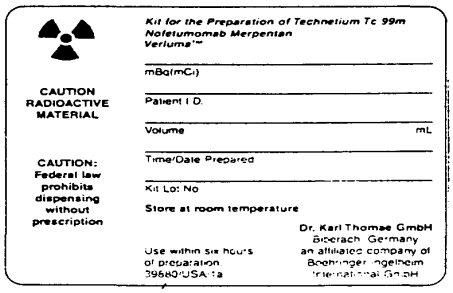
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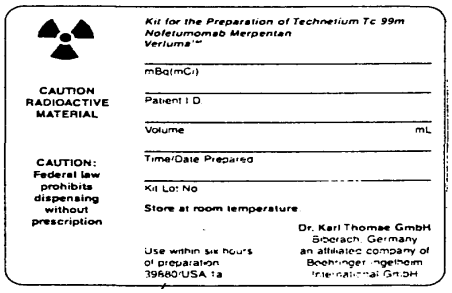
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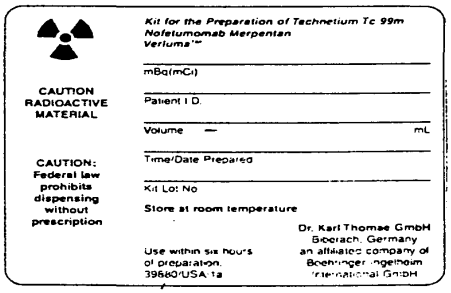
DuPont Radiopharmaceutical Division
The DuPont Merck Pharmaceutical Company
331 Treble Cove Road
Billerica, Massachusetts 01862

For Ordering Tel. Toll Free: 800-225-1572
All other business: 800-362-2668
(For Massachusetts and International, call 508-667-9531)

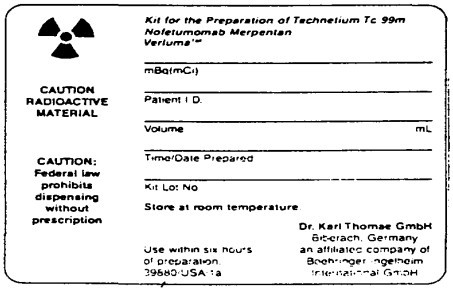
Samsung Bioepis Exhibit No. 1002
Page 2710

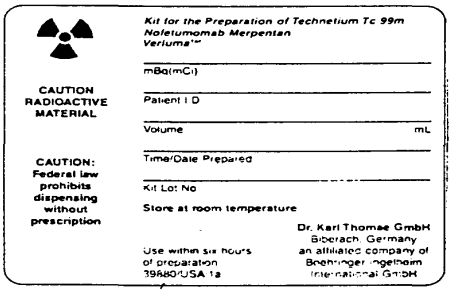


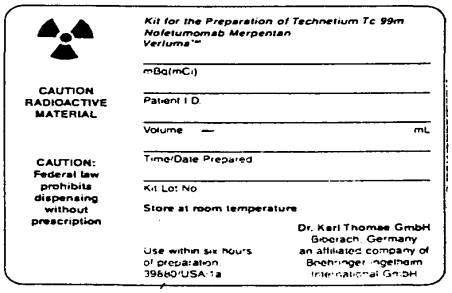




Samsung Bioepis Exhibit No. 1002
Page 2711







Samsung Bioepis Exhibit No. 1002
Page 2712

CAUTION
RADIOACTIVE MATERIAL

CAUTION
RADIOACTIVE MATERIAL

CAUTION
RADIOACTIVE MATERIAL

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CAUTION
RADIOACTIVE MATERIAL

Prior to adding the Technetium Tc 99m injection to the vial, write the estimated activity, date, and time of preparation in the space provided on the vial shield label. Then tear off a radiation symbol and attach it to the neck of the vial.

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Samsung Bioepis Exhibit No. 1002
Page 2713

OKT3 - muromonab-cd3 injection, solution
Centocor Ortho Biotech, L.P.

ORTHOCLONE OKT3® STERILE SOLUTION
(muromonab-CD3)

For Intravenous Use Only

WARNING

Only physicians experienced in immunosuppressive therapy and management of solid organ transplant patients should use ORTHOCLONE OKT3 (muromonab-CD3). Patients treated with ORTHOCLONE OKT3 must be managed in a facility equipped and staffed for cardiopulmonary resuscitation and where the patient can be closely monitored for an appropriate period based on his or her health status.

Anaphylactic and anaphylactoid reactions may occur following administration of any dose or course of ORTHOCLONE OKT3. In addition, serious, occasionally life-threatening or lethal, systemic, cardiovascular, and central nervous system reactions have been reported following administration of ORTHOCLONE OKT3. These have included: pulmonary edema, especially in patients with volume overload; shock, cardiovascular collapse, cardiac or respiratory arrest, seizures, coma, cerebral edema, cerebral herniation, blindness, and paralysis. Fluid status should be carefully monitored prior to and during ORTHOCLONE OKT3 administration. Pretreatment with methylprednisolone is recommended to minimize symptoms of Cytokine Release Syndrome. (See: WARNINGS: Cytokine Release Syndrome, Central Nervous System Events, Anaphylactic Reactions; DOSAGE AND ADMINISTRATION).

DESCRIPTION

ORTHOCLONE OKT3 (muromonab-CD3) Sterile Solution is a murine monoclonal antibody to the CD3 antigen of human T cells which functions as an immunosuppressant. It is for intravenous use only. The antibody is a biochemically purified IgG2a immunoglobulin with a heavy chain of approximately 50,000 daltons and a light chain of approximately 25,000 daltons. It is directed to a glycoprotein with a molecular weight of 20,000 in the human T cell surface which is essential for T cell functions. Because it is a monoclonal antibody preparation, ORTHOCLONE OKT3 Sterile Solution is a homogeneous, reproducible antibody product with consistent, measurable reactivity to human T cells.

Each 5 mL ampule of ORTHOCLONE OKT3 Sterile Solution contains 5 mg (1 mg/mL) of muromonab-CD3 in a clear colorless solution which may contain a few fine translucent protein particles. Each ampule contains a buffered solution (pH 7.0 ± 0.5) of monobasic sodium phosphate (2.25 mg), dibasic sodium phosphate (9.0 mg), sodium chloride (43 mg), and polysorbate 80 (1.0 mg) in water for injection.

The proper name, muromonab-CD3, is derived from the descriptive term murine monoclonal antibody. The CD3 designation identifies the specificity of the antibody as the Cell Differentiation (CD) cluster 3 defined by the First International Workshop on Human Leukocyte Differentiation Antigens.

CLINICAL PHARMACOLOGY

ORTHOCLONE OKT3 reverses graft rejection, probably by blocking the function of T cells which play a major role in acute allograft rejection. OTHOCLONE OKT3 reacts with and blocks the function of a 20,000 dalton molecule (CD3) in the membrane of human T cells that has been associated in vitro with the antigen recognition structure of T cells and is essential for signal transduction. In in vitro cytolytic assays, ORTHOCLONE OKT3 blocks both the generation and function of effector cells. Binding of ORTHOCLONE OKT3 to T lymphocytes results in early activation of T cells, which leads to cytokine release, followed by blocking T cell functions. After termination of ORTHOCLONE OKT3 therapy, T cell function usually returns to normal within one week.

In vivo, ORTHOCLONE OKT3 reacts with most peripheral blood T cells and T cells in body tissues, but has not been found to react with other hematopoietic elements or other tissues of the body.

A rapid and concomitant decrease in the number of circulating CD3 positive cells, including those that are CD2, CD4, or CD8 positive has been observed in patients studied within minutes after the administration of ORTHOCLONE OKT3. This decrease in the number of CD3 positive T cells results from the specific interaction between ORTHOCLONE OKT3 and the CD3 antigen on the surface of all T lymphocytes. T cell activation results in the release of numerous cytokines/lymphokines, which are felt to be responsible for many of the acute clinical manifestations seen following ORTHOCLONE OKT3 administration. (See: WARNINGS: Cytokine Release Syndrome, Central Nervous System Events.)

While CD3 positive cells are not detectable between days two and seven, increasing numbers of circulating CD2, CD4, and CD8 positive cells have been observed. The presence of these CD2, CD4, and CD8 positive cells has not been shown to affect reversal of rejection. After termination of ORTHOCLONE OKT3 therapy, CD3 positive cells reappear rapidly and reach pre-treatment levels within a week. In some patients however, increasing numbers of CD3 positive cells have been observed prior to termination of ORTHOCLONE OKT3 therapy. This reappearance of CD3 positive cells has been attributed to the development of neutralizing antibodies to ORTHOCLONE OKT3, which in turn block its ability to bind to the CD3 antigen on T lymphocytes. (See: PRECAUTIONS: Sensitization.)

Pediatric patients are known to have higher CD3 lymphocyte counts than adults. Pediatric patients receiving ORTHOCLONE OKT®3 therapy often require progressively higher doses of ORTHOCLONE OKT3 to achieve depletion of CD3 positive cells (<25 cells/mm3) and ensure therapeutic ORTHOCLONE OKT3 serum concentrations (>800 ng/mL). (See: DOSAGE AND ADMINISTRATION; PRECAUTIONS: Laboratory Tests.)

Serum levels of ORTHOCLONE OKT3 are measurable using an enzyme-linked immunosorbent assay (ELISA). During the initial clinical trials in renal allograft rejection, in patients treated with 5 mg per day for 14 days, mean serum trough levels of the drug rose over the first three days and then averaged 900 ng/mL on days 3 to 14. Serum concentrations measured daily during treatment with ORTHOCLONE OKT3 in renal, hepatic, and cardiac allograft

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Samsung Bioepis Exhibit No. 1002
Page 2714

recipients revealed that pediatric patients less than 10 years of age have higher levels than patients 10–50 years of age. Subsequent clinical experience has demonstrated that serum levels greater than or equal to 800 ng/mL of ORTHOCLONE OKT3 blocks the function of cytotoxic T cells in vitro and in vivo. Reduced T cell clearance or low plasma ORTHOCLONE OKT3 levels provide a basis for adjusting ORTHOCLONE OKT3 dosage or for discontinuing therapy. (See: WARNINGS: Anaphylactic Reactions; PRECAUTIONS: Laboratory Tests; ADVERSE EVENTS: Hypersensitivity Reactions; DOSAGE AND ADMINISTRATION.)

Following administration of ORTHOCLONE OKT3 in vivo, leukocytes have been observed in cerebrospinal and peritoneal fluids. The mechanism for this effect is not completely understood, but probably is related to cytokines altering membrane permeability, rather than an active inflammatory process. (See: WARNINGS: Cytokine Release Syndrome, Central Nervous System Events.)

CLINICAL STUDIES

Acute Renal Rejection

In a controlled randomized clinical trial, ORTHOCLONE OKT3 was compared with conventional high-dose steroid therapy in reversing acute renal allograft rejection. In this trial, 122 evaluable patients undergoing acute rejection of cadaveric renal transplants were treated either with ORTHOCLONE OKT3 daily for a mean of 14 days, with concomitant lowering of the dosage of azathioprine and maintenance steroids (62 patients), or with conventional high-dose steroids (60 patients). ORTHOCLONE OKT3 reversed 94% of the rejections compared to a 75% reversal rate obtained with conventional high-dose steroid treatment (p=0.006). The one year Kaplan-Meier (actuarial) estimates of graft survival rates for these patients who had acute rejection were 62% and 45% for ORTHOCLONE OKT3 and steroid-treated patients, respectively (p=0.04). At two years the rates were 56% and 42%, respectively (p=0.06).

One- and two-year patient survivals were not significantly different between the two groups, being 85% and 75% for ORTHOCLONE OKT3 treated patients and 90% and 85% for steroid-treated patients.

In additional open clinical trials, the observed rate of reversal of acute renal allograft rejection was 92% (n=126) for ORTHOCLONE OKT3 therapy. ORTHOCLONE OKT3 was also effective in reversing acute renal allograft rejections in 65% (n=225) of cases where steroids and lymphocyte immune globulin preparations were contraindicated or were not successful.

The effectiveness of ORTHOCLONE OKT3 for prophylaxis of renal allograft rejection has not been established.

Acute Cardiac or Hepatic Allograft Rejection

ORTHOCLONE OKT3 was studied for use in reversing acute cardiac and hepatic allograft rejection in patients who are unresponsive to high-doses of steroids. The rate of reversal in acute cardiac allograft rejection was 90% (n = 61) and was 83% for hepatic allograft rejection (n = 124) in patients unresponsive to treatment with steroids.

Controlled randomized trials have not been conducted to evaluate the effectiveness of ORTHOCLONE OKT3 compared to conventional therapy as first line treatment for acute cardiac and hepatic allograft rejection.

INDICATIONS AND USAGE

ORTHOCLONE OKT3 is indicated for the treatment of acute allograft rejection in renal transplant patients.

ORTHOCLONE OKT3 is indicated for the treatment of steroid-resistant acute allograft rejection in cardiac and hepatic transplant patients.

The dosage of other immunosuppressive agents used in conjunction with ORTHOCLONE OKT3 should be reduced to the lowest level compatible with an effective therapeutic response. (See: WARNINGS and ADVERSE EVENTS: Infections, Neoplasia; DOSAGE AND ADMINISTRATION.)

CONTRAINDICATIONS

ORTHOCLONE OKT3 should not be given to patients who:

- are hypersensitive to this or any other product of murine origin;
- have anti-mouse antibody titers ≥1:1000;
- are in (uncompensated) heart failure or in fluid overload, as evidenced by chest X-ray or a greater than 3 percent weight gain within the week prior to planned ORTHOCLONE OKT3 administration;
- have uncontrolled hypertension;
- have a history of seizures, or are predisposed to seizures;
- are determined or suspected to be pregnant, or who are breast-feeding. (See: PRECAUTIONS: Pregnancy, Nursing Mothers.)

WARNINGS

SEE BOXED WARNING

Cytokine Release Syndrome

Samsung Bioepis Exhibit No. 1002
Page 2715

Most patients develop an acute clinical syndrome [i.e., Cytokine Release Syndrome (CRS)] that has been attributed to the release of cytokines by activated lymphocytes or monocytes and is temporally associated with the administration of the first few doses of ORTHOCLONE OKT®3 (particularly, the first two to three doses). This clinical syndrome has ranged from a more frequently reported mild, self-limited, "flu-like" illness to a less frequently reported severe, life-threatening shock-like reaction, which may include serious cardiovascular and central nervous system manifestations. The syndrome typically begins approximately 30 to 60 minutes after administration of a dose of ORTHOCLONE OKT3 (but may occur later) and may persist for several hours. The frequency and severity of this symptom complex is usually greatest with the first dose. With each successive dose of ORTHOCLONE OKT3, both the frequency and severity of the Cytokine Release Syndrome tends to diminish. Increasing the amount of ORTHOCLONE OKT3 or resuming treatment after a hiatus may result in a reappearance of the CRS.

Common clinical manifestations of CRS may include: high fever (often spiking, up to 107°F), chills/rigors, headache, tremor, nausea/vomiting, diarrhea, abdominal pain, malaise, muscle/joint aches and pains, and generalized weakness. Less frequently reported adverse experiences include: minor dermatologic reactions (e.g., rash, pruritus, etc.) and a spectrum of often serious, occasionally fatal, cardiorespiratory and central nervous system adverse experiences.

Cardiorespiratory findings may include: dyspnea, shortness of breath, bronchospasm/wheezing, tachypnea, respiratory arrest/failure/distress, cardiovascular collapse, cardiac arrest, angina/myocardial infarction, chest pain/tightness, tachycardia (including ventricular), hypertension, hemodynamic instability, hypotension including profound shock, heart failure, pulmonary edema (cardiogenic and non-cardiogenic), adult respiratory distress syndrome, hypoxemia, apnea, and arrhythmias. (See: BOXED WARNING; PRECAUTIONS; ADVERSE EVENTS.)

In the initial studies of renal allograft rejection, potentially fatal, severe pulmonary edema occurred in 5% of the initial 107 patients. Fluid overload was present before treatment in all of these cases. It occurred in none of the subsequent 311 patients treated with first-dose volume/weight restrictions. In subsequent trials and in post-marketing experience, severe pulmonary edema has occurred in patients who appeared to be euvolemic. The pathogenesis of pulmonary edema may involve all or some of the following: volume overload; increased pulmonary vascular permeability; and/or reduced left ventricular compliance/contractility. During the first 1 to 3 days of ORTHOCLONE OKT3 therapy, some patients have experienced an acute and transient decline in the glomerular filtration rate (GFR) and diminished urine output with a resulting increase in the level of serum creatinine. Massive release of cytokines appears to lead to reversible renal functional impairment and/or delayed renal allograft function. Similarly, transient elevations in hepatic transaminases have been reported following administration of the first few doses of ORTHOCLONE OKT3.

Patients at risk for more serious complications of CRS may include those with the following conditions: unstable angina; recent myocardial infarction or symptomatic ischemic heart disease; heart failure of any etiology; pulmonary edema of any etiology; any form of chronic obstructive pulmonary disease; intravascular volume overload or depletion of any etiology (e.g., excessive dialysis, recent intensive diuresis, blood loss, etc.); cerebrovascular disease; patients with advanced symptomatic vascular disease or neuropathy; a history of seizures; and septic shock. Efforts should be made to correct or stabilize background conditions prior to the initiation of therapy. (See: PRECAUTIONS.)

Prior to administration of ORTHOCLONE OKT3, the patient's volume (fluid) status and a chest X-ray should be assessed to rule out volume overload, uncontrolled hypertension, or uncompensated heart failure. Patients should not weigh >3% above their minimum weight during the week prior to injection.

The Cytokine Release Syndrome is associated with increased serum levels of cytokines (e.g., TNF-α, IL-2, IL-6, IFN-γ) that peak between 1 and 4 hours following administration of ORTHOCLONE OKT3. The serum levels of cytokines and the manifestations of CRS may be reduced by pretreatment with 8 mg/kg of methylprednisolone (i.e., high-dose steroids), given 1 to 4 hours prior to administration of the first dose of ORTHOCLONE OKT3, and by closely following recommendations for dosage and treatment duration. (See: DOSAGE AND ADMINISTRATION.) It is not known if corticosteroid pretreatment decreases organ damage and sequelae associated with CRS. For example, increased intracranial pressure and cerebral herniation have occurred despite pretreatment with currently recommended doses and schedules of methylprednisolone.

If any of the more serious presentations of the Cytokine Release Syndrome occur, intensive treatment including oxygen, intravenous fluids, corticosteroids, pressor amines, antihistamines, intubation, etc., may be required.

Central Nervous System Events

Seizures, encephalopathy, cerebral edema, aseptic meningitis, and headache have been reported, even following the first dose, during therapy with ORTHOCLONE OKT®3. Seizures, some accompanied by loss of consciousness or cardiorespiratory arrest, or death, have occurred independently or in conjunction with any of the neurologic syndromes described below.

A few cases of fatal cerebral herniations subsequent to cerebral edema have been reported. All patients, particularly pediatric patients, must be carefully evaluated for fluid retention and hypertension before the initiation of ORTHOCLONE OKT3 therapy. Close monitoring for neurologic symptoms must be performed during the first twenty - four (24) hours following each of the first few doses of ORTHOCLONE OKT3 injection.

Patients should be closely monitored for convulsions and manifestations of encephalopathy, including: impaired cognition, confusion, obtundation, altered mental status, disorientation, auditory/visual hallucinations, psychosis (delirium, paranoia), mood changes (e.g., mania, agitation, combativeness, etc.), diffuse hypotonus, hyperreflexia, myoclonus, tremor, asterixis, involuntary movements, major motor seizures, lethargy/stupor/coma, and diffuse weakness. Approximately one-third of patients with a diagnosis of encephalopathy may have had coexisting aseptic meningitis syndrome.

Signs and symptoms of the aseptic meningitis syndrome described in association with the use of ORTHOCLONE OKT3 have included: fever, headache, meningismus (stiff neck), and photophobia. Diagnosis is confirmed by cerebrospinal fluid (CSF) analysis demonstrating leukocytosis with pleocytosis, elevated protein and normal or decreased glucose, with negative viral, bacterial, and fungal cultures. The possibility of infection should be evaluated in any immunosuppressed transplant patient with clinical findings suggesting meningitis. Approximately one-third of the patients with a diagnosis of aseptic meningitis had coexisting signs and symptoms of encephalopathy. Most patients with the aseptic meningitis syndrome had a benign course and recovered without any permanent sequelae during therapy or subsequent to its completion or discontinuation. However, because meningitis is a frequent infection encountered in pediatric allograft recipients, and the immunosuppression associated with transplantation increases the risk of opportunistic infection, pediatric patients with signs or symptoms suggestive of meningeal irritation while receiving ORTHOCLONE OKT3 should have lumbar punctures performed to rule out an infectious etiology. (See: PRECAUTIONS: Pediatric Use.)

Samsung Bioepis Exhibit No. 1002
Page 2716

Signs or symptoms of encephalopathy, meningitis, seizures, and cerebral edema, with or without headache, typically have been reversible. Headache, aseptic meningitis, seizures, and less severe forms of encephalopathy resolved in most patients despite continued treatment with ORTHOCLONE OKT3. However, some events resulted in permanent neurologic impairment.

The following additional central nervous system events have each been reported: irreversible blindness, impaired vision, quadri-or paraparesis/plegia, cerebrovascular accident (hemiparesis/plegia), aphasia, transient ischemic attack, subarachnoid hemorrhage, palsy of the VI cranial nerve, hearing decrease, and deafness.

Patients who may be at greater risk for CNS adverse experiences include those: with known or suspected CNS disorders (e.g., history of seizure disorder, etc.); with cerebrovascular disease (small or large vessel); with conditions having associated neurologic problems (e.g., head trauma, uremia, infection, fluid and electrolyte disturbance, etc.); with underlying vascular diseases; or who are receiving a medication concomitantly that may, by itself, affect the central nervous system. (See: WARNINGS, PRECAUTIONS and ADVERSE EVENTS: Cytokine Release Syndrome.)

Anaphylactic Reactions

Serious and occasionally fatal, immediate (usually within 10 minutes) hypersensitivity (anaphylactic) reactions have been reported in patients treated with ORTHOCLONE OKT3. Manifestations of anaphylaxis may appear similar to manifestations of the Cytokine Release Syndrome (described above). It may be impossible to determine the mechanism responsible for any systemic reaction(s). Reactions attributed to hypersensitivity have been reported less frequently than those attributed to cytokine release. Acute hypersensitivity reactions may be characterized by: cardiovascular collapse, cardiorespiratory arrest, loss of consciousness, hypotension/shock, tachycardia, tingling, angioedema (including laryngeal, pharyngeal, or facial edema), airway obstruction, bronchospasm, dyspnea, urticaria, and pruritus.

Serious allergic events, including anaphylactic or anaphylactoid reactions, have been reported in patients re-exposed to ORTHOCLONE OKT3 subsequent to their initial course of therapy. Pretreatment with antihistamines and/or steroids may not reliably prevent anaphylaxis in this setting. Possible allergic hazards of retreatment should be weighed against expected therapeutic benefits and alternatives. If a patient is retreated with ORTHOCLONE OKT3, it is particularly important that epinephrine and other emergency life-support equipment should be immediately available.

If hypersensitivity is suspected, discontinue the drug immediately; do not resume therapy or re-expose the patient to ORTHOCLONE OKT3. Serious acute hypersensitivity reactions may require emergency treatment with 0.3 mL to 0.5 mL aqueous epinephrine (1:1000 dilution) subcutaneously and other resuscitative measures including oxygen, intravenous fluids, antihistamines, corticosteroids, pressor amines, and airway management, as clinically indicated. (See: PRECAUTIONS: Cytokine Release Syndrome vs. Anaphylactic Reactions; ADVERSE EVENTS: Hypersensitivity Reactions.)

Consequences of Immunosuppression

Serious and sometimes fatal infections and neoplasias have been reported in association with all immunosuppressive therapies, including those regimens containing ORTHOCLONE OKT®3.

Infections

ORTHOCLONE OKT3 is usually added to immunosuppressive therapeutic regimens, thereby augmenting the degree of immunosuppression. This increase in the total amount of immunosuppression may alter the spectrum of infections observed and increase the risk, the severity, and the morbidity of infectious complications. During the first month post-transplant, patients are at greatest risk for the following infections: (1) those present prior to transplant, perhaps exacerbated by post-transplant immunosuppression; (2) infection conveyed by the donor organ; and (3) the usual post-operative urinary tract, intravenous line related, wound, or pulmonary infections due to bacterial pathogens. (See: ADVERSE EVENTS: Infections.)

Approximately one to six months post-transplant, patients are at risk for viral infections [e.g., cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus (HSV), etc.] which produce serious systemic disease and which also increase the overall state of immunosuppression.

Reactivation (1 to 4 months post-transplant) of EBV and CMV has been reported. When administration of an anti-lymphocyte antibody, including ORTHOCLONE OKT3, is followed by an immunosuppressive regimen including cyclosporine, there is an increased risk of reactivating CMV and impaired ability to limit its proliferation, resulting in symptomatic and disseminated disease. EBV infection, either primary or reactivated, may play an important role in the development of post-transplant lymphoproliferative disorders. (See: WARNINGS and ADVERSE EVENTS: Neoplasia.)

In the pediatric transplant population, viral infections often include pathogens uncommon in adults, such as varicella zoster virus (VZV), adenovirus, and respiratory syncytial virus (RSV). A large proportion of pediatric patients have not been infected with the herpes viruses prior to transplantation and, therefore, are susceptible to developing primary infections from the grafted organ and/or blood products.

Geriatric patients may have a reduced capability to overcome infections during intense immunosuppression. There is no information on the use of OKT3 in geriatric patients. In an age-stratification analysis, no overall difference in the safety of OKT3 was noted between older (51 to 64 years) and younger (≤30 years) patients. Caution should be used when prescribing immunosuppressive agents to elderly patients.

Anti-infective prophylaxis may reduce the morbidity associated with certain potential pathogens and should be considered for pediatric and other high-risk patients. Judicious use of immunosuppressive drugs, including type, dosage, and duration, may limit the risk and seriousness of some opportunistic infections. It is also possible to reduce the risk of serious CMV or EBV infection by avoiding transplantation of a CMV-seropositive (donor) and/or EBV-seropositive (donor) organ into a seronegative patient.

Neoplasia

As a result of depressed cell-mediated immunity from immunosuppressive agents, organ transplant patients have an increased risk of developing malignancies. This risk is evidenced almost exclusively by the occurrence of lymphoproliferative disorders, squamous cell carcinomas of the skin and lip, and sarcomas. In immunosuppressed patients, T cell cytotoxicity is impaired allowing for transformation and proliferation of EBV-infected B lymphocytes. Transformed B lymphocytes are thought to initiate oncogenesis, which ultimately culminates in the development of most post-transplant lymphoproliferative disorders. Patients, especially pediatric patients, with primary EBV infection may be at a higher risk for the development of EBV-

Samsung Bioepis Exhibit No. 1002
Page 2717

associated lymphoproliferative disorders. Data support an association between the development of lymphoproliferative disorders at the time of active EBV infection and ORTHOCLONE OKT3 administration in pediatric liver allograft recipients. (See: ADVERSE EVENTS, Infections, Neoplasia.)

Following the initiation of ORTHOCLONE OKT3 therapy, patients should be continuously monitored for evidence of lymphoproliferative disorders through physical examination and histological evaluation of any suspect lymphoid tissue. Close surveillance is advised, since early detection with subsequent reduction of total immunosuppression may result in regression of some of these lymphoproliferative disorders. Since the potential for the development of lymphoproliferative disorders is related to the duration and extent (intensity) of total immunosuppression, physicians are advised: to adhere to the recommended dosage and duration of ORTHOCLONE OKT3 therapy; to limit the number of courses of ORTHOCLONE OKT3 and other anti- T lymphocyte antibody preparations administered within a short period of time; and, if appropriate, to reduce the dosage(s) of immunosuppressive drugs used concomitantly to the lowest level compatible with an effective therapeutic response. (See: DOSAGE AND ADMINISTRATION.)

A recent study examined the incidence of non-Hodgkin's lymphoma (NHL) among 45,000 kidney transplant recipients and over 7,500 heart transplant recipients. This study suggested that all transplant patients, regardless of the immunosuppressive regimen employed, are at increased risk of NHL over the general population. The relative risk was highest among those receiving the most aggressive regimens.

The long-term risk of neoplastic events in patients being treated with ORTHOCLONE OKT3 has not been determined.

PRECAUTIONS

General

When using combinations of immunosuppressive agents, the dose of each agent, including ORTHOCLONE OKT®3, should be reduced to the lowest level compatible with an effective therapeutic response so as to reduce the potential for and severity of infections and malignant transformations.

Fever

If the temperature of the patient exceeds 37.8°C (100°F), it should be lowered by antipyretics before administration of each dose of ORTHOCLONE OKT3. The possibility of infection should be evaluated.

Severe Cytokine Release Syndrome Versus Anaphylactic Reactions

It may not be possible to distinguish between an acute hypersensitivity reaction (e.g., anaphylaxis, angioedema, etc.) and the Cytokine Release Syndrome. Potentially serious sigus and symptoms having an immediate onset (usually within 10 minutes) following administration of ORTHOCLONE OKT3 are probably due to acute hypersensitivity. If hypersensitivity is suspected, discontinue the drug immediately; do not resume therapy or re-expose the patient to ORTHOCLONE OKT3. Clinical manifestations beginning approximately 30 to 60 minutes (or later) following administration of ORTHOCLONE OKT3 are more likely cytokine-mediated. (See: WARNINGS: Cytokine Release Syndrome, Anaphylactic Reactions.)

Central Nervous System Events

Since some seizures (and other serious central nervous system events) following ORTHOCLONE OKT3 administration have been life-threatening, anti-seizure precautions (e.g., an airway ready for use, if needed) should be taken. (See: WARNINGS and ADVERSE EVENTS: Central Nervous System Events.)

Infection/Viral-Induced Lymphoproliferative Disorders

If infection or a viral induced lymphoproliferative disorder occurs, culture or biopsy as soon as possible, promptly institute appropriate anti-infective therapy, and (if possible) reduce /discontinue immunosuppressive therapy. (See: WARNINGS, ADVERSE EVENTS.)

Low Protein-Binding Filter

Use a low protein-binding 0.2 or 0.22 micrometer (μm) filter to prepare the injections. (See: ADMINISTRATION INSTRUCTIONS.)

Sensitization

ORTHOCLONE OKT3 is a mouse (immunoglobulin) protein that can induce human anti-mouse antibody production (i.e., sensitization) in some patients following exposure; a titer ≥1:1000 is a contraindication for use. (See: WARNINGS, ADVERSE EVENTS.)

In the initial clinical trials using low doses of prednisone and azathioprine during ORTHOCLONE OKT3 therapy for renal allograft rejection, antibodies to ORTHOCLONE OKT3 were observed with an incidence of 21% (n=43) for IgM, 86% (n=43) for IgG and 29% (n=35) for IgE. The mean time of appearance of IgG antibodies was 20 ± 2 days (mean ± SD). Early IgG antibodies appeared towards the end of the second week of treatment in 3% (n=86) of the patients.

Subsequent clinical experience has shown that the dose, duration, and type of immunosuppressive medications used in combination with ORTHOCLONE OKT3 may affect both the incidence and magnitude of the host antibody response. Furthermore, immunosuppressive agents used concomitantly with ORTHOCLONE OKT3 (i.e., steroids, azathioprine, prednisone, or cyclosporine) have altered the time course of anti-mouse antibody development and the specificity of the antibodies formed (i.e., idiotypic, isotypic, allotypic).

Thrombosis

As with other immunosuppressive therapies, arterial, venous, and capillary thromboses of allografts and other vascular beds (e.g., heart, lungs, brain, bowel, etc.) have been reported in patients treated with ORTHOCLONE OKT3. In addition, microangiopathic changes (e.g., platelet microthrombi) in the

Samsung Bioepis Exhibit No. 1002
Page 2718

renal allograft associated in some patients with microangiopathic hemolytic anemia have been reported. This was observed in 5 of 93 (5%) patients receiving doses above the recommended dose. The relationship to dose remains uncertain; however, the relative risk appears to be greater with doses above the recommended dose. Patients with a history of thrombosis or underlying vascular disease should be given ORTHOCLONE OKT3 only when the potential benefits clearly outweigh the increased risks of therapy.

Information for Patients

Patients should be advised:

- of the signs and symptoms associated with the Cytokine Release Syndrome and the potentially serious nature of this syndrome (e.g., systemic, cardiovascular, central nervous system events).
- to seek medical attention for skin rash, urticaria, rapid heart beat, respiratory distress, dysphagia, or any swelling suggesting an allergic reaction or angioedema.
- that ORTHOCLONE OKT3 may impair mental alertness and coordination and may effect the ability to operate an automobile or machinery.
- of other risks associated with the use of ORTHOCLONE OKT3. (See: BOXED WARNING; WARNINGS; PRECAUTIONS; ADVERSE EVENTS.)

Laboratory Tests

The following tests should be monitored prior to and during ORTHOCLONE OKT®3 therapy:

- Renal: BUN, serum creatinine, etc.;
- Hepatic: transaminases, alkaline phosphatase, bilirubin;
- Hematopoietic: WBCs and differential, platelet count, etc.;
- Chest X-ray within 24 hours before initiating ORTHOCLONE OKT3 treatment to rule out heart failure or fluid overload.
- Blood Tests: Periodic assessment of organ system functions (renal, hepatic, and hematopoietic) should be performed.

During therapy with ORTHOCLONE OKT3: In adults, periodic monitoring to ensure plasma ORTHOCLONE OKT3 levels (≥800 ng/mL) or T cell clearance (CD3 positive T cells <25 cells/mm3) is recommended. In pediatric patients, both plasma ORTHOCLONE OKT3 levels (≥800 ng/mL) and T cell clearance (CD3 positive T cells <25 cells/mm3) should be monitored daily. (See: CLINICAL PHARMACOLOGY.)

Carcinogenesis

Long-term studies have not been performed in laboratory animals to evaluate the carcinogenic potential of ORTHOCLONE OKT3; however, neoplasia has been reported in patients receiving this product. (See: WARNINGS and ADVERSE EVENTS: Neoplasia.)

Pregnancy Category C

Animal reproductive studies have not been conducted with ORTHOCLONE OKT3. It is also not known whether ORTHOCLONE OKT3 can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. However, ORTHOCLONE OKT3 is an IgG antibody and may cross the human placenta. The effect on the fetus of the release of cytokines and/or immunosuppression after treatment with ORTHOCLONE OKT3 is not known. ORTHOCLONE OKT3 should be given to a pregnant woman only if clearly needed. If this drug is used during pregnancy, or the patient becomes pregnant while taking this drug, the patient should be apprised of the potential hazard to the fetus. (See: CONTRAINDICATIONS, WARNINGS, and ADVERSE EVENTS.)

Nursing Mothers

It is not known whether ORTHOCLONE OKT3 is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse events/oncogenesis shown for ORTHOCLONE OKT3 in human studies, a decision should be made to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother. (See: CONTRAINDICATIONS.)

Pediatric Use

Safety and effectiveness have been established in infants (1 mo. up to 2 yr.); children (2 yr. up to 12 yr.); and adolescents (12 yr. up to 16 yr.). Use of ORTHOCLONE OKT3 in these age groups is supported by clinical studies that included adults and pediatric patients. In those studies, the safety and efficacy of ORTHOCLONE OKT3 in pediatric patients receiving renal or hepatic transplants was similar to that in the overall cohort. There were insufficient data to compare the safety and efficacy of ORTHOCLONE OKT3 in pediatric patients in a study of patients receiving cardiac transplants. Additional pharmacokinetic, pharmacodynamic, and clinical studies in infants, children, and adolescents have been reported in published literature.

Pediatric patients are known to have higher CD3 lymphocyte counts than adults; therefore, progressively higher doses of ORTHOCLONE OKT3 are often required to achieve therapeutic levels of lymphocyte clearance. (See: DOSAGE AND ADMINISTRATION.)

Specific Safety Concerns in Pediatric Patients

Deaths Due to Cerebral Herniation

The postmarketing data base indicates that pediatric patients may be at increased risk of developing cerebral edema with or without herniation compared to adults. In the period between 1986 and 1996, twenty-five cases (6 in pediatric patients) of cerebral edema were identified with subsequent cerebral herniation and death in five cases (4 in pediatric patients). Herniation in the pediatric patients and one 19 year old subject occurred within a few hours to one day after the first dose (2.5 or 5 mg) of ORTHOCLONE OKT3 administered in the investigational setting for prophylaxis of renal allograft rejection.

Samsung Bioepis Exhibit No. 1002
Page 2719

All pediatric patients and especially those receiving a renal allograft must be carefully evaluated for fluid retention and hypertension before the initiation of ORTHOCLONE OKT3 therapy. (See: WARNINGS: Cytokine Release Syndrome; DOSAGE AND ADMINISTRATION: General.) Patients should be closely monitored for neurologic symptoms during the first twenty four (24) hours following each of the first few doses of ORTHOCLONE OKT3 injection.

Other Serious Central Nervous System Adverse Events

Other significant neurologic complications reported in pediatric transplant recipients receiving ORTHOCLONE OKT3 include status epilepticus, cerebral edema, diffuse encephalopathy, cerebritis, seizures, cortical dysfunction, and intracranial hemorrhage. Permanent neurologic impairments (e.g., blindness, deafness, paralysis) have been reported rarely. Because meningitis is a frequent infection encountered in pediatric allograft recipients, and the immunosuppression associated with transplantation increases the risk of opportunistic infection, patients with meningeal irritation following treatment with ORTHOCLONE OKT3 therapy should be evaluated with lumbar puncture as early as possible to rule out an infectious etiology.

Viral Infection

The overall incidence of infections appeared to be similar in pediatric patients compared to the overall population studied. In the pediatric population, viral infections often include pathogens uncommon in adults, such as varicella zoster virus (VZV), adenovirus, enterovirus, parainfluenza virus, and respiratory syncytial virus (RSV). In addition, many viral diseases often manifest differently in pediatric patients than they do in adults. Because a large proportion of pediatric patients have not been infected by herpes viruses (e.g., EBV, HSV, CMV) prior to transplantation they may be more susceptible to acquiring primary infections from the grafted organ and/or blood products when immunosuppressed. Antiviral prophylactic therapy may be particularly useful in these high risk pediatric patients. (See: ADVERSE EVENTS: Infections.)

Neoplasia

Patients with primary EBV infection may be at higher risk for the development of EBV-associated lymphoproliferative disorders. There are data to support an association between the development of lymphoproliferative disorders at the time of active EBV infection and ORTHOCLONE OKT®3 administration in pediatric liver allograft recipients. Antiviral prophylactic therapy may be particularly useful in these high risk pediatric patients.

Gastrointestinal Fluid Losses

Parenteral hydration may be required for gastrointestinal fluid loss secondary to diarrhea and/or vomiting resulting from the "Cytokine Release Syndrome."

Thrombosis

Pediatric patients may be at an increased risk of thrombosis. Pediatric patients weighing less than 15 kg are at high-risk for hepatic artery thrombosis. Thrombosis has been reported in pediatric transplant recipients treated with ORTHOCLONE OKT3. A number of factors, including surgical technique, the presence of a hypercoagnable state, and the absence of prior dialysis experience may be relevant to the pathophysiology of the increased risk of thrombosis. (See: BOXED WARNING; WARNINGS; PRECAUTIONS; ADVERSE EVENTS; DOSAGE AND ADMINISTRATION.)

Geriatric Use

There is no information on the use of OKT3 in geriatric patients. Caution should be used when prescribing immunosuppressive agents to elderly patients.

ADVERSE EVENTS

Cytokine Release Syndrome

In controlled clinical trials for treatment of acute renal allograft rejection, patients treated with ORTHOCLONE OKT3 plus concomitant low-dose immunosuppressive therapy (primarily azathioprine and corticosteroids) were observed to have an increased incidence of adverse experiences during the first two days of treatment, as compared with the group of patients receiving azathioprine and high-dose steroid therapy. During this period the majority of patients experienced pyrexia (90%), of which 19% were 40.0°C (104°F) or above, and chills (59%). In addition, other adverse experiences occurring in 8% or more of the patients during the first two days of ORTHOCLONE OKT3 therapy included: dyspnea (21%), nausea (19%), vomiting (19%), chest pain (14%), diarrhea (14%), tremor (13%), wheezing (13%), headache (11%), tachycardia (10%), rigor (8%), and hypertension (8%). A similar spectrum of clinical manifestations has been observed in open clinical studies and in post-marketing experience involving patients treated with ORTHOCLONE OKT3 for rejection following renal, cardiac, and hepatic transplantation.

Additional serious and occasionally fatal cardiorespiratory manifestations have been reported following any of the first few doses. (See: WARNINGS: Cytokine Release Syndrome; ADVERSE EVENTS: Cardiovascular, Respiratory.)

In the acute renal allograft rejection trials, potentially fatal pulmonary edema had been reported following the first two doses in less than 2% of the patients treated with ORTHOCLONE OKT3. Pulmonary edema was usually associated with fluid overload. However, post-marketing experience revealed that pulmonary edema has occurred in patients who appeared to be euvolemic, presumably as a consequence of cytokine-mediated increased vascular permeability ("leaky capillaries") and/or reduced myocardial contractility/compliance (i.e., left ventricular dysfunction). (See: WARNINGS: Cytokine Release Syndrome; DOSAGE AND ADMINISTRATION.)

Infections

In the controlled randomized renal allograft rejection trial conducted before cyclosporine was marketed, the most common infections during the first 45 days of ORTHOCLONE OKT3 therapy were due to herpes simplex virus (27%) and cytomegalovirus (19%). Other severe and life-threatening infections

were Staphylococcus epidermidis (5%), Pneumocystis carinii (3%), Legionella (2%), Cryptococcus (2%), Serratia (2%) and gram-negative bacteria (2%). The incidence of infections was similar in patients treated with ORTHOCLONE OKT3 and in patients treated with high-dose steroids.

In a clinical trial of acute hepatic allograft rejection, refractory to conventional treatment, the most common infections reported in patients treated with ORTHOCLONE OKT3 during the first 45 days of the study were cytomegalovirus (16% of patients, of which 43% of infections were severe), fungal infections (15% of patients, of which 30% were severe), and herpes simplex virus (8% of patients, of which 10% were severe). Other severe and life-threatening infections were gram-positive infections (9% of patients), gram-negative infections (8% of patients), viral infections (2% of patients), and Legionella (1% of patients). In another trial studying the use of ORTHOCLONE OKT®3 in patients with hepatic allografts, the incidence of fungal infections was 34% and infections with the herpes simplex virus was 31%.

In a clinical trial studying the use of ORTHOCLONE OKT3 in patients with acute cardiac rejection refractory to conventional treatment, the most common infections in the ORTHOCLONE OKT3 group reported during the first 45 days of the study were herpes simplex virus (5% of patients, of which 20% were severe), fungal infections (4% of patients, of which 75% were severe), and cytomegalovirus (3% of patients, of which 33% were severe). No other severe or life-threatening infections were reported during this period.

In a retrospective analysis of pediatric patients treated for acute hepatic rejection, the most common infections reported in patients treated with ORTHOCLONE OKT3 therapy were due to bacterial infections (47%), fungal infections (21%), cytomegalovirus (19%), herpes simplex virus (15%), adenovirus (8%), and Epstein-Barr virus (8%). The overall rates of viral, fungal, and bacterial infections were similar in patients treated with ORTHOCLONE OKT3 (n = 53) and in patients whose rejection was treated with steroids alone (n = 27). In another study of 149 pediatric liver allograft patients where 59 episodes of steroid-resistant rejection were treated with ORTHOCLONE OKT3, the incidence of invasive cytomegalovirus infection was higher in patients receiving ORTHOCLONE OKT3 than in those receiving steroids alone.

Clinically significant infections (e.g., pneumonia, sepsis, etc.) due to the following pathogens have been reported:

Bacterial:	Clostridium species (including perfringens), Corynebacterium, Enterococcus, Enterobacter aerogenes, Escherichia coli, Klebsiella species, Lactobacillus, Legionella, Listeria monocytogenes, Mycobacteria species, Nocardia asteroides, Proteus species, Providencia species, Pseudomonas aeruginosa, Serratia species, Staphylococcus species, Streptococcus species, Yersinia enterocolitica, and other gram-negative bacteria.
Fungal: *	Aspergillus, Candida, Cryptococcus, Dermatophytes.
Protozoa:	Pneumocystis carinii, Toxoplasma gondii.
Viral:	cytomegalovirus* (CMV), Epstein-Barr virus* (EBV), herpes simplex virus* (HSV), hepatitis viruses, varicella zoster virus (VZV), adenovirus, enterovirus, respiratory syncytial virus (RSV), parainfluenza virus.

As a consequence of being a potent immunosuppressive, the incidence and severity of infections with designated(*) pathogens, especially the herpes family of viruses, may be increased. (See: WARNINGS: Infections.)

Neoplasia

In patients treated with ORTHOCLONE OKT3, post-transplant lymphoproliferative disorders have ranged from lymphadenopathy or benign polyclonal B cell hyperplasias to malignant and often fatal monoclonal B cell lymphomas. In post-marketing experience, approximately one-third of the lymphoproliferations reported were benign and two-thirds were malignant. Lymphoma types included: B cell, large cell, polyclonal, non-Hodgkin's, lymphocytic, T cell, Burkitt's. The majority were not histologically classified. Malignant lymphomas appear to develop early after transplantation, the majority within the first four months post-treatment. Many of these have been rapidly progressive. Some were fulminant, involving the allografted organ and were widely disseminated at the time of diagnosis. Carcinomas of the skin included: basal cell, squamous cell, sarcoma, melanoma, and keratoacanthoma. Other neoplasms infrequently reported include: multiple myeloma, leukemia, carcinoma of the breast, adenocarcinoma, cholangiocarcinoma, and recurrences of pre-existing hepatoma and renal cell carcinoma. (See: WARNINGS: Neoplasia.)

Hypersensitivity Reactions

Reported adverse reactions resulting from the formation of antibodies to ORTHOCLONE OKT3 have included antigen-antibody (immune complex) mediated syndromes and IgE-mediated reactions. Hypersensitivity reactions have ranged from a mild, self-limited rash or pruritus to severe, life-threatening anaphylactic reactions/shock or angioedema (including: swelling of lips, eyelids, laryngeal spasm and airway obstruction with hypoxia). (See: WARNINGS: Anaphylactic Reactions.)

Other hypersensitivity reactions have included: ineffectiveness of treatment, serum sickness, arthritis, allergic interstitial nephritis, immune complex deposition resulting in glomerulonephritis, vasculitis (including temporal and retinal), and eosinophilia.

Adverse Reactions by Body System

Adverse events reported in greater than or equal to 1% of clinical trial patients treated with ORTHOCLONE OKT3 (n=393) are shown in Table 1:

Table 1: Adverse Events Reported in Clinical Trials (≥1% incidence, n=393)

Body System	Incidence (%)
Autonomic Nervous System Disorders	
Diaphoresis	7
Vasodilation	7
Body as a Whole, General Disorders	
Anorexia	4
Asthenia	10
Chills	43

Samsung Bioepis Exhibit No. 1002
Page 2721

Body System	Incidence (%)
Fatigue	9
Lethargy	6
Malaise	5
Pain, trunk	6
Pyrexia	77
Cardiovascular Disorders, General	
Arrhythmia	4
Bradycardia	4
Hypertension	19
Hypotension	25
Pain, chest	9
Tachycardia	26
Vascular Occlusion	2
Central and Peripheral Nervous System Disorders	
Convulsions	1
Dizziness	6
Headache	28
Meningitis	1
Tremor	14
Gastrointestinal System Disorders	
Diarrhea	37
Nausea	32
Pain, abdominal	6
Pain, GI	7
Vomiting	25
Hematopoietic Disorders	
Anemia	2
Leukocytosis	1
Thrombocytopenia	2
Metabolic and Nutritional Disorders	
Edema	12
Musculoskeletal System Disorders	
Arthralgia	7
Myalgia	1
Psychiatric Disorders	
Confusion	6
Depression	3
Nervousness	5
Somnolence	2
Renal Disorders	
Renal Dysfunction	3
Respiratory System Disorders	
Abnormal Chest Sound	10
Dyspnea	16
Hyperventilation	7
Hypoxia	1
Pneumonia	1
Pulmonary Edema	2
Respiratory Congestion	4
Wheezing	6
Skin and Appendages Disorders	
Pruritus	7
Rash	14
Rash Erythematous	2
Special Senses	
Photophobia	1
Tinnitus	1

Samsung Bioepis Exhibit No. 1002
Page 2722

Body System	Incidence (%)
White Cell and Reticuloendothelial System Disorders	
Leukopenia	7

Selected Adverse Events Reported In Clinical Trials

Cardiovascular Disorders, General: Angina, Cardiac Arrest, Fluctuation in Blood Pressure, Heart Failure, Myocardial Infarction, Shock, Thrombosis.

Central and Peripheral Nervous System Disorders: Coma, Encephalopathy, Epilepsy, Hypotonia.

Gastrointestinal Disorders: Gastrointestinal Hemorrhage.

Hemapoietic Disorders: Coagulation Disorder, Lymphadenopathy, Lymphopenia.

Hepatobiliary: Hepatitis, SGOT Increased, SGPT Increased.

Psychiatric Disorders: Hallucinations, Mood Changes, Paranoia, Psychosis.

Renal Disorders: Anuria, Oliguria.

Respiratory System Disorders: Apnea, Pneumonitis.

Special Senses: Conjunctivitis, Hearing Decrease.

Worldwide Postmarketing Experience - Body Systems/Events Listed Alphabetically

Body as a Whole, General Disorders: Fever (including spiking temperatures as high as 107°F), Flu-like Syndrome.

Cardiovascular Disorders: Cardiovascular Collapse, Hemodynamic Instability, Left Ventricular Dysfunction.

Central and Peripheral Nervous System Disorders: Agitation, Aphasia, Asterixis, Cerebritis, Cerebral Edema, Cerebral Herniation, Cerebrovascular Accident, CNS Infection, CNS Malignancy, Cranial Nerve VI Palsy, Encephalitis, Hyperreflexia, Involuntary Movements, Intracranial Hemorrhage, Impaired Cognition, Myoclonus, Obnubilation, Paresis/plegia including quadriparesis/plegia, Status Epilepticus, Stupor, Transient Ischemic Attack, Vertigo.

In a post-marketing survey involving 214 renal transplant patients, the incidence of aseptic meningitis syndrome was 6%. Fever (89%), headache (44%), neck stiffness (14%), and photophobia (10%) were the most commonly reported symptoms; a combination of these four symptoms occurred in 5% of patients.

Between 1987 and 1992, 75 post-marketing reports have described seizures, averaging about 12 per year, and including 23 fatalities. More than two-thirds of these reports (53) were of domestic spontaneous origin, and their age and sex distributions were broad. Post-licensure reports generally provide insufficient data to allow accurate estimation of risk or of incidence.

Gastrointestinal Disorders: Bowel Infarction.

Hematopoietic Disorders: Aplastic anemia, Arterial, Venous and Capillary Thrombosis of allografts and other vascular beds e.g., heart, lung, brain and bowel etc., Disseminated Intravascular Coagulation, Microangiopathic Changes (e.g., platelet microthrombi), Microangiopathic Hemolytic Anemia, Neutropenia, Pancytopenia.

Hepatobiliary: Hepatitis or Hepato/splenomegaly, usually secondary to viral infection or lymphoma.

Musculoskeletal Disorders: Arthritis, Stiffness/Aches/Pains.

Renal Disorders: Azotemia, Abnormal Urinary Cytology including exfoliation of damaged lymphocytes, collecting duct cells and cellular casts, Delayed Graft Function, Renal Insufficiency/Renal Failure, usually transient and reversible and occasionally in association with Cytokine Release Syndrome.

Respiratory System Disorders: Adult Respiratory Distress Syndrome, Respiratory Arrest, Respiratory Failure.

Skin and Appendages: Erythema, Flushing, Stevens-Johnson Syndrome, Urticaria.

Special Senses: Blindness, Blurred Vision, Deafness, Diplopia, Otitis Media, Nasal and Ear Stuffiness, Papilledema.

OVERDOSAGE

Symptoms of overdosage with ORTHOCLONE OKT®3 may include hyperthermia, severe chills, myalgia, vomiting, diarrhea, edema, oliguria, pulmonary edema, and acute renal failure. A high incidence (5%) of microangiopathic hemolytic anemia/HUS syndrome in patients receiving 10 mg per day of ORTHOCLONE OKT3 was also reported. In the event of acute overdosage with ORTHOCLONE OKT3, the patient should be carefully observed and given symptomatic and supportive treatment.

DOSAGE AND ADMINISTRATION

Samsung Bioepis Exhibit No. 1002
Page 2723

Adults

The recommended dose of ORTHOCLONE OKT3 for the treatment of acute renal, steroid-resistant cardiac, or steroid-resistant hepatic allograft rejection is 5 mg per day in a single (bolus) intravenous injection in less than one minute for 10 to 14 days. For acute renal rejection, treatment should begin upon diagnosis. For steroid-resistant cardiac or hepatic allograft rejection, treatment should begin when the treating physician deems a rejection has not been reversed by an adequate course of corticosteroid therapy. (See: CLINICAL PHARMACOLOGY; PRECAUTIONS: Sensitization, Laboratory Tests.)

Pediatric Patients

The initial recommended dose is 2.5 mg per day in pediatric patients weighing less than or equal to 30 kg and 5 mg per day in pediatric patients weighing greater than 30 kg in a single (bolus) intravenous injection in less than one minute for 10 to 14 days. Daily increases in ORTHOCLONE OKT3 doses (i.e., 2.5 mg increments) may be required to achieve depletion of CD3 positive cells (<25 cells/mm3) and ensure therapeutic ORTHOCLONE OKT3 serum concentrations (> 800 ng/mL). Pediatric patients may require augmentation of the ORTHOCLONE OKT3 dose. For acute renal rejection, treatment should begin upon diagnosis. For steroid-resistant cardiac or hepatic allograft rejection, treatment should begin when the treating physician deems a rejection has not been reversed by an adequate course of corticosteroid therapy. (See: CLINICAL PHARMACOLOGY; PRECAUTIONS: Laboratory Tests; Pediatric Use.)

General

For the first few doses, patients should be monitored in a facility equipped and staffed for cardiopulmonary resuscitation (CPR). Patients receiving subsequent doses of ORTHOCLONE OKT3, should also be monitored in a facility equipped and staffed for CPR. Vital signs should be monitored frequently. Patients receiving ORTHOCLONE OKT3 should also be carefully monitored for signs and symptoms of Cytokine Release Syndrome, particularly after the first few doses but also after a treatment hiatus with resumption of therapy. The patient's temperature should be lowered to <37.8°C (100°F) before the administration of any dose of ORTHOCLONE OKT3.

Prior to administration of ORTHOCLONE OKT3, the patient's volume status should be assessed carefully. It is imperative, especially prior to the first few doses, that there be no clinical evidence of volume overload, uncontrolled hypertension, or uncompensated heart failure. Patients should have a clear chest X-ray and should not weigh more than 3% above their minimum weight during the week prior to injection.

To decrease the incidence and severity of Cytokine Release Syndrome, associated with the first dose of ORTHOCLONE OKT3, it is strongly recommended that methylprednisolone sodium succinate 8.0 mg/kg be administered intravenously 1 to 4 hours prior to the initial dose of ORTHOCLONE OKT3. Acetaminophen and antihistamines given concomitantly with ORTHOCLONE OKT3 may also help to reduce some early reactions. (See: WARNINGS and ADVERSE EVENTS: Cytokine Release Syndrome.)

When using concomitant immunosuppressive drugs, the dose of each should be reduced to the lowest level compatible with an effective therapeutic response in order to reduce the potential for malignancy and infections. Maintenance immunosuppression should be resumed approximately three days prior to the cessation of ORTHOCLONE OKT®3 therapy. (See: WARNINGS and ADVERSE EVENTS: Infection, Neoplasia.)

Reduced T cell clearance or low plasma ORTHOCLONE OKT3 levels provide a basis for adjusting ORTHOCLONE OKT3 dosage or for discontinuing therapy. (See: WARNINGS: Anaphylactic Reactions; PRECAUTIONS: Laboratory Tests; ADVERSE EVENTS: Hypersensitivity Reactions.)

Administration Instructions

1. Before administration, ORTHOCLONE OKT3 should be inspected for particulate matter and discoloration. Because ORTHOCLONE OKT3 is a protein solution, it may develop fine translucent particles (shown not to affect potency).
2. No bacteriostatic agent is present in this product. Adherence to aseptic technique is advised. Once the ampule is opened, use immediately and discard the unused portion.
3. Prepare ORTHOCLONE OKT3 for injection by drawing solution into a syringe through a low protein-binding 0.2 or 0.22 micrometer (μm) filter. Detach filter and attach a new needle for a single intravenous (bolus) injection.
4. Because no data is available on compatibility of ORTHOCLONE OKT3 with other intravenous substances or additives, other medications/substances should not be added or infused simultaneously through the same intravenous line. If the same intravenous line is used for sequential infusion of several different drugs, the line should be flushed with saline before and after injection of ORTHOCLONE OKT3.
5. Administer ORTHOCLONE OKT3 as a single intravenous (bolus) injection in less than one minute. Do not administer by intravenous infusion or in conjunction with other drug solutions.

HOW SUPPLIED

ORTHOCLONE OKT3 is supplied as a sterile solution in packages of 5 ampules (NDC 59676-101-01). Each 5 mL ampule contains 5 mg of muromonab-CD3.

Storage

Store in a refrigerator at 2° to 8°C (36° to 46°F).

DO NOT FREEZE OR SHAKE.

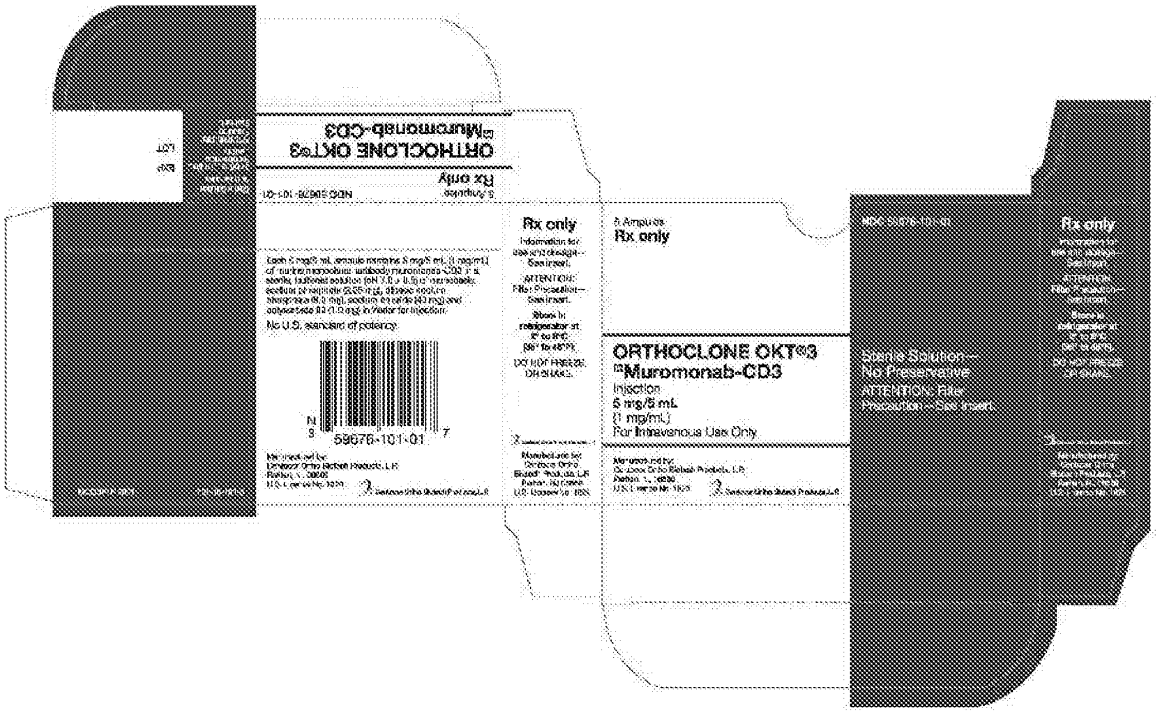
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Samsung Bioepis Exhibit No. 1002
Page 2724

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Raritan, New Jersey 08869
U.S.A.
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Revised: March 2010



PRINCIPAL DISPLAY PANEL - 5mg/5ml

NDC 59676–101–01

5 Ampules

Rx only

ORTHOCLONE OKT®3

PrMuromonab-CD3

Injection

5 mg/5 mL

(1mg/mL)

For Intravenous Use Only

Manufactured by:

Centocor Ortho Biotech Products, L.P.

Raritan, NJ 08869

U.S. License No. 1824

Centocor Ortho Biotech Products, L.P.

Sterile Solution-

No Preservative

ATTENTION: Filter

Precaution-See Insert.

OKT3
muromonab-cd3 injection, solution

Product Information			
Product Type	HUMAN PRESCRIPTION DRUG	NDC Product Code (Source)	59676-101
Route of Administration	INTRAVENOUS	DEA Schedule	

Active Ingredient/Active Moiety

Ingredient Name	Basis of Strength	Strength
MUROMONAB-CD3 (MUROMONAB-CD3)	MUROMONAB-CD3	5 mg in 5 mL

Inactive Ingredients

Ingredient Name	Strength
sodium chloride	43 mg in 5 mL
SODIUM PHOSPHATE, MONOBASIC	2.25 mg in 5 mL
WATER	
SODIUM PHOSPHATE, DIBASIC	9.0 mg in 5 mL
polysorbate 80	1.0 mg in 5 mL

Product Characteristics

Color		Score	
Shape		Size	
Flavor		Imprint Code	
Contains			

Packaging

#	NDC	Package Description	Multilevel Packaging
1	59676-101-01	5 AMPULE In 1 CARTON	contains a AMPULE (59676-101-00)
1	59676-101-00	5 mL In 1 AMPULE	This package is contained within the CARTON (59676-101-01)

Marketing Information

Marketing Category	Application Number or Monograph Citation	Marketing Start Date	Marketing End Date
BLA	BLA103463	07/19/1986	

Labeler - Centocor Ortho Biotech, L.P. (804684207)

Establishment

Name	Address	ID/FEI	Operations

Samsung Bioepis Exhibit No. 1002
Page 2726

Centocor Ortho Biotech Products, LP	804684207	API MANUFACTURE, MANUFACTURE

Establishment Name	Address	ID/FEI	Operations
Ortho-McNeil-Janssen Pharmaceuticals, Inc.		010779978	ANALYSIS

Revised: 02/2010 Centocor Ortho Biotech, L.P.

Samsung Bioepis Exhibit No. 1002
Page 2727

REMICADE™

INFLIXIMAB

for IV Injection

DESCRIPTION:

Remicade (Infliximab) is a chimeric IgG1κ monoclonal antibody with an approximate molecular weight of 149,100 daltons. It is composed of human constant and murine variable regions. Infliximab binds specifically to human tumor necrosis factor alpha (TNFα) with an association constant of 10^{10} M^{-1}. Infliximab is produced by a recombinant cell line cultured by continuous perfusion and is purified by a series of steps that includes measures to inactivate and remove viruses.

Remicade is supplied as a sterile, white, lyophilized powder for intravenous infusion. Following reconstitution with 10 mL of Sterile Water for Injection, USP, the resulting pH is approximately 7.2. Each single-use vial contains 100 mg Infliximab, 500 mg sucrose, 0.5 mg polysorbate 80, 2.2 mg monobasic sodium phosphate and 6.1 mg dibasic sodium phosphate. No preservatives are present.

CLINICAL PHARMACOLOGY:

General

Infliximab neutralizes the biological activity of TNFα by binding with high affinity to the soluble and transmembrane forms of TNFα and inhibits binding of TNFα with its receptors.[1-3] Infliximab does not neutralize TNFβ (lymphotoxin α),[1] a related cytokine that utilizes the same receptors as TNFα. Biological activities attributed to TNFα include: induction of pro-inflammatory cytokines such as IL-1 and IL-6, enhancement of leukocyte migration by increasing endothelial layer permeability and expression of adhesion molecules by endothelial cells and leukocytes, activation of neutrophil and eosinophil functional activity, and induction of acute phase and other liver proteins.[4] Cells expressing transmembrane TNFα bound by Infliximab can be lysed *in vitro* by complement or effector cells.[2] Anti-TNFα antibodies reduce disease activity in a cotton-top tamarin colitis model.[5] Infliximab inhibits the functional activity of TNFα in a wide variety of *in vitro* bioassays utilizing human fibroblasts, endothelial cells, neutrophils,[3] B and T lymphocytes[6,7] and epithelial cells.[8]

Pharmacodynamics

Elevated concentrations of TNFα have been found in the stools of Crohn's disease patients and correlate with elevated disease activity.[9] Treatment with Infliximab reduced infiltration of inflammatory cells and TNFα production in inflamed areas of the intestine,[8] and reduced the proportion of mononuclear cells from the lamina propria able to express TNFα and interferon γ.[10] After treatment with Infliximab, patients with Crohn's disease have decreased levels of serum IL-6 and C-reactive protein compared to baseline.[8] Peripheral blood lymphocytes from Infliximab-treated patients, however, showed no decrease in proliferative responses to *in vitro* mitogenic stimulation when

Samsung Bioepis Exhibit No. 1002
Page 2728

compared to cells from untreated patients.[8]

Pharmacokinetics

Data from a study of single intravenous infusions of 1, 5, 10 or 20 mg/kg showed a direct and linear relationship between the dose administered and the maximum serum concentration (C_{max}) and area under the concentration-time curve. The volume of distribution at steady state (V_d), clearance and mean residence time were independent of the administered dose. Infliximab has a prolonged terminal half-life and is predominantly distributed within the vascular compartment. A single infusion of the recommended dose of 5 mg/kg resulted in a median C_{max} of 118 µg/mL, a median V_d equal to 3.0 liters and a terminal half-life of 9.5 days. During the clinical studies, no pharmacokinetic differences were observed in patient subgroups defined by gender, age, weight or hepatic or renal function. Corticosteroid use significantly increased the V_d of Infliximab from 2.8 to 3.3 liters (a 17% increase, possibly secondary to corticosteroid-mediated changes in electrolyte balance and fluid retention). No evidence of accumulation was observed after repeated dosing in patients with fistulizing disease given 5 mg/kg Infliximab at weeks 0, 2 and 6, or in patients with moderate or severe Crohn's disease retreated with 4 infusions of 10 mg/kg Infliximab at 8-week intervals.

CLINICAL STUDIES:

Active Crohn's Disease

The safety and efficacy of Infliximab were assessed in a randomized, double-blind, placebo-controlled dose ranging study of 108 patients with moderate to severe active Crohn's disease[11] [Crohn's Disease Activity Index (CDAI) ≥220≤400].[12] All patients had experienced an inadequate response to prior conventional therapies, including corticosteroids (60% of patients), 5-aminosalicylates (5-ASA)(60%) and/or 6-mercaptopurine/azathioprine (6-MP/AZA)(37%). Concurrent use of stable dose regimens of corticosteroids, 5-ASA, 6-MP and/or AZA was permitted and 92% of patients continued to receive at least one of these medications.

The study was divided into three phases. In the first phase, patients were randomized to receive a single intravenous (IV) dose of either placebo, 5, 10 or 20 mg/kg of Infliximab. The primary endpoint was the proportion of patients who experienced a clinical response, defined as a decrease in CDAI by ≥70 points from baseline at the 4-week evaluation and without an increase in Crohn's disease medications or surgery for Crohn's disease. Patients who responded at week 4 were followed to week 12. Secondary endpoints included the proportion of patients who were in clinical remission at week 4 (CDAI <150), and clinical response over time.

At week 4, four of twenty-five (16%) of the placebo patients achieved a clinical response vs. twenty-two of twenty-seven (82%) of the patients at 5 mg/kg Infliximab ($p < 0.001$, two-sided, Fisher's Exact test). One of twenty-five (4%) placebo patients and thirteen of twenty-seven (48%) patients receiving 5 mg/kg Infliximab achieved a CDAI <150 at week 4. The maximum response to any dose of Infliximab was observed within 2 to 4 weeks. The proportion of patients in response gradually diminished over the 12 weeks of the evaluation period. There was no evidence of a dose response; doses higher than 5 mg/kg did not result in a greater proportion of responders. Results are shown in Figure 1.

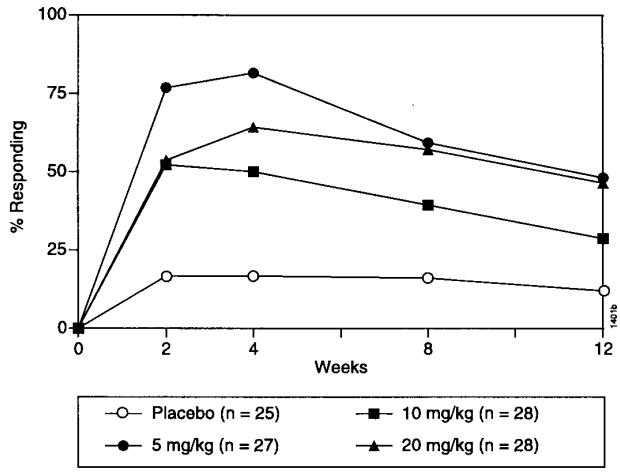


Figure 1. Response (≥70 point decrease in CDAI) to a Single IV Infliximab or Placebo Dose

During the 12-week period following infusion, patients treated with Infliximab compared to placebo demonstrated improvement in quality of life as measured by the Inflammatory Bowel Disease Questionaire.[13]

In the second phase, 29 patients who did not respond to the single dose of 5, 10 or 20 mg/kg of Infliximab entered the open label phase and received a single 10 mg/kg dose of Infliximab 4 weeks after the initial dose. Ten of twenty-nine (34%) patients experienced a response 4 weeks after receiving the second dose.

Patients, who remained in clinical response at week 8 during the first or second phase, were eligible for the retreatment phase. Seventy-three patients were re-randomized at week 12 to receive 4 infusions of placebo or 10 mg/kg Infliximab at 8-week intervals (weeks 12, 20, 28, 36) and were followed to week 48. In the limited data set available, no significant differences were observed between the Infliximab and placebo-treated groups..

Fistulizing Crohn's Disease

The safety and efficacy of Infliximab were assessed in a randomized, double-blind, placebo controlled study of 94 patients with fistulizing Crohn's disease with fistula(s) that were of at least 3 months duration. Concurrent use of stable doses of corticosteroids, 5-ASA, antibiotics, methotrexate, 6-MP and/or AZA was permitted, and 83% of patients continued to receive at least one of these medications. Fifty-two (55%) had multiple cutaneously draining fistulas; 90% of patients had fistula(s) in the perianal area and 10% had abdominal fistula(s).

Patients received 3 doses of either placebo, 5 or 10 mg/kg Infliximab at weeks 0, 2 and 6 and were followed up to 26 weeks. The primary endpoint was the proportion of patients who experienced a clinical response, defined as ≥50% reduction from baseline in the number of fistula(s) draining upon gentle compression, on at least two consecutive visits, without an increase in medication for Crohn's disease, or surgery for Crohn's disease.

Eight of thirty-one (26%) patients in the placebo arm achieved a clinical response vs. twenty-one of the thirty-one (68%) patients in the 5 mg/kg Infliximab arm (p = 0.002, two-sided, Fisher's Exact test).

Eighteen of thirty-two (56%) patients in the 10 mg/kg arm achieved a clinical response.

The median time to onset of response in the Infliximab-treated group was 2 weeks. The median duration of response was 12 weeks; after 22 weeks there was no difference between either dose of Infliximab and placebo in the proportion of patients in response (Figure 2). New fistula(s) developed in approximately 15% of both Infliximab and placebo-treated patients.

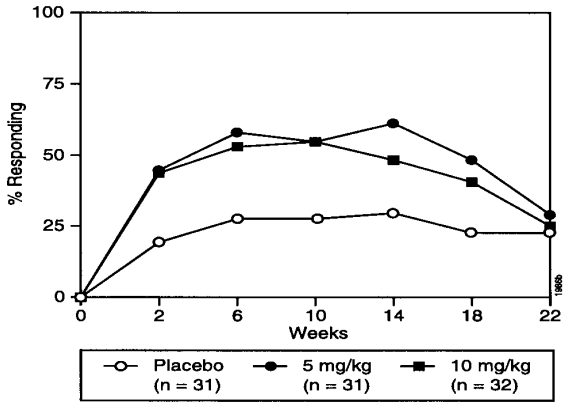


Figure 2. Response [fistula(s) closure] with Three Doses of Infliximab or Placebo

Seven of sixty (12%) evaluable Infliximab-treated patients, compared to one of thirty-one (3.5%) placebo-treated patients, developed an abscess in the area of fistulas between 8 and 16 weeks after the last infusion of Infliximab. Six of the Infliximab patients who developed an abscess had experienced a clinical response. (See *ADVERSE REACTIONS, Infections*).

Dose regimens other than dosing at weeks 0, 2 and 6 have not been studied. Studies have not been done to assess the effects of Infliximab on healing of the internal fistular canal, on closure of non-cutaneously draining fistulas (e.g., entero-entero), or on cutaneously draining fistulas in locations other than perianal and periabdominal.

INDICATIONS AND USAGE:

Infliximab is indicated for:

1. treatment of moderately to severely active Crohn's disease for the reduction of the signs and symptoms, in patients who have an inadequate response to conventional therapy.

   The safety and efficacy of therapy continued beyond a single dose have not been established (See *DOSAGE AND ADMINISTRATION*).

2. treatment of patients with fistulizing Crohn's disease for the reduction in the number of draining enterocutaneous fistula(s).

   The safety and efficacy of therapy continued beyond three doses have not been studied

(See *DOSAGE AND ADMINISTRATION*).

CONTRAINDICATIONS:

Infliximab should not be administered to patients with known hypersensitivity to any murine proteins or other component of the product.

WARNINGS:

Hypersensitivity

Infliximab has been associated with hypersensitivity reactions. Urticaria, dyspnea and hypotension have occurred in association with Infliximab infusion. Infliximab should be discontinued for severe reactions. Medications for the treatment of hypersensitivity reactions (e.g., acetaminophen, antihistamines, corticosteroids and/or epinephrine) should be available for immediate use in the event of a reaction (see *ADVERSE REACTIONS, Infusion-related Reactions).*

Autoimmunity

Anti-TNF therapy may result in the formation of autoimmune antibodies and, rarely, in the development of a lupus-like syndrome. If a patient develops symptoms suggestive of a lupus-like syndrome following treatment with Infliximab and is positive for antibodies against double-stranded DNA, treatment should be discontinued (see *ADVERSE REACTIONS, Auto-antibodies/Lupus-like Syndrome).* In clinical trials, patients who developed anti-double-stranded DNA (dsDNA) antibodies and/or symptoms suggestive of a lupus-like syndrome have had resolution of symptoms and disappearance of the anti-dsDNA after discontinuation of Infliximab therapy.

PRECAUTIONS:

Immunosuppression

TNFα mediates inflammation and modulates cellular immune response; therefore, the possibility exists for anti-TNF therapies, including Infliximab, to affect normal immune responses.

Malignancy/Infection

Patients with long duration of Crohn's disease and chronic exposure to immunosuppressant therapies are more prone to develop lymphomas and infections (see *ADVERSE REACTIONS, Lymphoproliferative Disorders and Infections).* The impact of Infliximab treatment on these phenomena is unknown.

Human Antichimeric Antibody (HACA) Development

One-hundred thirty-four of the 199 Crohn's disease patients treated with Infliximab were evaluated for HACA; 18 (13%) were HACA-positive (the majority at low titer, ≤1:20). Patients who were HACA-positive were more likely to experience an infusion reaction (see *ADVERSE REACTIONS, Infusion-related Reactions*). The incidence of positive HACA responses was lower among Crohn's disease patients receiving immunosuppressant therapies such as 6-MP, AZA or corticosteroids [10/99 (10%)

of these patients developed positive HACA responses] than among those not receiving these agents [8/35 (23%) of these patients developed positive HACA responses].

Drug Interactions

Specific studies on drug interactions with Infliximab have not been conducted. The majority of patients in Crohn's disease clinical trials received one or more of the following concomitant medications: antibiotics, antivirals, corticosteroids, 6-MP/AZA and aminosalicylates, all medications commonly used in Crohn's disease. Patients receiving immunosuppressants tended to experience fewer infusion reactions as compared to patients on no immunosuppressants (see *PRECAUTIONS, HACA Development* and *ADVERSE REACTIONS, Infusion-related Reactions).*

Carcinogenesis, Mutagenesis and Impairment of Fertility

Long-term studies in animals have not been performed to evaluate the carcinogenic potential or effects on potential impairment of fertility in male and female animals. No clastogenic or mutagenic effects of Infliximab were observed in the *in vivo* mouse micronucleus test or the *Salmonella-Escherichia coli* (Ames) assay, respectively. Chromosomal aberrations were not observed in an assay performed using human lymphocytes.

Pregnancy Category C

Since Infliximab does not cross-react with TNFα in species other than humans and chimpanzees, animal reproduction studies have not been conducted with Infliximab. It is not known whether Infliximab can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Infliximab should be given to a pregnant woman only if clearly needed. In a developmental toxicity study conducted in mice using an analogous antibody that selectively inhibits the functional activity of mouse TNFα, no evidence of maternal toxicity, embryotoxicity or teratogenicity was observed.

Nursing Mothers

It is not known whether Infliximab is excreted in human milk or absorbed systemically after ingestion. Because many drugs and immunoglobulins are excreted in human milk, and because of the potential for serious adverse reactions in nursing infants from Infliximab, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.

Pediatric Use

Safety and effectiveness of Infliximab in pediatric patients have not been established.

Geriatric Use

Clinical studies of Infliximab did not include sufficient numbers of Crohn's disease patients aged 65 and over to determine whether they respond differently from patients aged 18 to 65. As the impact of Infliximab treatment on the incidence of infections is unknown and there is a higher incidence of infections in the elderly population in general, caution should be used in treating the elderly[14] (see

ADVERSE REACTIONS, Infections).

ADVERSE REACTIONS:

In clinical trials, a total of 533 patients were treated with Infliximab doses up to 20 mg/kg; 199 in Crohn's disease trials, 334 in investigational trials for other disease. Approximately one-third of the patients received only a single infusion of Infliximab while the remaining patients received multiple infusions, up to a maximum of five. One-hundred fifty-eight patients were followed for at least 2 years, of whom only 30 patients were in Crohn's disease trials.

In studies in Crohn's disease, approximately 5% of patients discontinued scheduled Infliximab infusions due to an adverse experience. The most common reasons for discontinuation of treatment were infusion reactions and infections.

Infusion-related Reactions

An infusion reaction was defined as any adverse event occurring during the infusion or the 2-hour post-infusion observation period. Sixteen percent of Infliximab-treated patients in all clinical trials experienced an infusion reaction compared to 6% of placebo-treated patients. Among the 1,207 Infliximab infusions, 5% (58/1207) were accompanied by nonspecific symptoms such as fever or chills, 1% were accompanied by pruritus or urticaria, 1% were accompanied by cardiopulmonary reactions (primarily chest pain, hypotension, hypertension or dyspnea), and 0.2% were accompanied by combined symptoms of pruritus/urticaria and cardiopulmonary reactions. Nine infusion reactions resulted in discontinuation of Infliximab; all patients recovered with treatment and/or discontinuation of infusion. Seven percent of patients experienced an infusion reaction during the initial infusion and 10% experienced an infusion reaction during the second Infliximab infusion. Subsequent Infliximab infusions beyond two were not associated with a higher incidence of reactions.

Patients who became positive for human antichimeric antibodies (HACA) were more likely to develop infusion reactions than those who remained HACA negative (36% vs. 11% respectively). Use of concomitant immunosuppressant agents appeared to reduce the frequency of infusion

reactions (see *PRECAUTIONS, Human Antichimeric Antibody Development and Drug Interactions).*

Infections

Among all placebo-controlled trials evaluating Infliximab, infections were reported by 21% of Infliximab-treated patients (average of 22 weeks of follow-up) and by 11% of placebo-treated patients (average of 12 weeks of follow-up). Fifteen Infliximab-treated patients (3%) reported serious infections, including: suspected pneumonia; cellulitis; and infection at a central venous catheter, sepsis, cholecystitis, endophthalmitis, and furunculosis. Two placebo-treated patients (2%) reported serious infections requiring antibiotic therapy. (See *PRECAUTIONS, Malignancy/Infection*).

Twelve percent of patients with fistulizing Crohn's disease, developed a new abscess 8 to 16 weeks after the last infusion with Infliximab (see *CLINICAL STUDIES, Fistulizing Crohn's Disease*).

Auto-Antibodies /Lupus-like Syndrome

Samsung Bioepis Exhibit No. 1002
Page 2734

Of the Infliximab-treated patients evaluated for antinuclear antibodies (ANA), the percentage of patients positive for ANA increased from 24% (85/357) at screening to 36% (128/357) at the last evaluation. Anti-dsDNA antibodies developed in approximately 9% of patients treated with Infliximab. No association of total Infliximab exposure with the development of anti-dsDNA antibodies was noted. However, particularly in Crohn's disease patients, baseline therapy with an immunosuppressant of any type reduced development of anti-dsDNA antibodies [3% (4/115) of patients compared to 21% (10/48) of patients not receiving any immunosuppressant]. Crohn's disease patients were approximately 2 times more likely to develop anti-dsDNA antibodies if they were ANA-positive at study entry.

Two patients developed clinical signs consistent with a lupus-like syndrome. One of these, a non-Crohn's disease patient, developed dyspnea and pleuropericarditis with resolution of symptoms within 6 to 8 weeks of initiation of treatment with oral corticosteroids. The other, a Crohn's disease patient, developed lupus arthritis, also responsive to corticosteroids, with symptoms resolving within 6 months after the last Infliximab infusion (see *WARNINGS, Autoimmunity*).

No other autoimmune disorders were reported in patients followed for 6 months to 3 years after receiving their last infusion.

Lymphoproliferative Disorders

In 394 Infliximab-treated patients (from all studies) who were followed for 6 months to 3 years after receiving their last infusion, one case of lymphoma occurred in a Crohn's disease patient and 2 cases occurred in non-Crohn's patients. One case of myeloma occurred in a non-Crohn's disease patient. One additional case of lymphoma occurred in a non-Crohn's patient with HIV/AIDS during a shorter period of follow up. The data are insufficient to determine if an association exists between the occurrence of lymphomas and the dose or duration of exposure to Infliximab. The lymphomas reported occurred in patients with a long duration of disease and chronic exposure to immunosuppressant therapies, a population at greater risk for development of malignancy[15,16] (see *PRECAUTIONS, Malignancy/Infection*).

Other Adverse Reactions

Table 1 lists the adverse reactions occurring at a frequency of at least 5% in the Crohn's disease trials.

Table 1
ADVERSE REACTIONS IN CROHN'S DISEASE TRIALS

	Placebo (n =56)	Infliximab (n = 199)
Avg. wks of follow-up	14.7	27.0
Pts with ≥ 1 AE	35 (62.5%)	168 (84.4%)
WHOART preferred term		
Headache	12 (21.4%)	45 (22.6%)
Nausea	2 (3.6%)	33 (16.6%)
Upper Respiratory Tract Infection	5 (8.9%)	32 (16.1%)
Abdominal Pain	2 (3.6%)	24 (12.1%)
Fatigue	3 (5.4%)	21 (10.6%)
Fever	4 (7.1%)	20 (10.1%)

Samsung Bioepis Exhibit No. 1002
Page 2735

Pharyngitis	3 (5.4%)	17 (8.5%)
Vomiting	0 (0.0%)	17 (8.5%)
Dizziness	5 (8.9%)	16 (8.0%)
Pain	3 (5.4%)	17 (8.5%)
Bronchitis	1 (1.8%)	14 (7.0%)
Rash	3 (5.4%)	12 (6.0%)
Rhinitis	2 (3.6%)	12 (6.0%)
Chest Pain	3 (5.4%)	11 (5.5%)
Coughing	0 (0.0%)	10 (5.0%)
Pruritus	1 (1.8%)	10 (5.0%)
Sinusitis	1 (1.8%)	10 (5.0%)
Myalgia	3 (5.4%)	10 (5.0%)
Back Pain	2 (3.6%)	10 (5.0%)
Moniliasis	0 (0.0%)	10 (5.0%)

In general, the adverse event rates in the combined data set (Crohn's disease and non-Crohn's disease) were similar to those observed in Crohn's disease trials.

Adverse events by WHOART body system that have occurred in all Infliximab-treated patients at frequencies less than 5% but more than 1% are as follows:

Body as a Whole: chills, edema peripheral, fall, hot flushes, malaise
Cardiovascular Disorders, General: hypertension, hypotension
Central & Peripheral Nervous System Disorders: muscle contractions involuntary, paresthesia, vertigo
Eye and Vision Disorders: conjunctivitis
Gastrointestinal System Disorders: constipation, dyspepsia, flatulence, intestinal obstruction, oral pain, stomatitis ulcerative, toothache
Heart Rate and Rhythm Disorders: tachycardia
Liver and Biliary System Disorders: hepatic enzymes increased
Musculoskeletal System Disorders: arthralgia, arthritis
Psychiatric Disorders: anxiety, depression, insomnia
Red and Blood Cell Disorders: anemia
Resistance Mechanism Disorders: abscess, flu syndrome, herpes simplex, herpes zoster
Skin and Appendages Disorders: acne, alopecia, dermatitis fungal, eczema, erythema, rash erythematous, rash maculopapular, rash papular, skin dry, sweating increased, urticaria
Urinary System Disorders: dysuria, micturition frequency
Vascular (Extracardiac) Disorders: ecchymosis, flushing, hematoma

Among Infliximab-treated patients with Crohn's disease, serious adverse events occurred at a frequency < 2% with the majority occurring at a frequency of ≤ 0.5%. These were abdominal pain, abdominal hernia, abscess, adult respiratory distress syndrome, back pain, chest pain, cholecystitis, Crohn's disease, dehydration, diarrhea, dyspnea, dysuria, fall, fever, furunculosis, hypertension, hypotension, infection bacterial, intestinal obstruction, intestinal perforation, intestinal stenosis, kidney infarction, lymphoma, lupus erythematosus syndrome, nausea, palpitation, pneumonia, proctalgia, sepsis, splenic infarction, splenomegaly, syncope, tendon injury, thrombocytopenia, ureteral obstruction and vomiting.

In the Infliximab-treated non-Crohn's disease patients, all serious events except fever (1.2%) occurred at a frequency of <1% with the majority at ≤ 0.4%. These were arthralgia, arthritis rheumatoid, bone fracture, breast neoplasm malignant, bronchitis, cardiac failure, cellulitis, coughing, depression, dizziness, dyspnea, endophthalmitis, fall, fever, headache, injection site inflammation, myalgia, nausea, pleurisy, pneumonia, rash erythematous, renal calculus, rheumatoid nodules, sepsis, somnolence, sweating increased, tachycardia and vomiting.

OVERDOSAGE:

Single doses up to 20 mg/kg have been administered without any direct toxic effect. In case of overdosage, it is recommended that the patient be monitored for any signs or symptoms of adverse reactions or effects and appropriate symptomatic treatment instituted immediately.

DOSAGE AND ADMINISTRATION:

The recommended dose of Infliximab is 5 mg/kg given as a single intravenous infusion for treatment of moderately to severely active Crohn's disease in patients who have had an inadequate response to conventional therapy. In patients with fistulizing disease, an initial 5 mg/kg dose should be followed with additional 5 mg/kg doses at 2 and 6 weeks after the first infusion.

There are insufficient safety and efficacy data for the use of Infliximab beyond the recommended duration (see *INDICATIONS AND USAGE).*

Preparation and administration instructions: Use aseptic technique.

Remicade vials do not contain antibacterial preservatives. Therefore, the vials after reconstitution should be used immediately, not re-entered or stored. The diluent to be used for reconstitution is 10 mL of Sterile Water for Injection, USP. The total dose of the reconstituted product must be further diluted to 250 mL with 0.9% Sodium Chloride Injection, USP. The infusion concentration should range between 0.4 mg/mL and 4 mg/mL The Remicade infusion should begin within 3 hours of preparation.

Studies have indicated that reconstituted Remicade and diluted Remicade infusion solution are incompatible with plasticized PVC (polyvinylchloride) equipment or devices. Diluted Remicade solutions should be prepared only in glass infusion bottles or polypropylene or polyolefin infusion bags and administered through polyethylene-lined administration sets.

1. Calculate the dose and the number of Remicade vials needed. Each Remicade vial contains 100 mg of Infliximab. Calculate the total volume of reconstituted Remicade solution required.

2. Reconstitute each Remicade vial with 10 mL of Sterile Water for Injection, USP, using a syringe equipped with a 21-gauge or smaller needle. Remove the flip-top from the vial and wipe the top with an alcohol swab. Insert the syringe needle into the vial through the center of the rubber stopper and direct the stream of Sterile Water for Injection, USP, to the glass wall of the vial. Do not use the vial if the vacuum is not present. Gently swirl the solution by rotating the vial to dissolve the lyophilized powder. Avoid prolonged or vigorous agitation. DO NOT SHAKE. Foaming of the solution on reconstitution is not unusual. Allow the reconstituted solution to stand for 5 minutes. The solution should be colorless to light yellow and opalescent, and the solution may develop a few translucent particles as Infliximab is a protein. Do not use if opaque particles, discoloration, or other foreign particles are present.

3. Dilute the total volume of the reconstituted Remicade solution dose to 250 mL with 0.9% Sodium Chloride Injection, USP, by withdrawing a volume of 0.9% Sodium Chloride Injection, USP, equal to the volume of reconstituted Remicade from the 0.9% Sodium Chloride Injection, USP, 250 mL glass bottle or polypropylene or polyolefin bag. Slowly add the total volume of

reconstituted Remicade solution to the 250 mL infusion bottle or bag. Gently mix.

4. The infusion solution must be administered over a period of not less than 2 hours and must use a polyethylene-lined infusion set with an in-line, sterile, non-pyrogenic, low-protein-binding filter (pore size of 1.2-μm or less). Any unused portion of the infusion solution should not be stored for reuse.

5. No physical biochemical compatibility studies have been conducted to evaluate the co-administration of Remicade with other agents. Remicade should not be infused concomitantly in the same intravenous line with other agents.

6. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit. If visibly opaque particles, discoloration or other foreign particulates are observed, the solution should not be used.

Storage

Store the lyophilized product under refrigeration at 2°C to 8°C (36°F to 46°F). Do not freeze. Do not use beyond the expiration date. This product contains no preservative.

HOW SUPPLIED:

Remicade (Infliximab) lyophilized concentrate for injection is supplied in individually-boxed single-use vials in the following strength:

NDC 57894-030-01 100 mg infliximab in a 20-mL vial

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Rx Only

BEXXAR®

Tositumomab and Iodine I 131 Tositumomab

WARNINGS

Hypersensitivity Reactions, including Anaphylaxis: Medications for the treatment of severe hypersensitivity reactions should be available for immediate use. Patients who develop severe hypersensitivity reactions should have infusions of the BEXXAR therapeutic regimen discontinued and receive medical attention (See **WARNINGS**).

Prolonged and Severe Cytopenias: The majority of patients who received the BEXXAR therapeutic regimen experienced severe thrombocytopenia and neutropenia. The BEXXAR therapeutic regimen should not be administered to patients with >25% lymphoma marrow involvement and/or impaired bone marrow reserve (See **WARNINGS** and **ADVERSE REACTIONS**).

Pregnancy Category X: The BEXXAR therapeutic regimen can cause fetal harm when administered to a pregnant woman.

Special requirements: The BEXXAR therapeutic regimen (Tositumomab and Iodine I 131 Tositumomab) contains a radioactive component and should be administered only by physicians and other health care professionals qualified by training in the safe use and handling of therapeutic radionuclides. The BEXXAR therapeutic regimen should be administered only by physicians who are in the process of being or have been certified by Corixa Corporation in dose calculation and administration of the BEXXAR therapeutic regimen.

DESCRIPTION

The BEXXAR therapeutic regimen (Tositumomab and Iodine I 131 Tositumomab) is an anti-neoplastic radioimmunotherapeutic monoclonal

Samsung Bioepis Exhibit No. 1002
Page 2740

antibody-based regimen composed of the monoclonal antibody, Tositumomab, and the radiolabeled monoclonal antibody, Iodine I 131 Tositumomab.

Tositumomab

Tositumomab is a murine IgG_{2a} lambda monoclonal antibody directed against the CD20 antigen, which is found on the surface of normal and malignant B lymphocytes. Tositumomab is produced in an antibiotic-free culture of mammalian cells and is composed of two murine gamma 2a heavy chains of 451 amino acids each and two lambda light chains of 220 amino acids each. The approximate molecular weight of Tositumomab is 150 kD.

Tositumomab is supplied as a sterile, pyrogen-free, clear to opalescent, colorless to slightly yellow, preservative-free liquid concentrate. It is supplied at a nominal concentration of 14 mg/mL Tositumomab in 35 mg and 225 mg single-use vials. The formulation contains 10% (w/v) maltose, 145 mM sodium chloride, 10 mM phosphate, and Water for Injection, USP. The pH is approximately 7.2.

Iodine I 131 Tositumomab

Iodine I 131 Tositumomab is a radio-iodinated derivative of Tositumomab that has been covalently linked to Iodine-131. Unbound radio-iodine and other reactants have been removed by chromatographic purification steps. Iodine I 131 Tositumomab is supplied as a sterile, clear, preservative-free liquid for IV administration. The dosimetric dosage form is supplied at nominal protein and activity concentrations of 0.1 mg/mL and 0.61 mCi/mL (at date of calibration), respectively. The therapeutic dosage form is supplied at nominal protein and activity concentrations of 1.1 mg/mL and 5.6 mCi/mL (at date of calibration), respectively. The formulation for the dosimetric and the therapeutic dosage forms contains 5.0%□6.0% (w/v) povidone, 1□2 mg/mL maltose (dosimetric dose) or 9□15 mg/mL maltose (therapeutic dose), 0.85□0.95 mg/mL sodium chloride, and 0.9□1.3 mg/mL ascorbic acid. The pH is approximately 7.0.

BEXXAR Therapeutic Regimen

The BEXXAR therapeutic regimen is administered in two discrete steps: the dosimetric and therapeutic steps. Each step consists of a sequential infusion of Tositumomab followed by Iodine I 131 Tositumomab. The therapeutic step is administered 7-14 days after the dosimetric step. The Bexxar therapeutic regimen is supplied in two distinct package configurations as follows:

BEXXAR Dosimetric Packaging

- A carton containing two single-use 225 mg vials and one single-use 35 mg vial of Tositumomab supplied by McKesson Biosciences and
- A package containing a single-use vial of Iodine I 131 Tositumomab (0.61mCi/mL at calibration), supplied by MDS Nordion.

BEXXAR Therapeutic Packaging

- A carton containing two single-use 225 mg vials and one single-use 35 mg vial of Tositumomab, supplied by McKesson Biosciences and
- A package containing one or two single-use vials of Iodine I 131 Tositumomab (5.6 mCi/mL at calibration), supplied by MDS Nordion.

Physical/Radiochemical Characteristics of Iodine-131

Iodine-131 decays with beta and gamma emissions with a physical half-life of 8.04 days. The principal beta emission has a mean energy of 191.6 keV and the principal gamma emission has an energy of 364.5 keV (Ref 1).

External Radiation: The specific gamma ray constant for Iodine-131 is 2.2 R/millicurie hour at 1 cm. The first half-value layer is 0.24 cm lead (Pb) shielding. A range of values is shown in Table 1 for the relative attenuation of the radiation emitted by this radionuclide that results from interposition of various thicknesses of Pb. To facilitate control of the

radiation exposure from this radionucide, the use of a 2.55 cm thickness of Pb will attenuate the radiation emitted by a factor of about 1,000.

Table 1
Radiation Attenuation by Lead Shielding

Shield Thickness (Pb) cm	Attenuation Factor
0.24	0.5
0.89	10^{-1}
1.60	10^{-2}
2.55	10^{-3}
3.7	10^{-4}

The fraction of Iodine-131 radioactivity that remains in the vial after the date of calibration is calculated as follows:

Fraction of remaining radioactivity of Iodine-131 after x days $=2^{-(x/8.04)}$.
Physical decay is presented in Table 2.

Table 2
Physical Decay Chart: Iodine-131: Half-Life 8.04 Days

Days	Fraction Remaining
0*	1.000
1	0.917
2	0.842
3	0.772
4	0.708
5	0.650
6	0.596
7	0.547
8	0.502
9	0.460
10	0.422
11	0.387
12	0.355
13	0.326
14	0.299

*(Calibration day)

CLINICAL PHARMACOLOGY

General Pharmacology

Tositumomab binds specifically to the CD20 (human B-lymphocyte restricted differentiation antigen, Bp 35 or B1) antigen. This antigen is a transmembrane phosphoprotein expressed on pre-B lymphocytes and at higher density on mature B lymphocytes (Ref. 2). The antigen is also expressed on >90% of B-cell non-Hodgkin's lymphomas (NHL) (Ref. 3). The recognition epitope for Tositumomab is found within the extracellular domain of the CD20 antigen. CD20 does not shed from the cell surface and does not internalize following antibody binding (Ref. 4).

Mechanism of Action: Possible mechanisms of action of the BEXXAR therapeutic regimen include induction of apoptosis (Ref. 5), complement-dependent cytotoxicity (CDC) (Ref. 6), and antibody-dependent cellular cytotoxicity (ADCC) (Ref. 5) mediated by the antibody. Additionally, cell death is associated with ionizing radiation from the radioisotope.

Pharmacokinetics/Pharmacodynamics

The phase 1 study of Iodine I 131 Tositumomab determined that a 475 mg predose of unlabeled antibody decreased splenic targeting and increased the terminal half-life of the radiolabeled antibody. The median blood clearance following administration of 485 mg of Tositumomab in 110 patients with NHL was 68.2 mg/hr (range: 30.2 □260.8 mg/hr). Patients with high tumor burden, splenomegaly, or bone marrow involvement were noted to have a faster clearance, shorter terminal half-life, and larger volume of distribution. The total body clearance, as measured by total body gamma camera counts, was dependent on the same factors noted for blood clearance. Patient-specific dosing, based on total body clearance, provided a consistent radiation dose, despite variable pharmacokinetics, by allowing each patient's administered activity to be adjusted for individual patient variables.

Elimination of Iodine-131 occurs by decay (see Table 2) and excretion in the urine. Urine was collected for 49 dosimetric doses. After 5 days, the whole body clearance was 67% of the injected dose. Ninety-eight percent of the clearance was accounted for in the urine.

Administration of the BEXXAR therapeutic regimen results in sustained depletion of circulating CD20 positive cells. The impact of administration of the BEXXAR therapeutic regimen on circulating CD20 positive cells was assessed in two clinical studies, one conducted in chemotherapy naïve patients and one in heavily pretreated patients. The assessment of circulating lymphocytes did not distinguish normal from malignant cells. Consequently, assessment of recovery of normal B cell function was not directly assessed. At seven weeks, the median number of circulating CD20 positive cells was zero (range: 0 - 490 cells/ mm3). Lymphocyte recovery began at approximately 12 weeks following treatment. Among patients who had CD20 positive cell counts recorded at baseline and at 6 months, 8 of 58 (14%) chemotherapy naïve patients had CD20 positive cell counts below normal limits at six months and 6 of 19 (32%) heavily pretreated patients had CD20 positive cell counts below normal limits at six months. There was no consistent effect of the BEXXAR therapeutic regimen on post-treatment serum IgG, IgA, or IgM levels.

Radiation Dosimetry

Estimations of radiation-absorbed doses for Iodine I 131 Tositumomab were performed using sequential whole body images and the MIRDOSE 3 software program. Patients with apparent thyroid, stomach, or intestinal imaging were selected for organ dosimetry analyses. The estimated radiation-absorbed doses to organs and marrow from a course of the BEXXAR therapeutic regimen are presented in Table 3.

164

165 **Table 3**

166 **Estimated Radiation-Absorbed Organ Doses**

From Organ ROIs		BEXXAR mGy/MBq Median	BEXXAR mGy/MBq Range
	Thyroid	2.71	1.4 - 6.2
	Kidneys	1.96	1.5 - 2.5
	ULI Wall	1.34	0.8 - 1.7
	LLI Wall	1.30	0.8 - 1.6
	Heart Wall	1.25	0.5 - 1.8
	Spleen	1.14	0.7 - 5.4
	Testes	0.83	0.3 - 1.3
	Liver	0.82	0.6 - 1.3
	Lungs	0.79	0.5 - 1.1
	Red Marrow	0.65	0.5 - 1.1
	Stomach Wall	0.40	0.2 - 0.8
From Whole Body ROIs			
	Urine Bladder Wall	0.64	0.6 - 0.9
	Bone Surfaces	0.41	0.4 - 0.6
	Pancreas	0.31	0.2 - 0.4
	Gall Bladder Wall	0.29	0.2 - 0.3
	Adrenals	0.28	0.2 - 0.3
	Ovaries	0.25	0.2 - 0.3
	Small Intestine	0.23	0.2 - 0.3
	Thymus	0.22	0.1 - 0.3
	Uterus	0.20	0.2 - 0.2
	Muscle	0.18	0.1 - 0.2
	Breasts	0.16	0.1 - 0.2
	Skin	0.13	0.1 - 0.2
	Brain	0.13	0.1 - 0.2
	Total Body	0.24	0.2 - 0.3

CLINICAL STUDIES

The efficacy of the BEXXAR therapeutic regimen was evaluated in a multi-center, single-arm study in patients with low grade or transformed low-grade or follicular large-cell lymphoma whose disease had not responded to or had progressed after Rituximab therapy. Determination of clinical benefit of the BEXXAR therapeutic regimen was based on evidence of durable responses without evidence of an effect on survival. All patients in the study were required to have received prior treatment with at least four doses of Rituximab without an objective response, or to have progressed following treatment. Patients were also required to have a platelet count $\geq$100,000/mm^3; an average of $\leq$25% of the intratrabecular marrow space involved by lymphoma, and no evidence of progressive disease arising in a field irradiated with >3500 cGy within 1 year of completion of irradiation.

Forty patients initiated treatment with the BEXXAR therapeutic regimen. The median age was 57 (range: 35–78); the median time from diagnosis to protocol entry was 50 months (range: 11–70); and the median number of prior chemotherapy regimens was 4 (range: 1–11). Twenty-four patients had disease that did not respond to their last treatment with Rituximab, 11 patients had disease that responded to Rituximab for less than 6 months, and five patients had disease that responded to Rituximab, with a duration of response of 6 months or greater. Overall, 35 of the 40 patients met the definition of "Rituximab refractory", defined as no response or a response of less than 6 months duration. Table 4 summarizes efficacy outcome data from this study, as determined by an independent panel that reviewed patient records and radiologic studies. The median duration of follow-up was 26 months for all patients and 26 months for the Rituximab-refractory subset.

Table 4

Efficacy Outcomes Patients

	Objective Responses to the BEXXAR Therapeutic Regimen in Patients Refractory to Rituximab		Objective Responses to the BEXXAR Therapeutic Regimen in All Patients	
	Response Rate (%) (95% CI[a]) (n=35)	Median duration of response (Mos) (Range)	Response Rate (%) (95% CI[a]) (n=40)	Median Duration of Response (Mos) (Range)
Overall Response	63% (45%, 79%)	25 (4+, 35+)	68% (51%, 81%)	16 (1+, 35+)
Complete Response[c]	29% (15%, 46%)	NR[b] (4, 35+)	33% (19%, 49%)	NR (4, 35+)

[a] C.I. = Confidence Interval
[b] NR = Not reached
[c] Complete response rate = Pathologic and clinical complete responses

The results of this study were supported by demonstration of durable objective responses in four single arm studies enrolling 190 patients evaluable for efficacy with Rituximab-naïve, follicular non-Hodgkin's lymphoma with or without transformation, who had relapsed following or were refractory to chemotherapy. In these studies, the overall response rates ranged from 47% to 64% and the median durations of response ranged from 12 to 18 months.

INDICATIONS AND USAGE

The BEXXAR therapeutic regimen (Tositumomab and Iodine I 131 Tositumomab) is indicated for the treatment of patients with CD20 positive, follicular, non-Hodgkin's lymphoma, with and without transformation, whose disease is refractory to Rituximab and has relapsed following chemotherapy. The BEXXAR therapeutic regimen is not indicated for the initial treatment of patients with CD20 positive non-Hodgkin's lymphoma.

The BEXXAR therapeutic regimen is intended as a single course of treatment. The safety of multiple courses of the BEXXAR therapeutic

regimen, or combination of this regimen with other forms of irradiation or chemotherapy, has not been evaluated.

CONTRAINDICATIONS

The BEXXAR therapeutic regimen is contraindicated in patients with known hypersensitivity to murine proteins or any other component of the BEXXAR therapeutic regimen.

PREGNANCY CATEGORY X

Iodine I 131 Tositumomab (a component of the BEXXAR therapeutic regimen) is contraindicated for use in women who are pregnant. Iodine-131 may cause harm to the fetal thyroid gland when administered to pregnant women. Review of the literature has shown that transplacental passage of radioiodide may cause severe, and possibly irreversible, hypothyroidism in neonates. While there are no adequate and well-controlled studies of the BEXXAR therapeutic regimen in pregnant animals or humans, use of the BEXXAR therapeutic regimen in women of childbearing age should be deferred until the possibility of pregnancy has been ruled out. If the patient becomes pregnant while being treated with the BEXXAR therapeutic regimen, the patient should be apprised of the potential hazard to the fetus. (See **BOXED WARNING**, **Pregnancy Category X**).

WARNINGS

Prolonged and Severe Cytopenias (See BOXED WARNINGS; ADVERSE REACTIONS, Hematologic Events):

The most common adverse reactions associated with the BEXXAR therapeutic regimen were severe or life-threatening cytopenias (NCI CTC grade 3 or 4) with 71% of the 230 patients enrolled in clinical studies experiencing grade 3 or 4 cytopenias. These consisted primarily of grade 3 or 4 thrombocytopenia (53%) and grade 3 or 4 neutropenia (63%). The time to nadir was 4 to 7 weeks and the duration of cytopenias was approximately 30 days. Thrombocytopenia, neutropenia, and anemia persisted for more than 90 days following administration of the BEXXAR

therapeutic regimen in 16 (7%), 15 (7%), and 12 (5%) patients respectively (this includes patients with transient recovery followed by recurrent cytopenia). Due to the variable nature in the onset of cytopenias, complete blood counts should be obtained weekly for 10-12 weeks. The sequelae of severe cytopenias were commonly observed in the clinical studies and included infections (45% of patients), hemorrhage (12%), a requirement for growth factors (12% G- or GM-CSF; 7% Epoetin alfa) and blood product support (15% platelet transfusions; 16% red blood cell transfusions). Prolonged cytopenias may also influence subsequent treatment decisions.

The safety of the BEXXAR therapeutic regimen has not been established in patients with >25% lymphoma marrow involvement, platelet count <100,000 cells/mm^3 or neutrophil count <1,500 cells/mm^3.

Hypersensitivity Reactions Including Anaphylaxis (See BOXED WARNINGS; ADVERSE REACTIONS, Immunogenicity): Hypersensitivity reactions, including anaphylaxis, were reported during and following administration of the BEXXAR therapeutic regimen. Emergency supplies including medications for the treatment of hypersensitivity reactions, e.g., epinephrine, antihistamines and corticosteroids, should be available for immediate use in the event of an allergic reaction during administration of the BEXXAR therapeutic regimen. Patients who have received murine proteins should be screened for human anti-mouse antibodies (HAMA). Patients who are positive for HAMA may be at increased risk of anaphylaxis and serious hypersensitivity reactions during administration of the BEXXAR therapeutic regimen.

Secondary Malignancies: Myelodysplastic syndrome (MDS) and/or acute leukemia were reported in 8% (19/230) of patients enrolled in the clinical studies and 2% (13/765) of patients included in expanded access programs, with median follow-up of 35 and 20 months, respectively. Among the 32 reported new cases, the median time to development of MDS/leukemia was 27 months following treatment; however, the cumulative rate continues to increase. The pretreatment characteristics (e.g., median age, number of prior chemotherapy regimens) were similar

in patients developing MDS/secondary leukemias as compared with those who did not. Additional malignancies were also reported in 52 of the 995 patients enrolled in clinical studies or included in the expanded access program. Approximately half of these were non-melanomatous skin cancers. The remainder which occurred in 2 or more patients included breast cancer, lung cancer, bladder cancer, head and neck cancer, colon cancer and melanoma, in order of decreasing incidence. The relative risk of developing secondary malignancies in patients receiving the BEXXAR therapeutic regimen over the background rate in this population cannot be determined, due to the absence of controlled studies (See **ADVERSE REACTIONS**).

Pregnancy Category X: (See **BOXED WARNINGS**; **CONTRAINDICATIONS**).

Hypothyroidism: Administration of the BEXXAR therapeutic regimen may result in hypothyroidism (See **ADVERSE REACTIONS, Hypothyroidism**). Thyroid-blocking medications should be initiated at least 24 hours before receiving the dosimetric dose and continued until 14 days after the therapeutic dose (see **DOSAGE and ADMINISTRATION**). All patients must receive thyroid blocking agents; any patient who is unable to tolerate thyroid blocking agents should not receive the BEXXAR therapeutic regimen. Patients should be evaluated for signs and symptoms of hypothyroidism and screened for biochemical evidence of hypothyroidism annually.

PRECAUTIONS

Radionuclide Precautions: Iodine I 131 Tositumomab is radioactive. Care should be taken, consistent with the institutional radiation safety practices and applicable federal guidelines, to minimize exposure of medical personnel and other patients.

Renal Function: Iodine I 131 Tositumomab and Iodine-131 are excreted primarily by the kidneys. Impaired renal function may decrease the rate of excretion of the radiolabeled iodine and increase patient exposure to the radioactive component of the BEXXAR therapeutic regimen. There are no

data regarding the safety of administration of the BEXXAR therapeutic regimen in patients with impaired renal function.

Immunization: The safety of immunization with live viral vaccines following administration of the BEXXAR therapeutic regimen has not been studied. The ability of patients who have received the BEXXAR therapeutic regimen to generate a primary or anamnestic humoral response to any vaccine has not been studied.

Information for Patients: Prior to administration of the BEXXAR therapeutic regimen, patients should be advised that they will have a radioactive material in their body for several days upon their release from the hospital or clinic. After discharge, patients should be provided with both oral and written instructions for minimizing exposure of family members, friends and the general public. Patients should be given a copy of the written instructions for use as a reference for the recommended precautionary actions.

The pregnancy status of women of childbearing potential should be assessed and these women should be advised of the potential risks to the fetus (See **CONTRAINDICATIONS**). Women who are breastfeeding should be instructed to discontinue breastfeeding and should be apprised of the resultant potential harmful effects to the infant if these instructions are not followed.

Patients should be advised of the potential risk of toxic effects on the male and female gonads following the BEXXAR therapeutic regimen, and be instructed to use effective contraceptive methods during treatment and for 12 months following the administration of the BEXXAR therapeutic regimen.

Patients should be informed of the risks of hypothyroidism and be advised of the importance of compliance with thyroid blocking agents and need for life-long monitoring.

Patients should be informed of the possibility of developing a HAMA immune response and that HAMA may affect the results of *in vitro* and

in vivo diagnostic tests as well as results of therapies that rely on murine antibody technology.

Patients should be informed of the risks of cytopenias and symptoms associated with cytopenia, the need for frequent monitoring for up to 12 weeks after treatment, and the potential for persistent cytopenias beyond 12 weeks.

Patients should be informed that certain anti-neoplastic agents used in the treatment of malignancy, e.g., alkylating agents, topoisomerase II inhibitors, and ionizing radiation, have been associated with the development of MDS, secondary leukemia and solid tumors. Patients should be informed that MDS, secondary leukemia, and solid tumors have also been observed in patients receiving the BEXXAR therapeutic regimen.

Laboratory Monitoring: A complete blood count (CBC) with differential and platelet count should be obtained prior to, and at least weekly following administration of the BEXXAR therapeutic regimen. Weekly monitoring of blood counts should continue for a minimum of 10 weeks or, if persistent, until severe cytopenias have completely resolved. More frequent monitoring is indicated in patients with evidence of moderate or more severe cytopenias (see **BOXED WARNINGS** and **WARNINGS**). Thyroid stimulating hormone (TSH) level should be monitored before treatment and annually thereafter. Serum creatinine levels should be measured immediately prior to administration of the BEXXAR therapeutic regimen.

Drug Interactions: No formal drug interaction studies have been performed. Due to the frequent occurrence of severe and prolonged thrombocytopenia, the potential benefits of medications that interfere with platelet function and/or anticoagulation should be weighed against the potential increased risk of bleeding and hemorrhage.

Drug/Laboratory Test Interactions: Administration of the BEXXAR therapeutic regimen may result in the development of human anti-murine

antibodies (HAMA). The presence of HAMA may affect the accuracy of the results of *in vitro* and *in vivo* diagnostic tests and may affect the toxicity profile and efficacy of therapeutic agents that rely on murine antibody technology. Patients who are HAMA positive may be at increased risk for serious allergic reactions and other side effects if they undergo *in vivo* diagnostic testing or treatment with murine monoclonal antibodies.

Carcinogenesis, Mutagenesis, Impairment of Fertility: No long-term animal studies have been performed to establish the carcinogenic or mutagenic potential of the BEXXAR therapeutic regimen or to determine its effects on fertility in males or females. However, radiation is a potential carcinogen and mutagen. Administration of the BEXXAR therapeutic regimen results in delivery of a significant radiation dose to the testes. The radiation dose to the ovaries has not been established. There have been no studies to evaluate whether administration of the BEXXAR therapeutic regimen causes hypogonadism, premature menopause, azoospermia and/or mutagenic alterations to germ cells. There is a potential risk that the BEXXAR therapeutic regimen may cause toxic effects on the male and female gonads. Effective contraceptive methods should be used during treatment and for 12 months following administration of the BEXXAR therapeutic regimen.

Pregnancy Category X: See CONTRAINDICATIONS; WARNINGS.

Nursing Mothers: Radioiodine is excreted in breast milk and may reach concentrations equal to or greater than maternal plasma concentrations. Immunoglobulins are also known to be excreted in breast milk. The absorption potential and potential for adverse effects of the monoclonal antibody component (Tositumomab) in the infant are not known. Therefore, formula feedings should be substituted for breast feedings before starting treatment. Women should be advised to discontinue nursing.

Pediatric Use: The safety and effectiveness of the BEXXAR therapeutic regimen in children have not been established.

Geriatric Use: Clinical studies of the BEXXAR therapeutic regimen did not include sufficient numbers of patients aged 65 and over to determine whether they respond differently from younger patients. In clinical studies, 230 patients received the BEXXAR therapeutic regimen at the recommended dose. Of these, 27% (61 patients) were age 65 or older and 4% (10 patients) were age 75 or older. Across all studies, the overall response rate was lower in patients age 65 and over (41% vs. 61%) and the duration of responses were shorter (10 months vs. 16 months), however these findings are primarily derived from 2 of the 5 studies. While the incidence of severe hematologic toxicity was lower, the duration of severe hematologic toxicity was longer in those age 65 or older as compared to patients less than 65 years of age. Due to the limited experience greater sensitivity of some older individuals cannot be ruled out.

ADVERSE REACTIONS

The most serious adverse reactions observed in the clinical trials were severe and prolonged cytopenias and the sequelae of cytopenias which included infections (sepsis), and hemorrhage in thrombocytopenic patients, allergic reactions (bronchospasm and angioedema), secondary leukemia and myelodysplasia. (See BOXED WARNINGS and WARNINGS).

The most common adverse reactions occurring in the clinical trials included neutropenia, thromobocytopenia, and anemia that are both prolonged and severe. Less common but severe adverse reactions included pneumonia, pleural effusion and dehydration.

Data regarding adverse events were primarily obtained in 230 patients with non-Hodgkin's lymphoma enrolled in five clinical trials using the recommended dose and schedule. Patients had a median follow-up of 35 months and 79% of the patients were followed at least 12 months for survival and selected adverse events. Patients had a median of 3 prior chemotherapy regimens, a median age of 55 years, 60% male, 27% had transformation to a higher grade histology, 29% were intermediate grade and 2% high grade histology (IWF) and 68% had Ann Arbor stage IV

disease. Patients enrolled in these studies were not permitted to have prior hematopoietic stem cell transplantation or irradiation to more than 25% of the red marrow. In the expanded access program, which included 765 patients, data regarding clinical serious adverse events and HAMA and TSH levels were used to supplement the characterization of delayed adverse events. (See **ADVERSE REACTIONS, Hypothyroidism, Secondary Leukemia and Myelodysplastic Syndrome, Immunogenicity**.)

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice. The adverse reaction information from clinical trials does, however, provide a basis for identifying the adverse events that appear to be related to drug use and for approximating rates.

Hematologic Events: Hematologic toxicity was the most frequently observed adverse event in clinical trials with the BEXXAR therapeutic regimen (Table 6). Sixty-three (27%) of 230 patients received one or more hematologic supportive care measures following the therapeutic dose: 12% received G-CSF; 7% received Epoetin alfa; 15% received platelet transfusions; and 16% received packed red blood cell transfusions. Twenty-eight (12%) patients experienced hemorrhagic events; the majority were mild to moderate.

Infectious Events: One hundred and four of the 230 (45%) patients experienced one or more adverse events possibly related to infection. The majority were viral (e.g. rhinitis, pharyngitis, flu symptoms, or herpes) or other minor infections. Nineteen of 230 (8%) patients experienced infections that were considered serious because the patient was hospitalized to manage the infection. Documented infections included pneumonia, bacteremia, septicemia, bronchitis, and skin infections.

Hypersensitivity Reactions: Fourteen patients (6%) experienced one or more of the following adverse events: allergic reaction, face edema,

injection site hypersensitivity, anaphylactoid reaction, laryngismus, and serum sickness.

Gastrointestinal toxicity: Eighty-seven patients (38%) experienced one or more gastrointestinal adverse events, including nausea, emesis, abdominal pain, and diarrhea. These events were temporally related to the infusion of the antibody. Nausea, vomiting, and abdominal pain were often reported within days of infusion, whereas diarrhea was generally reported days to weeks after infusion.

Infusional Toxicity: A constellation of symptoms, including fever, rigors or chills, sweating, hypotension, dyspnea, bronchospasm, and nausea, have been reported during or within 48 hours of infusion. Sixty-seven patients (29%) reported fever, rigors/chills, or sweating within 14 days following the dosimetric dose. Although all patients in the clinical studies received pretreatment with acetaminophen and an antihistamine, the value of premedication in preventing infusion-related toxicity was not evaluated in any of the clinical studies. Infusional toxicities were managed by slowing and/or temporarily interrupting the infusion. Symptomatic management was required in more severe cases. Adjustment of the rate of infusion to control adverse reactions occurred in 16 patients (7%); seven patients required adjustments for only the dosimetric infusion, two required adjustments for only the therapeutic infusion, and seven required adjustments for both the dosimetric and the therapeutic infusions. Adjustments included reduction in the rate of infusion by 50%, temporary interruption of the infusion, and in 2 patients, infusion was permanently discontinued.

Table 5 lists clinical adverse events that occurred in ≥5% of patients. Table 6 provides a detailed description of the hematologic toxicity.

509 **Table 5**
510 **Incidence of Clinical Adverse Experiences Regardless of Relationship to**
511 **Study Drug Occurring in ≥5% of the Patients Treated with BEXXAR**
512 **Therapeutic Regimen[a]**
513 **(N = 230)**

Body System Preferred Term	All Grades	Grade 3/4
Total	**(96%)**	**(48%)**
Non-Hematologic AEs		
Body as a Whole	81%	12%
Asthenia	46%	2%
Fever	37%	2%
Infection[b]	21%	<1%
Pain	19%	1%
Chills	18%	1%
Headache	16%	0%
Abdominal pain	15%	3%
Back pain	8%	1%
Chest pain	7%	0%
Neck pain	6%	1%
Cardiovascular System	26%	3%
Hypotension	7%	1%
Vasodilatation	5%	0%
Digestive System	56%	9%
Nausea	36%	3%
Vomiting	15%	1%
Anorexia	14%	0%
Diarrhea	12%	0%
Constipation	6%	1%
Dyspepsia	6%	<1%
Endocrine System	7%	0%
Hypothyroidism	7%	0%
Metabolic and Nutritional Disorders	21%	3%
Peripheral edema	9%	0%
Weight loss	6%	<1%
Musculoskeletal System	23%	3%
Myalgia	13%	<1%
Arthralgia	10%	1%
Nervous System	26%	3%
Dizziness	5%	0%
Somnolence	5%	0%

514

Table 5 (cont'd)
Incidence of Clinical Adverse Experiences Regardless of Relationship to Study Drug Occurring in ≥5% of the Patients Treated with BEXXAR Therapeutic Regimen[a]
(N =230)

Respiratory System	44%	8%
Cough increased	21%	1%
Pharyngitis	12%	0%
Dyspnea	11%	3%
Rhinitis	10%	0%
Pneumonia	6%	0%
Skin and Appendages	44%	5%
Rash	17%	<1%
Pruritus	10%	0%
Sweating	8%	<1%

[a] Excludes laboratory derived hematologic adverse events (See Table 6).

[b] The COSTART term for infection includes a subset of infections (e.g., upper respiratory infection). Other terms are mapped to preferred terms (e.g., pneumonia and sepsis). For a more inclusive summary see ADVERSE REACTIONS, Infectious Events.

Table 6
Hematologic Toxicity[a] (N=230)

Endpoint	Values
Platelets	
Median nadir (cells/mm^3)	43,000
Per patient incidence[a] platelets <50,000/mm^3	53% (n=123)
Median[b] duration of platelets <50,000/mm^3 (days)	32
Grade 3/4 without recovery to Grade 2, N (%)	16 (7%)
Per patient incidence[c] platelets <25,000/mm^3	21% (n=47)
ANC	
Median nadir (cells/mm^3)	690
Per patient incidence[a] ANC<1,000 cells/mm^3 (%)	63% (n=145)
Median[b] duration of ANC<1,000 cells/mm^3 (days)	31
Grade 3/4 without recovery to Grade 2, N (%)	15 (7%)
Per patient incidence[c] ANC< 500 cells/mm^3, N (%)	25% (n=57)
Hemoglobin	
Median nadir (gm/dL)	10
Per patient incidence[a] < 8 gm/dL	29% (n=66)
Median[b] duration of hemoglobin < 8.0 gm/dL (days)	23
Grade 3/4 without recovery to Grade 2, N (%)	12 (5%)
Per patient incidence[c] hemoglobin <6.5 gm/dL, N (%)	5% (n=11)

[a] Grade 3/4 toxicity was assumed if patient was missing 2 or more weeks of hematology data between Week 5 and Week 9.

[b] Duration of grade 3/4 of 1000+ days (censored) was assumed for those patients with undocumented grade 3/4 and no hematologic data on or after Week 9.

[c] Grade 4 toxicity was assumed if patient had documented Grade 3 toxicity and was missing 2 or more weeks of hematology data between Week 5 and Week 9.

Delayed Adverse Reactions

Delayed adverse reactions, including hypothyroidism, HAMA, and myelodysplasia/leukemia, were assessed in 230 patients included in clinical studies and 765 patients included in expanded access programs. The entry characteristics of patients included from the expanded access

programs were similar to the characteristics of patients enrolled in the clinical studies, except that the median number of prior chemotherapy regimens was fewer (2 vs. 3) and the proportion with low-grade histology was higher (77% vs. 70%) in patients from the expanded access programs.

Secondary Leukemia and Myelodysplastic Syndrome (MDS): There were 32 new cases of MDS/secondary leukemia reported among 994 (3.2%) patients included in clinical studies and expanded access programs, with a median follow-up of 21 months. The overall incidence of MDS/secondary leukemia among the 229 patients included in the clinical studies, was 8.3% (19/229), with a median follow-up of 35 months and a median time to development of MDS of 30 months. The cumulative incidence of MDS/secondary leukemia was 4.2% at 2 years and 10.7% at 4 years. Among the 765 patients included in the expanded access program, where the median duration of follow-up was shorter (20 months), the overall incidence of MDS/secondary leukemia was 1.7% (13/765) and the median time to development of MDS was 23 months. In the expanded access population, the cumulative incidence of MDS/secondary leukemia was 1.4% at 2 years and 4.8% at 4 years.

Secondary Malignancies: There were 52 reports of second malignancies, excluding secondary leukemias. The most common included non-melanomatous skin cancers, breast, lung, bladder, and head and neck cancers. Some of these events included recurrence of an earlier diagnosis of cancer.

Hypothyroidism: Twelve percent (27/230) of the patients included from the clinical studies had an elevated TSH level (8%) or no TSH level obtained (4%) prior to treatment. Of the 203 patients documented to be euthyroid at entry, 137 (67%) patients had at least one follow-up TSH value. The overall incidence of hypothyroidism, in the clinical study patients was 14% with cumulative incidences of 4.2% at 6 months and 8.1%, 12.6%, and 15.0% at 1, 2, and 4 years, respectively. New events have been observed up to 72 months post treatment. Twelve percent (117/990) of the patients included in clinical studies or the expanded access programs had an elevated TSH level (8%) or a history of

hypothyroidism (4%) prior to treatment and 5 patients had no baseline information. Of the 873 who were euthyroid at entry, 583 (67%) had at least one post-treatment TSH value obtained. With a median observation period of 18 months, 54 patients (9%) became hypothyroid as determined by elevated TSH. The cumulative incidence of hypothyroidism in the combined populations was 9.1% and 17.4% at 2 and 4 years, respectively.

Immunogenicity: Two percent (4/230) of the chemotherapy-relapsed or refractory patients included in the clinical studies had a positive serology for HAMA prior to treatment and six patients had no baseline assessment for HAMA. Of the 220 patients who were seronegative prior to treatment, 219 (99.5%) had at least one post-treatment HAMA value obtained. With a median observation period for HAMA seroconversion of 6 months, 23 patients (11%) seroconverted to HAMA positivity. The median time to development of HAMA was 6 months. In a study of 77 patients who were chemotherapy-naïve, the incidence of conversion to HAMA seropositivity was 70%, with a median time to development of HAMA of 27 days.

One percent (11/989) of the chemotherapy-relapsed or refractory patients included in the clinical studies or the expanded access program had a positive serology for HAMA prior to treatment and six patient had no baseline assessment for HAMA. Of the 978 patients who were seronegative for HAMA prior to treatment, 785 (80%) had at least one post-treatment HAMA value obtained. With a median observation period of 6 months, a total of 76 patients (10%) became seropositive for HAMA post-treatment. The median time of HAMA development was 148 days, with 45 (59%) patients seropositive for HAMA by 6 months. No patient became seropositive for HAMA more than 30 months after administration of the BEXXAR therapeutic regimen.

The data reflect the percentage of patients whose test results were considered positive for HAMA in an ELISA assay that detects antibodies to the Fc portion of IgG_1 murine immunoglobulin and are highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of antibody positivity in an assay may be influenced by several factors including sample handling, concomitant medications, and underlying disease. For these reasons, comparison of the incidence of

HAMA in patients treated with the BEXXAR therapeutic regimen with the incidence of HAMA in patients treated with other products may be misleading.

OVERDOSAGE

The maximum dose of the BEXXAR therapeutic regimen that was administered in clinical trials was 88 cGy. Three patients were treated with a total body dose of 85 cGy of Iodine I 131 Tositumomab in a dose escalation study. Two of the 3 patients developed Grade 4 toxicity of 5 weeks duration with subsequent recovery. In addition, accidental overdose of the BEXXAR therapeutic regimen occurred in one patient at total body doses of 88 cGy. The patient developed Grade 3 hematologic toxicity of 18 days duration. Patients who receive an accidental overdose of Iodine I 131 Tositumomab should be monitored closely for cytopenias and radiation-related toxicity. The effectiveness of hematopoietic stem cell transplantation as a supportive care measure for marrow injury has not been studied; however, the timing of such support should take into account the pharmacokinetics of the BEXXAR therapeutic regimen and decay rate of the Iodine-131 in order to minimize the possibility of irradiation of infused hematopoietic stem cells.

DOSAGE AND ADMINISTRATION

Recommended Dose

The BEXXAR therapeutic regimen consists of four components administered in two discrete steps: the dosimetric step, followed 7-14 days later by a therapeutic step.

Note: the safety of the BEXXAR therapeutic regimen was established only in the setting of patients receiving thyroid blocking agents and premedication to ameliorate/prevent infusion reactions (See **Concomitant Medications**).

Dosimetric step

- Tositumomab 450 mg intravenously in 50 ml 0.9% Sodium Chloride over 60 minutes. Reduce the rate of infusion by 50% for mild to moderate infusional toxicity; interrupt infusion for severe infusional toxicity. After complete resolution of severe infusional toxicity, infusion may be resumed with a 50% reduction in the rate of infusion.

- Iodine I 131 Tositumomab (containing 5.0 mCi I-131 and 35 mg tositumomab) intravenously in 30 ml 0.9% Sodium Chloride over 20 minutes. Reduce the rate of infusion by 50% for mild to moderate infusional toxicity; interrupt infusion for severe infusional toxicity. After complete resolution of severe infusional toxicity, infusion may be resumed with a 50% reduction in the rate of infusion.

Therapeutic step

Note: Do not administer the therapeutic step if biodistribution is altered. (See **Assessment of Biodistribution of Iodine I 131 Tositumomab)**

- Tositumomab 450 mg intravenously in 50 ml 0.9% Sodium Chloride over 60 minutes. Reduce the rate of infusion by 50% for mild to moderate infusional toxicity; interrupt infusion for severe infusional toxicity. After complete resolution of severe infusional toxicity, infusion may be resumed with a 50% reduction in the rate of infusion.

- Iodine I 131 Tositumomab (See **CALCULATION OF IODINE-131 ACTIVITY FOR THE THERAPEUTIC DOSE**). Reduce the rate of infusion by 50% for mild to moderate infusional toxicity; interrupt infusion for severe infusional toxicity. After complete resolution of severe infusional toxicity, infusion may be resumed with a 50% reduction in the rate of infusion.

    - Patients with $\geq$ 150,000 platelets/mm^3: The recommended dose is the activity of Iodine-131 calculated to deliver 75cGy total body irradiation and 35 mg Tositumomab, administered intravenously over 20 minutes.

    - Patients with NCI Grade 1 thrombocytopenia (platelet counts =100, 000 but <150,000 platelets/mm^3): the recommended dose is the

activity of Iodine-131 calculated to deliver 65 cGy total body irradiation and 35 mg Tositumomab, administered intravenously over 20 minutes.

Concomitant Medications: The safety of the BEXXAR therapeutic regimen was established in studies in which all patients received the following concurrent medications:

- Thyroid protective agents: Saturated solution of potassium iodide (SSKI) 4 drops orally t.i.d; Lugol's solution 20 drops orally t.i.d.; or Potassium iodide tablets 130 mg orally q.d. Thyroid protective agents should be initiated at least 24 hours prior to administration of the Iodine-131 Tositumomab dosimetric dose and continued until 2 weeks after administration of the Iodine I 131 Tositumomab therapeutic dose.

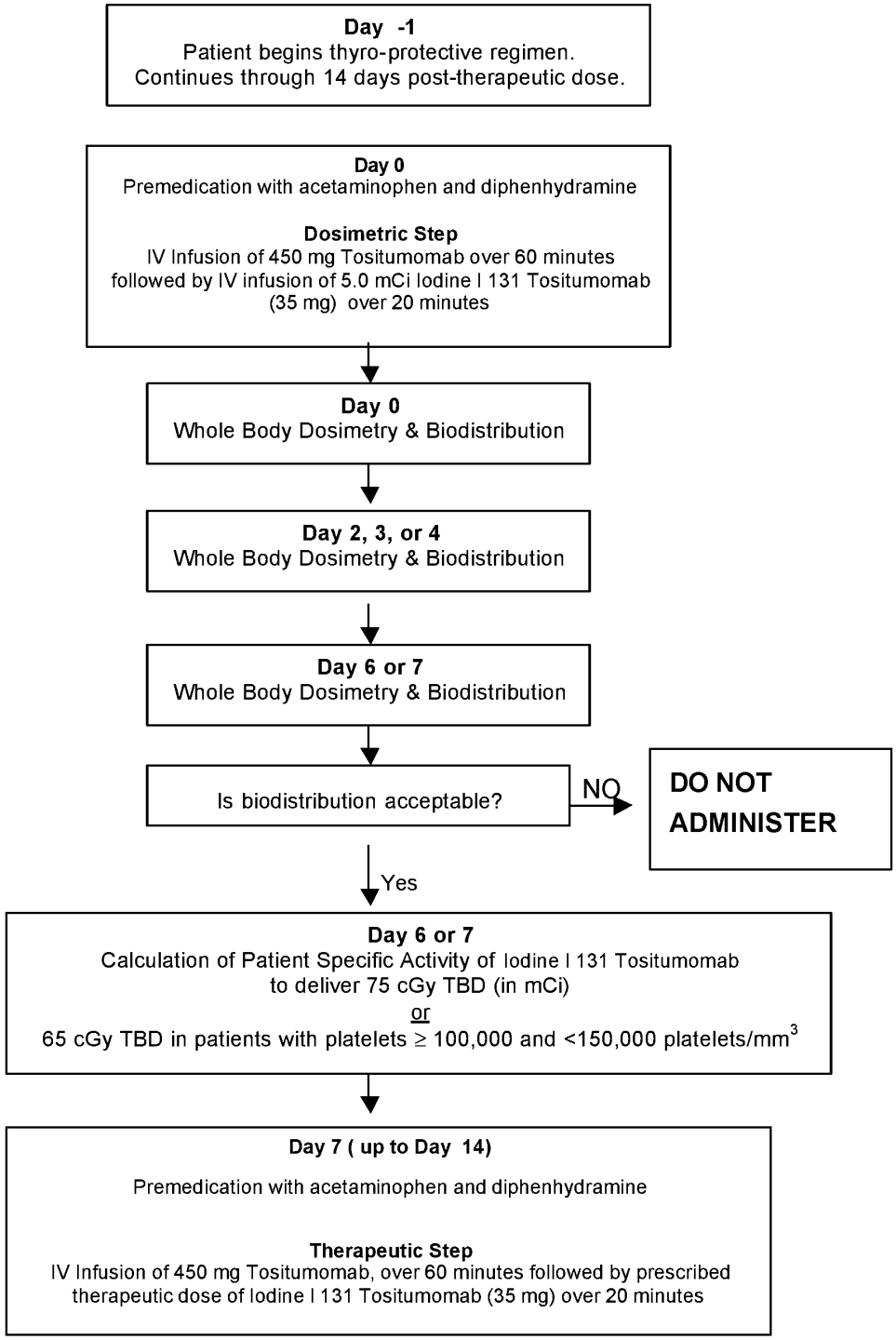
  Patients should not receive the dosimetric dose of Iodine I 131 Tositumomab if they have not yet received at least 3 doses of SSKI, three doses of Lugol's solution, or one dose of 130 mg potassium iodide tablet (at least 24 hours prior to the dosimetric dose).

- Acetaminophen 650 mg orally and diphenhydramine 50 mg orally 30 minutes prior to administration of Tositumomab in the dosimetric and therapeutic steps.

The BEXXAR therapeutic regimen is administered via an IV tubing set with an in-line 0.22 μ filter. **THE SAME IV TUBING SET AND FILTER MUST BE USED THROUGHOUT THE ENTIRE DOSIMETRIC OR THERAPEUTIC STEP. A CHANGE IN FILTER CAN RESULT IN LOSS OF DRUG.**

Figure 1 shows an overview of the dosing schedule.

Figure 1
Dosing Schedule



Samsung Bioepis Exhibit No. 1002
Page 2767

PREPARATION OF THE BEXXAR THERAPEUTIC REGIMEN

GENERAL

Read all directions thoroughly and assemble all materials before preparing the dose for administration.

The Iodine I 131 Tositumomab dosimetric and therapeutic doses should be measured by a suitable radioactivity calibration system immediately prior to administration. The dose calibrator must be operated in accordance with the manufacturer's specifications and quality control for the measurement of Iodine-131.

All supplies for preparation and administration of the BEXXAR therapeutic regimen should be sterile. Use appropriate aseptic technique and radiation precautions for the preparation of the components of the BEXXAR therapeutic regimen.

Waterproof gloves should be utilized in the preparation and administration of the product. Iodine I 131 Tositumomab doses should be prepared, assayed, and administered by personnel who are licensed to handle and/or administer radionuclides. Appropriate shielding should be used during preparation and administration of the product.

Restrictions on patient contact with others and release from the hospital must follow all applicable federal, state, and institutional regulations.

Preparation for the Dosimetric Step

Tositumomab Dose

Required materials not supplied

A. One 50 mL syringe with attached 18 gauge needle (to withdraw 450 mg of Tositumomab from two vials each containing 225 mg Tositumomab)

B. One 50 mL bag of sterile 0.9% Sodium Chloride for Injection, USP.

C. One 50 mL syringe for drawing up 32 mL of saline for disposal from the 50 mL bag of sterile 0.9% Sodium Chloride for Injection, USP

Method

1. Withdraw and dispose of 32 mL of saline from a 50 mL bag of sterile 0.9% Sodium Chloride for Injection, USP.

2. Withdraw the entire contents from each of the two 225-mg vials (a total of 450 mg Tositumomab in 32 mL) and transfer to the infusion bag containing 18 mL of 0.9% Sodium Chloride for Injection, USP to yield a final volume of 50 mL.

3. Gently mix the solution by inverting/rotating the bag. DO NOT SHAKE.

4. The diluted Tositumomab may be stored for up to 24 hours when stored refrigerated at 2°C □8°C (36°F □46°F) and for up to 8 hours at room temperature.

Note: Tositumomab solution may contain particulates that are generally white in nature. The product should appear clear to opalescent, colorless to slightly yellow.

Preparation of Iodine I 131 Tositumomab Dosimetric Dose

Required materials not supplied:

A. Lead shielding for preparation vial and syringe pump

B. Two 30 mL syringes with 18 gauge needles: one to withdraw the calculated volume of Iodine I 131 Tositumomab from the Iodine I 131 Tositumomab vial and one to withdraw the volume from the preparation vial into a syringe for administration.

C. One 20 mL syringe with attached needle, filled with 0.9% Sodium Chloride for Injection, USP

D. One 3 mL syringe with attached needle to withdraw Tositumomab from 35-mg vial

E. One sterile, 30 or 50 mL preparation vial

F. Two lead pots, both kept at room temperature. One pot is used to thaw the labeled antibody and the second pot is used to hold the preparation vial.

Method:

1. Allow approximately 60 minutes for thawing (at ambient temperature) of the Iodine I 131 Tositumomab dosimetric vial with appropriate lead shielding.

2. Based on the activity concentration of the vial (see actual product specification sheet for the vial supplied in the dosimetric package), calculate the volume required for an Iodine I 131 Tositumomab activity of 5.0 mCi.

3. Withdraw the calculated volume from the Iodine I 131 Tositumomab vial.

4. Transfer this volume to the shielded preparation vial.

5. Assay the dose to ensure that the appropriate activity (mCi) has been prepared.

    a. If the assayed dose is 5.0 mCi (+/- 10%) proceed with step 6.

    b. If the assayed dose does not contain 5.0 mCi (+/- 10%) recalculate the activity concentration of the Iodine I 131 Tositumomab at this time, based on the volume and the activity in the preparation vial. Recalculate the volume required for an Iodine I 131 Tositumomab activity of 5.0 mCi. Using the same 30 mL syringe, add or subtract the appropriate volume from the Iodine I 131 Tositumomab vial so that the preparation vial contains the volume required for an Iodine I 131 Tositumomab activity of 5.0 mCi (+/- 10%). Re-assay the preparation vial and proceed with step 6.

6. Calculate the amount of Tositumomab contained in the solution of Iodine I 131 Tositumomab in the shielded preparation vial, based on the volume and protein concentration (see actual product specification sheet supplied in the dosimetric package).

7. If the shielded preparation vial contains less than 35 mg, calculate the amount of additional Tositumomab needed to yield a total of 35 mg protein. Calculate the volume needed from the 35 mg vial of Tositumomab, based on the protein concentration. Withdraw the calculated volume of Tositumomab from the 35 mg vial of Tositumomab, and transfer this volume to the shielded preparation vial. The preparation vial should now contain a total of 35 mg of Tositumomab.

8. Using the 20 mL syringe containing 0.9% Sodium Chloride for Injection, USP, add a sufficient quantity to the shielded preparation vial to yield a final volume of 30 mL. Gently mix the solutions.

9. Withdraw the entire contents from the preparation vial into a 30 mL syringe using a large bore needle (18 gauge).

10. Assay and record the activity.

Administration of the Dosimetric Step

Required materials not supplied:

A. One I.V. Filter set (0.22 µm filter), 15 inch with injection site (port) and luer lock

B. One Primary IV infusion set

C. One 100 mL bag of sterile 0.9% Sodium Chloride for Injection, USP

D. Two Secondary I.V infusion sets

E. One I.V. Extension set, 30 inch luer lock

F. One 3-way stopcock

G. One 50 mL bag of sterile 0.9% Sodium Chloride for Injection, USP

H. One Infusion pump for Tositumomab infusion

I. One Syringe Pump for Iodine I 131 Tositumomab infusion

J. Lead shielding for use in the administration of the dosimetric dose

Tositumomab Infusion:

(See Figure 1 in the "**Workbook for Dosimetry Methodology and Administration Set-Up**" for diagrammatic illustration of the configuration of the infusion set components).

1. Attach a primary IV infusion set (Item B) to the 0.22 micron in-line filter set (Item A) and the 100 mL bag of sterile 0.9% Sodium Chloride for Injection, USP (Item C).

2. After priming the primary IV infusion set (Item B) and IV filter set (Item A), connect the infusion bag containing 450 mg Tositumomab (50 mL) via a secondary IV infusion set (Item D) to the primary IV infusion set (Item B) at a port distal to the 0.22 micron in-line filter. Infuse Tositumomab over 60 minutes.

3. After completion of the Tositumomab infusion, disconnect the secondary IV infusion set (Item D) and flush the primary IV infusion set (Item B) and the in-line IV filter set (Item A) with 0.9% Sodium Chloride. Discard the Tositumomab bag and secondary IV infusion set.

Iodine I 131 Tositumomab Dosimetric Infusion

(See Figure 2 in the "**Workbook for Dosimetry Methodology and Administration Set-Up**" for diagrammatic illustration of the configuration of the infusion set components).

1. Appropriate shielding should be used in the administration of the dosimetric dose.

2. The dosimetric dose is delivered in a 30 mL syringe.

3. Connect the extension set (Item E) to the 3-way stopcock (Item F).

4. Connect the 50 mL bag of sterile 0.9% Sodium Chloride for Injection, USP (Item G) to a secondary IV infusion set (Item D) and connect the infusion set to the 3-way stopcock (Item F). Prime the secondary IV infusion set (Item D) and the extension set (Item E). Connect the extension set (Item E) to a port in the primary IV infusion set (Item B), distal to the filter.

(**Note**: You **must** use the same primary infusion set (Item B) and IV filter set (Item A) with pre-wetted filter that was used for the Tositumomab infusion. A change in filter can result in loss of up to 7% of the Iodine I 131 Tositumomab dose.)

5. Attach the syringe filled with the Iodine I 131 Tositumomab to the 3-way stopcock (Item F).

6. Set syringe pump to deliver the entire 5.0 mCi (35 mg) dose of Iodine I 131 Tositumomab over 20 minutes.

7. After completion of the infusion of Iodine I 131 Tositumomab, close the stopcock (Item F) to the syringe. Flush the extension set (Item E) and the secondary IV infusion set (Item D) with 0.9% Sodium Chloride for Injection, USP from the 50 mL bag (Item G).

8. After the flush, disconnect the extension set (Item E), 3-way stopcock (Item F) and syringe. Disconnect the primary IV infusion set (Item B) and in-line filter set (Item A). Determine the combined residual activity of the syringe and infusion set components (stopcock, extension set, primary infusion set and in-line filter set) by assaying these items in a suitable radioactivity calibration system immediately following completion of administration of all components of the dosimetric step. Calculate and record the dose delivered to the patient by subtracting the residual activity in the syringe and the infusion set components from the activity of Iodine I 131 Tositumomab in the syringe prior to infusion.

9. Discard all materials used to deliver the Iodine I 131 Tositumomab (e.g., syringes, vials, in-line filter set, extension set and infusion sets) in accordance with local, state, and federal regulations governing radioactive and biohazardous waste.

Determination of Dose for the Therapeutic Step (See CALCULATION OF IODINE-131 ACTIVITY FOR THERAPEUTIC DOSE)

The methodology for determining and calculating the patient-specific dose of Iodine I 131 activity (mCi) to be administered in the therapeutic step involves the following steps:

1. Following infusion of the Iodine I 131 Tositumomab dosimetric dose, obtain total body gamma camera counts and whole body images at the following timepoints:
   a. Within one hour of infusion and prior to urination
   b. 2-4 days after infusion of the dosimetric dose, following urination
   c. 6-7 days after infusion of the dosimetric dose, following urination
2. Assess biodistribution. If biodistribution is altered, the therapeutic step should not be administered.
3. Determine total body residence time (See Graph 1, "**Determination of Residence Time**", in the "**Workbook for Dosimetry Methodology and Administration Set-Up**").
4. Determine activity hours, (See Table 2, "**Determination of Activity Hours**", in the "**Workbook for Dosimetry Methodology and Administration Set-Up**") according to gender. Use actual patient mass (in kg) or maximum effective mass (in kg) whichever is lower (See Table 1, "**Determination of Maximum Effective Mass**", in the "**Workbook for Dosimetry Methodology and Administration Set-Up**").

5. Determine whether the desired total body dose should be reduced (to 65 cGy) due to a platelet count of 100,000 to <150,000 cells/mm^3.

6. Based on the total body residence time and activity hours, calculate the Iodine-131 activity (mCi) to be administered to deliver the therapeutic dose of 65 or 75 cGy.

The following equation is used to calculate the activity of Iodine-131 required for delivery of the desired total body dose of radiation.

$$\textbf{Iodine-131 Activity (mCi)} = \frac{\text{Activity Hours (mCi hr)}}{\text{Residence Time (hr)}} \times \frac{\text{Desired Total Body Dose (cGy)}}{75\ \text{cGy}}$$

Preparation for the Therapeutic Step

Tositumomab Dose

Required materials not supplied

A. One 50 mL syringe with attached 18 gauge needle (to withdraw 450 mg of Tositumomab from two vials each containing 225 mg Tositumomab)

B. One 50 mL bag of sterile 0.9% Sodium Chloride for Injection, USP

C. One 50 mL syringe for drawing up 32 mL of saline for disposal from the 50 mL bag of sterile 0.9% Sodium Chloride for Injection USP

Method

1. Withdraw and dispose of 32 mL of saline from a 50 mL bag of sterile 0.9% Sodium Chloride for Injection, USP.

2. Withdraw the entire contents from each of the two 225-mg vials (a total of 450 mg Tositumomab in 32 mL) and transfer to the infusion bag containing 18 mL of 0.9% Sodium Chloride for Injection, USP to yield a final volume of 50 mL.

3. Gently mix the solutions by inverting/rotating the bag. DO NOT SHAKE.

4. The diluted Tositumomab may be stored for up to 24 hours when stored refrigerated at 2°C 8°C (36°F 46°F) and for up to 8 hours at room temperature.

Note: Tositumomab solution may contain particulates that are generally white in nature. The product should appear clear to opalescent, colorless to slightly yellow.

Preparation of Iodine I 131 Tositumomab Therapeutic Dose

Required materials not supplied:

A. Lead shielding for preparation vial and syringe pump

B. Two or four 30 mL syringes with 18 gauge needles: one or two to withdraw the calculated volume of Iodine I 131 Tositumomab from the Iodine I 131 Tositumomab vial(s) and one or two to withdraw the volume from the preparation vial into a syringe for administration.

C. One 20 mL syringe with attached needle filled with 0.9% Sodium Chloride for Injection, USP

D. One 3 mL sterile syringe with attached needle to draw up Tositumomab from the 35 mg vial

E. One sterile, 30 or 50 mL preparation vial

F. Two lead pots both kept at room temperature. One pot is used to thaw the labeled antibody, and the second pot is used to hold the preparation vial.

Method:

1. Allow approximately 60 minutes for thawing (at ambient temperature) of the Iodine I 131 Tositumomab therapeutic vial with appropriate lead shielding.

2. Calculate the dose of Iodine I 131 Tositumomab required (See **CALCULATION OF IODINE-131 ACTIVITY FOR THERAPEUTIC DOSE**)

3. Based on the activity concentration of the vial (see actual product specification sheet for each vial supplied in the therapeutic package), calculate the volume required for the Iodine I 131 Tositumomab activity required for the therapeutic dose.

4. Using one or more 30 mL syringes with an 18-gauge needle, withdraw the calculated volume from the Iodine I 131 Tositumomab vial.

5. Transfer this volume to the shielded preparation vial.

6. Assay the dose to ensure that the appropriate activity (mCi) has been prepared.

    a. If the assayed dose is the calculated dose (+/- 10%) needed for the therapeutic step, proceed with step 7.

    b. If the assayed dose does not contain the desired dose (+/- 10%), re-calculate the activity concentration of the Iodine I 131 Tositumomab at this time, based on the volume and the activity in the preparation vial. Re-calculate the volume required for an Iodine I 131 Tositumomab activity for the therapeutic dose. Using the same 30 mL syringe, add or subtract the appropriate volume from the Iodine I 131 Tositumomab vial so that the preparation vial contains the volume required for the Iodine I 131 Tositumomab activity required for the therapeutic dose. Re-assay the preparation vial. Proceed to step 7.

7. Calculate the amount of Tositumomab protein contained in the solution of Iodine I 131 Tositumomab in the shielded preparation vial, based on the volume and protein concentration. (See product specification sheet.)

8. If the shielded preparation vial contains less than 35 mg, calculate the amount of additional Tositumomab needed to yield a total of 35 mg protein. Calculate the volume needed from the 35 mg vial of Tositumomab, based on the protein concentration. Withdraw the calculated volume of Tositumomab from the 35 mg vial of Tositumomab, and transfer this volume to the shielded preparation vial. The preparation vial should now contain a total of 35 mg of Tositumomab.

    Note: If the dose of Iodine I 131 Tositumomab requires the use of 2 vials of Iodine I 131 Tositumomab or the entire contents of a single vial of Iodine I 131 Tositumomab, there may be no need to add protein from the 35 mg vial of Tositumomab.

9. Using the 20 mL syringe containing 0.9% Sodium Chloride for Injection, USP, add a sufficient volume (if needed) to the shielded preparation vial to yield a final volume of 30 mL. Gently mix the solution.

10. Withdraw the entire volume from the preparation vial into a one or more sterile 30 mL syringes using a large bore needle (18 gauge).

11. Assay and record the activity.

Administration of the Therapeutic Step

Note: Restrictions on patient contact with others and release from the hospital must follow all applicable federal, state, and institutional regulations.

Required materials not supplied:

A. One I.V. Filter set (0.22 µm filter), 15 inch with injection site (port) and luer lock

B. One Primary I.V. infusion set

C. One 100 mL bag of sterile 0.9% Sodium Chloride for Injection, USP

D. Two Secondary I.V. infusion sets

E. One I.V. extension set, 30 inch luer lock

F. One 3-way stopcock

G. One 50 mL bag of sterile 0.9% Sodium Chloride for Injection, USP

H. One Infusion pump for Tositumomab infusion

I. One Syringe Pump for Iodine I 131 Tositumomab infusion

J. Lead shielding for use in the administration of the therapeutic dose

Tositumomab Infusion:

(See Figure 1 in the "**Workbook for Dosimetry Methodology and Administration Set-Up**" for diagrammatic illustration of the configuration of the infusion set components).

1. Attach a primary IV infusion set (Item B) to the 0.22 micron in-line filter set (Item A) and a 100 mL bag of sterile 0.9% Sodium Chloride for Injection, USP (Item C).

2. After priming the primary IV infusion set (Item B) and filter set (Item A), connect the infusion bag containing 450 mg Tositumomab (50 mL) via a secondary IV infusion set (Item D) to the primary IV infusion set (Item B) at a port distal to the 0.22 micron in-line filter. Infuse Tositumomab over 60 minutes.

3. After completion of the Tositumomab infusion, disconnect the secondary IV infusion set (Item D) and flush the primary IV infusion set (Item B) and the IV filter set (Item A) with 0.9% Sodium Chloride. Discard the Tositumomab bag and secondary IV infusion set.

Iodine I 131 Tositumomab Therapeutic Infusion:

(See Figure 2 in the "**Workbook for Dosimetry Methodology and Administration Set-Up**" for diagrammatic illustration of the configuration of the infusion set components).

1. Appropriate shielding should be used in the administration of the therapeutic dose.

2. The therapeutic dose is delivered in one or more 30 mL syringes.

3. Connect the extension set (Item E) to the 3-way stopcock (Item F).

4. Connect the 50 mL bag of sterile 0.9% Sodium Chloride for Injection, USP (Item G) to a secondary IV infusion set (Item D) and connect the infusion set to the 3-way stopcock (Item F). Prime the secondary IV infusion set (Item D) and the extension set (Item E). Connect the extension set (Item E) to a port in the primary IV infusion set (Item B), distal to the filter.

(**Note**: You **must** use the same primary infusion set (Item B) and IV filter set (Item A) with pre-wetted filter that was used for the Tositumomab infusion. A change in filter can result in loss of up to 7% of the Iodine I 131 Tositumomab dose.)

5. Attach the syringe filled with the Iodine I 131 Tositumomab to the 3-way stopcock (Item F).

6. Set syringe pump to deliver the entire therapeutic dose of Iodine I 131 Tositumomab over 20 minutes. (Note: if more than one syringe is required, remove the syringe and repeat steps 5 and 6.)

7. After completion of the infusion of Iodine I 131 Tositumomab, close the stopcock (Item F) to the syringe. Flush the secondary IV infusion set (Item D) and the extension set (Item E) with 0.9% Sodium Chloride from the 50 mL bag of sterile, 0.9% Sodium Chloride for Injection, USP (Item G).

8. After the flush, disconnect the extension set (Item E), 3-way stopcock (Item F) and syringe. Disconnect the primary IV infusion set (Item B) and in-line filter set (Item A). Determine the combined residual activity of the syringe(s) and infusion set components (stopcock, extension set, primary infusion set and in-line filter set) by assaying these items in a suitable radioactivity calibration system immediately following completion of administration of all components of the therapeutic step. Calculate and record the dose delivered to the patient by subtracting the residual activity in the syringe and infusion set components from the activity of Iodine I 131 Tositumomab in the syringe prior to infusion.

9. Discard all materials used to deliver the Iodine I 131 Tositumomab (e.g., syringes, vials, in-line filter set, extension set and infusion sets) in accordance with local, state, and federal regulations governing radioactive and biohazardous waste.

DOSIMETRY

The following sections describe the procedures for image acquisition for collection of dosimetry data, interpretation of biodistribution images, calculation of residence time, and calculation of activity hours. Please read all sections carefully.

IMAGE ACQUISITION AND INTERPRETATION

Gamma Camera and Dose Calibrator Procedures

Manufacturer-specific quality control procedures should be followed for the gamma camera/computer system, the collimator, and the dose calibrator. Less than 20% variance between maximum and minimum pixel count values in the useful field of view is acceptable on Iodine-131 intrinsic flood fields and variability <10% is preferable. Iodine-131-specific camera uniformity corrections are strongly recommended, rather than applying lower energy correction to the Iodine-131 window. Camera extrinsic uniformity should be assessed at least monthly using 99mTc or 57 Co as a source with imaging at the appropriate window.

Additional (non-routine) quality control procedures are required. To assure the accuracy and precision of the patient total body counts, the gamma camera must undergo validation and daily quality control on each day it is used to collect patient images.

Use the same setup and region of interest (ROI) for calibration, determination of background, and whole body patient studies.

Gamma Camera Set-Up

The **same** camera, collimator, region of interest (ROI), scanning speed, energy window, and setup must be used for all studies. The gamma camera must be capable of whole body imaging and have a large or extra large field of view with a digital interface. It must be equipped with a parallel-hole collimator rated to at least 364 keV by the manufacturer with a septal penetration for Iodine-131 of <7%.

The camera and computer must be set up for scanning as follows:

- Parallel hole collimator rated to at least 364 keV with a septal penetration for Iodine-131 of <7%
- Symmetric window (20-25%) centered on the 364 keV photo peak of Iodine-131 (314 - 414 keV)
- Matrix: minimum 128 x 128
- Scanning speed: 10-30 cm/minute

Counts from Calibrated Source for Quality Control

Camera sensitivity for Iodine-131 must be determined each day. Determination of the gamma camera's sensitivity is obtained by scanning a calibrated activity of Iodine-131 (e.g., 200□250 µCi in at least 20 mL of saline within a sealed pharmaceutical vial). The radioactivity of the Iodine-131 source is first determined using a NIST-traceable-calibrated clinical dose calibrator at the Iodine-131 setting.

Background Counts

The background count is obtained from a scan with no radioactive source. This should be obtained following the count of the calibrated source and just prior to obtaining the patient count.

If abnormally high background counts are measured, the source should be identified and, if possible, removed. If abnormally low background counts are

measured, the camera energy window setting and collimator should be verified before repeating the background counts.

The counts per μCi are obtained by dividing the background-corrected source count by the calibrated activity for that day. For a specific camera and collimator, the counts per μCi should be relatively constant. When values vary more than 10% from the established ratio, the reason for the discrepancy should be ascertained and corrected and the source count repeated.

Patient Total Body Counts

The source and background counts are obtained first and the camera sensitivity (i.e., constant counting efficiency) is established prior to obtaining the patient count. The same rectangular region of interest (ROI) must be used for the whole body counts, the quality control counts of the radioactive source, and the background counts.

Acquire anterior and posterior whole body images for gamma camera counts. For any particular patient, the same gamma camera must be used for all scans. To obtain proper counts, extremities must be included in the images, and arms should not cross over the body. The scans should be centered on the midline of the patient. Record the time of the start of the radiolabeled dosimetric infusion and the time of the start of each count acquisition.

Gamma camera counts will be obtained at the three imaging time points:

- **Count 1:** ***Within an hour of end of the infusion*** of the Iodine I 131 Tositumomab dosimetric dose prior to patient voiding.

- **Count 2:** Two to 4 days after administration of the Iodine I 131 Tositumomab dosimetric dose and immediately following patient voiding.

- **Count 3:** Six to 7 days after the administration of the Iodine I 131 Tositumomab dosimetric dose and immediately following patient voiding

Assessment of Biodistribution of Iodine I 131 Tositumomab

The biodistribution of Iodine I 131 Tositumomab should be assessed by determination of total body residence time and by visual examination of whole

body camera images from the first image taken at the time of Count 1 (within an hour of the end of the infusion) and from the second image taken at the time of Count 2 (at 2 to 4 days after administration). To resolve ambiguities, an evaluation of the third image at the time of Count 3 (6 to 7 days after administration) may be necessary. If either of these methods indicates that the biodistribution is altered, the Iodine I 131 Tositumomab therapeutic dose should not be administered.

Expected Biodistribution

- On the first imaging timepoint: Most of the activity is in the blood pool (heart and major blood vessels) and the uptake in normal liver and spleen is less than in the heart.
- On the second and third imaging timepoints: The activity in the blood pool decreases significantly and there is decreased accumulation of activity in normal liver and spleen. Images may show uptake by thyroid, kidney, and urinary bladder and minimal uptake in the lungs. Tumor uptake in soft tissues and in normal organs is seen as areas of increased intensity.

Results Indicating Altered Biodistribution

- On the first imaging timepoint: If the blood pool is not visualized or if there is diffuse, intense tracer uptake in the liver and/or spleen or uptake suggestive of urinary obstruction the biodistribution is altered. Diffuse lung uptake greater than that of blood pool on the first day represents altered biodistribution.
- On the second and third imaging timepoints: uptake suggestive of urinary obstruction and diffuse lung uptake greater than that of the blood pool represent altered biodistribution.
- Total body residence times of less than 50 hours and more than 150 hours.

CALCULATION OF IODINE-131 ACTIVITY FOR THE THERAPEUTIC DOSE

The methods for determining the residence time (hr) and activity hours (mCi hr) are described below.

Residence Time (hr)

For each time point, calculate the background corrected total body count at each timepoint (defined as the geometric mean). The following equation is used:

Geometric mean of counts = $\sqrt{(C_A - C_{BA})(C_P - C_{BP})}$

In this equation, C_A = the anterior counts, C_{BA} = the anterior background counts, C_P= the posterior counts, and C_{BP} = the posterior background counts.

Once the geometric mean of the counts has been calculated for each of the 3 timepoints, the % injected activity remaining for each timepoint is calculated by dividing the geometric mean of the counts from that timepoint by the geometric mean of the counts from Day 0 and multiplying by 100.

The residence time (h) is then determined by plotting the time from the start of infusion and the % injected activity values for the 3 imaging timepoints on Graph 1 (See Worksheet "**Determination of Residence Time**" in the "**Workbook for Dosimetry Methodology and Administration Set-Up**" supplied with Dosimetric Dose Packaging). A best-fit line is then drawn from 100% (the pre-plotted Day 0 value) through the other 2 plotted points (if the line does not intersect the two points, one point must lie above the best-fit line and one point must lie below the best-fit line). The residence time (h) is read from the x-axis of the graph at the point where the fitted line intersects with the horizontal 37% injected activity line.

Activity Hours (mCi hr)

In order to determine the activity hours (mCi hr), look up the patient's maximum effective mass derived from the patient's sex and height (See Worksheet "**Determination of Maximum Effective Mass**" in the "**Workbook for Dosimetry Methodology and Administration Set-Up**" supplied with Dosimetric Dose Packaging). If the patient's actual weight is less than the maximum effective mass, the actual weight should be used in the activity hours table (See Worksheet "**Determination of Activity Hours**" in the

Workbook for Dosimetry Methodology and Administration Set-Up supplied with Dosimetric Dose Packaging). If the patient's actual weight is greater than the maximum effective mass, the mass from the worksheet for **Determination of Maximum Effective Mass** should be used.

Calculation of Iodine-131 Activity for the Therapeutic Dose

The following equation is used to calculate the activity of Iodine-131 required for delivery of the desired total body dose of radiation.

$$\textbf{Iodine-131 Activity (mCi)} = \frac{\text{Activity Hours (mCi hr)}}{\text{Residence Time (hr)}} \times \frac{\text{Desired Total Body Dose (cGy)}}{75\ \text{cGy}}$$

HOW SUPPLIED

TOSITUMOMAB DOSIMETRIC PACKAGING

The components of the dosimetric step will be shipped **ONLY** to individuals who are participating in the certification program or have been certified in the preparation and administration of the BEXXAR therapeutic regimen. The components are shipped from separate sites; when ordering, ensure that the components are scheduled to arrive on the same day. The components of the Tositumomab Dosimetric Step include:

1. Tositumomab: Two single-use 225 mg vials (16.1 mL) and one single-use 35 mg vial (2.5 mL) of Tositumomab at a protein concentration of 14 mg/mL supplied by McKesson Biosciences.

NDC 67800-101-31

2. Iodine I 131 Tositumomab: A single-use vial of Iodine I 131 Tositumomab within a lead pot, supplied by MDS Nordion. Each single-use vial contains not less than 20 mL of Iodine I 131 Tositumomab at nominal protein and activity concentrations of 0.1 mg/mL and 0.61 mCi/mL (at calibration), respectively. (Refer to the product specification sheet for the lot-specific protein concentration, activity concentration, total activity and expiration date.)

NDC 67800-111-10

TOSITUMOMAB THERAPEUTIC PACKAGING

The components of the therapeutic step will be shipped **ONLY** to individuals who are participating in the certification program or have been certified in the preparation and administration of the BEXXAR therapeutic regimen for an individual patient who has completed the Dosimetric Step. The components of the therapeutic step are shipped from separate sites; when ordering, ensure that the components are scheduled to arrive on the same day. The components of the Tositumomab Therapeutic Step include:

1. Tositumomab: Two single-use 225 mg vials (16.1 mL) and one single-use 35 mg vial (2.5 mL) of Tositumomab at a protein concentration of 14 mg/mL supplied by McKesson Biosciences.

NDC 67800-101-32

2. One or two single-use vials of Iodine I 131 Tositumomab within a lead pot, supplied by MDS Nordion. Each single-use vial contains not less than 20 mL of Iodine I 131 Tositumomab at nominal protein and activity concentrations of 1.1 mg/mL and 5.6 mCi/mL (at calibration), respectively. Refer to the product specification sheet for the lot-specific protein concentration, activity concentration, total activity and expiration date.

NDC 67800-121-10.

STABILITY AND STORAGE

TOSITUMOMAB

Vials of Tositumomab (35 mg and 225 mg) should be stored refrigerated at 2°C-8°C (36°F-46°F) prior to dilution. Do not use beyond expiration date. Protect from strong light. **DO NOT SHAKE**. Do not freeze. Discard any unused portions left in the vial.

Solutions of diluted Tositumomab are stable for up to 24 hours when stored refrigerated at 2°C □8°C (36°F□46°F) and for up to 8 hours at room temperature. However, it is recommended that the diluted solution be stored

refrigerated at 2°C 8°C (36°F 46°F) prior to administration because it does not contain preservatives. Any unused portion must be discarded. Do not freeze solutions of diluted Tositumomab.

IODINE I 131 TOSITUMOMAB

Store frozen in the original lead pots. The lead pot containing the product must be stored in a freezer at a temperature of -20°C or below until it is removed for thawing prior to administration to the patient. Do not use beyond the expiration date on the label of the lead pot.

Thawed dosimetric and therapeutic doses of Iodine I 131 Tositumomab are stable for up to 8 hours at 2°C 8°C (36°F 46°F) or at room temperature. Solutions of Iodine I 131 Tositumomab diluted for infusion contain no preservatives and should be stored refrigerated at 2°C 8°C (36°F 46°F) prior to administration (do not freeze). Any unused portion must be discarded according to federal and state laws.

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Samsung Bioepis Exhibit No. 1002
Page 2787

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ProstaScint™ Kit
(Capromab Pendetide)

Kit for the Preparation of Indium In 111 Capromab Pendetide
For Intravenous Use Only

DESCRIPTION

ProstaScint™ (Capromab Pendetide) is the murine monoclonal antibody, 7E11-C5.3, conjugated to the linker-chelator, glycyl-tyrosyl-(N, ε-diethylenetriaminepentaacetic acid)-lysine hydrochloride (GYK-DTPA-HCl). The 7E11-C5.3 antibody is of the IgG1, kappa subclass (IgG1K). This antibody is directed against a glycoprotein expressed by prostate epithelium known as Prostate Specific Membrane Antigen (PSMA). The PSMA epitope recognized by monoclonal antibody (MAb) 7E11-C5.3 is located in the cytoplasmic domain. Expression of this glycoprotein has not been demonstrated on any other adenocarcinomas or transitional cell cancers tested. The antibody is produced by serum-free *in vitro* cultivation of cells, and purified by sequential protein isolation and chromatographic separation procedures.

Each ProstaScint™ kit consists of two vials which contain all of the non-radioactive ingredients necessary to produce a single unit dose of Indium In 111 ProstaScint™, an immunoscintigraphic agent for administration by intravenous injection only. The ProstaScint™ vial contains 0.5 mg of capromab pendetide in 1 mL of sodium phosphate buffered saline solution adjusted to pH 6; a sterile, pyrogen-free, clear, colorless solution that may contain some translucent particles. The vial of sodium acetate buffer contains 82 mg of sodium acetate in 2 mL of Water for Injection adjusted to pH 5-7 with glacial acetic acid; it is a sterile, pyrogen-free, clear, and colorless solution. Neither solution contains a preservative. Each kit also includes one sterile 0.22 μm Millex® GV filter, prescribing information, and two identification labels.

The sodium acetate solution must be added to the sterile, non-pyrogenic high purity Indium In 111 Chloride solution to buffer it prior to radiolabeling ProstaScint™. The immunoscintigraphic agent Indium In 111 Capromab Pendetide (Indium In 111 ProstaScint™) is formed after radiolabeling with Indium In 111.

Physical Characteristics of Indium In 111

Indium In 111 decays by electron capture with a physical half-life of 67.2 hours (2.8 days).[1] The energies of the photons that are useful for detection and imaging studies are listed in TABLE 1.

<< TABLE Nº 1 >>

Samsung Bioepis Exhibit No. 1002
Page 2789

External Radiation

The exposure rate constant for 37 MBq (1 mCi) of Indium In 111 is 8.3×10^{-4} C/kg/hr (3.21 R/hr). The first half-value thickness of lead (Pb) for Indium In 111 is 0.023 cm. A range of values for the relative attenuation of the radiation emitted by this radionuclide that results from the interposition of various thicknesses of Pb is shown in TABLE 2. For example, the use of 0.834 cm of lead will decrease the external radiation exposure by a factor of about 1,000.

<< TABLE Nº 2>>

These estimates of attenuation do not take into consideration the presence of longer-lived contaminants with higher energy photons, namely Indium In 114m/114.

To allow correction for physical decay of Indium In 111, the fractions that remain at selected intervals before and after the time of calibration are shown in TABLE 3.

<< TABLE Nº 3>>

CLINICAL PHARMACOLOGY

Pharmacodynamics

Prostate Specific Membrane Antigen is expressed in many primary and metastatic prostate cancer lesions, and *in vitro* immunohistologic studies have shown 7E11-C5.3 to be reactive with over 95% of the prostate adenocarcinomas evaluated. In general, PSMA expression by prostate cancer cells is either unchanged or increased in patients treated with hormonal therapy (see PRECAUTIONS, Drug Interactions). The 7E11-C5.3 antibody is immunoreactive with normal and hypertrophic adult prostate tissue. In clinical studies of patients with prostate cancer, Indium In 111 ProstaScint™ localized to the prostate, and some known primary and metastatic tumor sites.

Non-antigen-dependent localization, suspected to be secondary to catabolism, has been observed in the liver, spleen, and bone marrow. Although there is variation among individuals, there may also be localization and imaging activity in the bowel, blood pool, kidneys, urinary bladder, and genitalia. Intracellular localization of 7E11-C5.3 has been observed in histochemically prepared tissue sections from normal adult skeletal and cardiac muscle, although primate studies revealed no specific localization to these tissues.

Pharmacokinetics

Based on data obtained from clinical studies, Indium In 111 ProstaScint™ demonstrated a monoexponential elimination pattern with a terminal-phase half life of 67 ± 11 hours (mean± SD). Approximately 10% of the administered radioisotope dose is excreted in the urine during the 72 hours following intravenous infusion. The pharmacokinetics of Indium In 111 ProstaScint™ are characterized by slow serum clearance rate (42 ± 22 ml/hr) and small volume of distribution (4 ± 2.1 L).

Samsung Bioepis Exhibit No. 1002
Page 2790

CLINICAL STUDIES

Indium In 111 ProstaScint™ has been administered in single doses to over 600 patients in clinical studies, and in repeat administrations (2 to 4 infusions) to 61 patients. A 0.5 mg dose was determined to be the lowest effective dose. The imaging performance of Indium In 111 ProstaScint™ was evaluated in a phase 2 and a phase 3 trial in each of two clinical settings: (1) patients with clinically-localized prostate cancer who were at high risk for metastases and (2) patients with a high clinical suspicion for occult recurrent or residual prostate cancer.

Imaging Performance in Newly-Diagnosed Patients

In one of two open label, multi-center, uncontrolled pivotal phase 3 trials, 180 patients with a tissue diagnosis of prostate cancer who were considered at high risk for lymph node metastases underwent Indium In 111 ProstaScint™ immunoscintigraphy prior to scheduled staging pelvic lymphadenectomy. High risk was defined as at least one of the following: (1) prostate specific antigen (PSA) ≥ 10x the upper limit of normal & Gleason score ≥7; (2) prostatic acid phosphatase above the upper limit of normal; (3) equivocal evidence of lymph node metastases on CT or ultrasound & PSA ≥8x the upper limit of normal; (4) Gleason score ≥8; or (5) clinical stage C & Gleason score ≥6. All patients had been evaluated for metastatic disease using standard non-invasive imaging techniques, and were considered to have clinically-localized prostate cancer. The Indium In 111 ProstaScint™ images were interpreted on-site, and the reader had access to all clinical data. The interpretations were correlated with the results of surgical staging; however, a correlation of specific areas of Indium In 111 ProstaScint™ uptake to specific sites of tumor involvement was not performed.

One hundred fifty-two patients had an interpretable scan and surgical staging. Forty scans were classified as true positive, 25 as false positive, 63 as true negative, and 24 as false negative. The results for immunoscintigraphy are summarized in TABLE 4.

<< TABLE Nº 4>>

Sixty-five patients (43%) had positive Indium In 111 ProstaScint™ images for pelvic lymph node metastases: Of these 38% (25 patients) did not have metastatic prostate cancer at surgery. Eighty-seven patients (57%) had negative Indium In 111 ProstaScint™ images: Of these 28% (24 patients) did have metastatic prostate cancer at surgery. The overall accuracy of Indium In 111 ProstaScint™ immunoscintigraphy, as measured against pelvic lymph node dissection, was 68% (103/152).

A retrospective subset analysis suggested that a positive Indium In 111 ProstaScint™ scan in patients with a Gleason score ≥7 and a PSA ≥ 40 contained additional information regarding the likelihood that tumor metastases would be found at the scheduled staging pelvic lymphadenectomy.

Samsung Bioepis Exhibit No. 1002
Page 2791

Imaging Performance in Patients with Occult Recurrent or Residual Disease

In the second open label, multi-center, uncontrolled pivotal phase 3 trial, 183 patients with a high clinical suspicion of residual or recurrent prostate cancer following radical prostatectomy were evaluated. Patients with a rising PSA, a negative bone scan, and negative or equivocal standard diagnostic techniques, (e.g. transrectal ultrasound, CT scan, or MRI) underwent Indium In 111 ProstaScint™ immunoscintigraphy prior to biopsy of the prostatic fossa. The Indium In 111 ProstaScint™ images were interpreted on-site, and the reader had access to all clinical data. The interpretations were correlated with the results of histopathologic analysis of the prostatic fossa biopsy specimens.

One hundred fifty-eight patients had an interpretable scan and prostatic fossa biopsy. Twenty-nine scans were classified as true positive, 29 as false positive, 70 as true negative, and 30 as false negative. The results are summarized in TABLE 5.

< TABLE Nº 5>>

Fifty-eight patients (37%) had positive Indium In 111 ProstaScint™ images in the prostatic fossa: Of these 50% (29 patients) did not have recurrent prostate cancer on biopsy. One hundred patients (63%) had negative Indium In 111 ProstaScint™ images: Of these 30% (30 patients) had recurrent prostate cancer on biopsy. The overall accuracy of Indium In 111 ProstaScint™ immunoscintigraphy, as measured against prostatic fossa biopsy, was 63% (99/158).

Indium In 111 ProstaScint™ localized to only the prostatic fossa in 29 (18%) patients, to prostatic fossa and extrafossa sites in 29 (18%) patients, and to only extrafossa sites in 39 (25%) patients. The study was not designed to evaluate extrafossa sites of uptake. Three extrafossa sites of uptake were biopsied, one of which was positive for metastatic prostate cancer.

ProstaScint™ Results in Patients with Distant Metastases

Clinical trials have not specifically studied the ability of Indium In 111 ProstaScint™ to image distant (extra-pelvic) metastases, and a limited number of patients with distant (primarily bone) metastases were enrolled. Thirteen patients out of 16 (81%) with CT evidence of distant soft tissue disease had positive extrafossa Indium In 111 ProstaScint™ scans. Thirty-five out of 61 patients (57%) with bone scan evidence of disease had positive Indium In 111 ProstaScint™ skeletal uptake; however, Indium In 111 ProstaScint™ imaging did not identify most sites of abnormal bone uptake on bone scan, nor did it demonstrate any new sites of metastasis that were not seen on bone scan. The Indium In 111 ProstaScint™ scan did, however, demonstrate sites of bone marrow metastases that were not seen on bone scan in 2 of 43 patients in the phase 1 study.

Samsung Bioepis Exhibit No. 1002
Page 2792

Repeat Scans

Sixty-one patients received a total of 74 repeat infusions of Indium In 111 ProstaScint™. The incidence of adverse reactions upon repeat infusion (5%) was comparable to that observed after single infusion (4 %). Human anti-mouse antibody (HAMA) levels were detected (at levels >8ng/mL) by radioimmune assay (RIA) after single infusion in 8% (20/239) of patients while 1% of patients had levels greater than 100 ng/mL. Serum HAMA levels were detected by RIA after repeat infusion in 19% (5/27) of patients.

Biodistribution was unaltered on 65 of 70 (93%) evaluable repeat scans. The efficacy of repeat Indium In 111 ProstaScint™ imaging was not evaluated.

INDICATIONS AND USAGE

Indium In 111 ProstaScint™ is indicated as a diagnostic imaging agent in newly-diagnosed patients with biopsy-proven prostate cancer, thought to be clinically-localized after standard diagnostic evaluation (e.g. chest x-ray, bone scan, CT scan, or MRI), who are at high-risk for pelvic lymph node metastases (see CLINICAL PHARMACOLOGY, Imaging Performance in Newly-Diagnosed Patients). It is not indicated in patients who are not at high risk.

Indium In 111 ProstaScint™ is also indicated as a diagnostic imaging agent in post-prostatectomy patients with a rising PSA and a negative or equivocal standard metastatic evaluation in whom there is a high clinical suspicion of occult metastatic disease. The imaging performance of Indium In 111 ProstaScint™ following radiation therapy has not been studied.

The information provided by Indium In 111 ProstaScint™ imaging should be considered in conjunction with other diagnostic information. Scans that are positive for metastatic disease should be confirmed histologically in patients who are otherwise candidates for surgery or radiation therapy unless medically contraindicated. Scans that are negative for metastatic disease should not be used in lieu of histological confirmation.

ProstaScint™ is not indicated as a screening tool for carcinoma of the prostate nor for readministration for the purpose of assessment of response to treatment.

CONTRAINDICATIONS

Indium In 111 ProstaScint™ should not be used in patients who are hypersensitive to this or any other product of murine origin or to Indium In 111 chloride.

WARNINGS

Patient management should not be based on Indium In 111 ProstaScint™ scan results

5

Samsung Bioepis Exhibit No. 1002
Page 2793

WITHOUT APPROPRIATE CONFIRMATORY STUDIES SINCE IN THE PIVOTAL TRIALS, THERE WAS A HIGH RATE OF FALSE POSITIVE AND FALSE NEGATIVE IMAGE INTERPRETATIONS (SEE PRECAUTIONS).

INDIUM IN 111 PROSTASCINT™ IMAGES SHOULD BE INTERPRETED ONLY BY PHYSICIANS WHO HAVE HAD SPECIFIC TRAINING IN INDIUM IN 111 PROSTASCINT™ IMAGE INTERPRETATION (SEE PRECAUTIONS, IMAGING PRECAUTIONS).

ALLERGIC REACTIONS, INCLUDING ANAPHYLAXIS, CAN OCCUR IN PATIENTS WHO RECEIVE MURINE ANTIBODIES. ALTHOUGH SERIOUS REACTIONS OF THIS TYPE HAVE NOT BEEN OBSERVED IN CLINICAL TRIALS AFTER INDIUM IN 111 PROSTASCINT™ ADMINISTRATION, MEDICATIONS FOR THE TREATMENT OF HYPERSENSITIVITY REACTIONS SHOULD BE AVAILABLE DURING ADMINISTRATION OF THIS AGENT.

INDIUM IN 111 PROSTASCINT™ MAY INDUCE HUMAN ANTI-MOUSE ANTIBODIES WHICH MAY INTERFERE WITH SOME IMMUNOASSAYS, INCLUDING THOSE USED TO ASSAY PSA AND DIGOXIN (SEE PRECAUTIONS, DRUG/LABORATORY TEST INTERACTIONS).

PRECAUTIONS

GENERAL

THERE WERE HIGH RATES OF FALSE POSITIVE AND FALSE NEGATIVE IMAGE INTERPRETATIONS IN THE PIVOTAL TRIALS (SEE CLINICAL STUDIES). FALSE POSITIVE SCAN INTERPRETATIONS MAY RESULT IN: (1) INAPPROPRIATE SURGICAL INTERVENTION TO CONFIRM SCAN RESULTS; (2) INAPPROPRIATE DENIAL OF CURATIVE THERAPY IF RESULTS ARE NOT CONFIRMED; OR (3) INADEQUATE SURGICAL STAGING IF ONLY AREAS OF UPTAKE ARE SAMPLED. SURGICAL SAMPLING SHOULD NOT BE LIMITED TO THE AREAS OF POSITIVE UPTAKE, UNLESS HISTOLOGIC EXAMINATION OF THESE AREAS IS DIAGNOSTIC. DUE TO THE POTENTIAL FOR FALSE NEGATIVE SCAN INTERPRETATIONS, NEGATIVE IMAGES SHOULD NOT BE USED IN LIEU OF HISTOLOGIC CONFIRMATION. PROPER PATIENT PREPARATION IS MANDATORY TO OBTAIN OPTIMAL IMAGES FOR INTERPRETATION (SEE IMAGING PRECAUTIONS, BELOW).

BONE SCANS ARE MORE SENSITIVE THAN PROSTASCINT™ FOR THE DETECTION OF METASTASES TO BONE, AND INDIUM IN 111 PROSTASCINT™ SHOULD NOT REPLACE BONE SCAN FOR THE EVALUATION OF SKELETAL METASTASES.

IMAGING PRECAUTIONS

RADIOPHARMACEUTICALS SHOULD BE USED ONLY BY PHYSICIANS AND OTHER PROFESSIONALS WHO ARE QUALIFIED BY TRAINING AND EXPERIENCE IN THE SAFE USE AND HANDLING OF RADIONUCLIDES. INDIUM IN 111 PROSTASCINT™ IMAGES SHOULD BE INTERPRETED ONLY BY PHYSICIANS WHO HAVE HAD SPECIFIC TRAINING IN THE INTERPRETATION OF INDIUM IN 111 PROSTASCINT™ IMAGES.

THERE MAY BE INDIUM IN 111 PROSTASCINT™ CLEARANCE AND IMAGING LOCALIZATION OBSERVED IN THE BOWEL, BLOOD POOL, KIDNEYS, AND URINARY BLADDER. WHEN OBTAINING ALL 72-120 HOUR PLANAR AND SINGLE-PHOTON EMISSION COMPUTED TOMOGRAPHY (SPECT) IMAGES, THE BLADDER SHOULD BE CATHETERIZED AND IRRIGATED. THE ADMINISTRATION OF A CATHARTIC IS REQUIRED THE EVENING BEFORE IMAGING THE PATIENT, AND A CLEANSING ENEMA SHOULD BE ADMINISTERED WITHIN AN HOUR PRIOR TO EACH 72-120 HOUR IMAGING SESSION.

Samsung Bioepis Exhibit No. 1002
Page 2794

The contents of the kit are not radioactive. However, after the Indium In 111 Chloride is added, appropriate shielding of Indium In 111 ProstaScint™ must be maintained. Care should be taken to minimize radiation exposure to patients and medical personnel, consistent with proper hospital and patient management procedures.

Each ProstaScint™ kit is a unit of use package. The contents of the kit are to be used only to prepare Indium In 111 ProstaScint™; unlabeled ProstaScint™ should NOT be administered directly to the patient. After radiolabeling with Indium In 111, the entire Indium In 111 ProstaScint™ dose must be administered to the patient for whom it was prescribed. Reducing the dose of Indium In 111, unlabeled ProstaScint™, or Indium In 111 ProstaScint™ may adversely impact imaging results and is not recommended.

The components of the kit are sterile and pyrogen-free and contain no preservative. Indium In 111 ProstaScint™ should be used within 8 hours after radiolabeling. It is essential to follow the directions for preparation carefully and to adhere to strict aseptic procedures during preparation of the radiolabeled product.

Information for Patients

Murine monoclonal antibodies (MAbs) are foreign proteins, and their administration can induce HAMA. While limited data exist concerning the clinical significance of HAMA, the presence of HAMA may interfere with murine-antibody based immunoassays, or could compromise the efficacy of diagnostic or therapeutic murine antibody-based agents and increase the risk of adverse reactions. For these reasons, patients should be informed that the use of this product could adversely affect the future ability to diagnose recurrence of their tumor, the ability to perform certain other laboratory tests, or to use other murine-based products. Patients should be advised to discuss prior use of murine-antibody based products with their physicians (see Heterologous Protein Administration, below).

Heterologous Protein Administration

Indium In 111 ProstaScint™ has been shown to induce HAMA to murine IgG infrequently and with low peak levels after single administration. HAMA levels were detected (at >8 ng/mL) by RIA after single infusion in 8% (20/239) of patients, while 1% of patients had levels greater than 100 ng/mL. In addition, serum HAMA levels were detected by RIA after repeat infusion in 19% (5/27) of the patients.

While limited data exist concerning the clinical significance of HAMA, detectable serum levels can alter the clearance and tissue biodistribution of MAbs. The development of persistently elevated serum HAMA levels could compromise the efficacy of diagnostic or therapeutic murine antibody-based agents. In repeat administration trials, 93% (65/70) of the evaluable repeat infusions were associated with normal tissue distribution of the MAb conjugate. Pre-infusion serum HAMA levels were generally not predictive of altered distribution.

When considering the administration of Indium In 111 ProstaScint™ to patients who have previously received other murine antibody-based products, physicians should be aware

7

Samsung Bioepis Exhibit No. 1002
Page 2795

of the potential for assay interference and increased clearance and altered biodistribution, which may interfere with the quality or sensitivity of the imaging study. Prior to administration of murine antibodies, including Indium In 111 ProstaScint™, the physician should review the patient history to determine whether the patient has previously received such products.

Drug Interactions

The effect of surgical and/or medical androgen ablation on the imaging performance of Indium In 111 ProstaScint™ has not been studied. Preliminary data suggest hormone ablation may increase PSMA expression, with concurrent decrease in tumor expression of PSA.[3] The use of ProstaScint™ in this patient population cannot be recommended at this time.

Drug/Laboratory Test Interactions

The presence of HAMA in serum as a result of ProstaScint™ may interfere with some antibody-based immunoassays (such as PSA and digoxin). When present, this interference generally results in falsely high values. When following PSA levels, assay methods resistant to HAMA interference should be utilized. PSA assays which were found to be resistant to HAMA interference were Hybritech Tandem-R and Abbott IMX.

When patients have received Indium In 111 ProstaScint™, the clinical laboratory should be notified to take appropriate measures to avoid interference by HAMA with clinical laboratory testing procedures. These methods include the use of non-murine-based immunoassays, HAMA removal by adsorption, or sample pre-treatment to block HAMA activity.

Carcinogenesis, Mutagenesis, Impairment of Fertility

Long-term animal studies have not been performed to evaluate the carcinogenic or mutagenic potential of Indium In 111 ProstaScint™ or to evaluate its effect on fertility.

Pregnancy

ProstaScint™ is not indicated for use in women.

Nursing Mothers and/or Lactating Women

ProstaScint™ is not indicated for use in women.

Pediatric Use

The safety and effectiveness of Indium In 111 ProstaScint™ in pediatric patients have not been established. ProstaScint™ is not indicated for use in children.

Samsung Bioepis Exhibit No. 1002
Page 2796

ADVERSE REACTIONS

ProstaScint™ was generally well tolerated in the clinical trials. After administration of 529 single doses of Indium In 111 ProstaScint™, adverse reactions were observed in 4% of patients. The most commonly reported adverse reactions were increases in bilirubin, hypotension, and hypertension, which occurred in 1% of patients. Elevated liver enzymes and injection site reactions occurred in slightly less than 1% of patients. Other adverse reactions, listed in order of decreasing frequency, were: pruritus, fever, rash, headache, myalgia, asthenia, burning sensation in thigh, shortness of breath, and alteration of taste. Most adverse reactions were mild and readily reversible. Data from repeat administration in 61 patients revealed a similar incidence of adverse reactions (5%). No deaths were attributable to Indium In 111 ProstaScint™ administration.

OVERDOSAGE

The maximum amount of Indium In 111 ProstaScint™ that can be safely administered has not been determined. In clinical studies, single doses of 10 mg of Indium In 111 ProstaScint™ were administered to 20 patients with prostate cancer; the type and frequency of adverse reactions at this dose were similar to those observed with lower doses. The maximum Indium In 111 dose administered with ProstaScint™ in a clinical study was 6.5 mCi.

DOSAGE AND ADMINISTRATION

The patient dose of the radiolabel must be measured in a dose calibrator prior to administration.

The recommended dose of ProstaScint™ is 0.5 mg radiolabeled with 5 mCi of Indium In 111 chloride. Each dose is administered intravenously over 5 minutes and should not be mixed with any other medication during its administration. Indium In 111 ProstaScint™ may be readministered following infiltration or a technically inadequate scan; however, it is not indicated for readministration for the purpose of assessment of response to treatment (see INDICATIONS AND USAGE).

Each ProstaScint™ kit is a unit dose package. After radiolabeling with Indium In 111, the entire Indium In 111 ProstaScint™ dose should be administered to the patient. Reducing the dose of Indium In 111, unlabeled ProstaScint™, or Indium In 111 ProstaScint™ may adversely impact imaging results and is, therefore, not recommended. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

Radiation Dosimetry

The estimated absorbed radiation doses to an average adult patient from an intravenous injection of ProstaScint™ labeled with 5 mCi of Indium In 111 are shown in TABLE 6. Total dose estimates include absorbed radiation doses from both Indium In 111 and the

Samsung Bioepis Exhibit No. 1002
Page 2797

INDIUM IN 111M RADIOCONTAMINANT. A LEVEL OF 0.06% OF INDIUM IN 111M WAS UTILIZED FOR THE DOSE ESTIMATES PRESENTED IN TABLE 6.

<< TABLE Nº 6>>

DIRECTIONS FOR RADIOLABELING PROSTASCINT™ WITH INDIUM IN 111 CHLORIDE

PROPER ASEPTIC TECHNIQUES AND PRECAUTIONS FOR HANDLING RADIOACTIVE MATERIALS SHOULD BE EMPLOYED. WATERPROOF GLOVES SHOULD BE WORN DURING THE RADIOLABELING PROCEDURE. THE PREPARATION OF THE PRODUCT SHOULD BE DONE BY THE FOLLOWING PROCEDURE.

..... 1. REQUIRED MATERIALS, NOT SUPPLIED:

A. INDIUM IN 111 CHLORIDE FROM AMERSHAM, INC. OR MALLINCKRODT, INC.
B. ONE STERILE 1 ML SYRINGE
C. VIAL SHIELD
D. DOSE CALIBRATOR SET FOR INDIUM IN 111
E. GELMAN ITLC-GC STRIPS
F. DEVELOPING CHAMBER FOR CHROMATOGRAPHY (E.G. SCINTILLATION VIAL)
G. 21-23 GAUGE STERILE NEEDLES
H. SHIELD FOR 10 ML SYRINGE
I. WATERPROOF GLOVES
J. ALCOHOL WIPE
K. WATER-SOLUBLE MARKER
L. 0.9% SODIUM CHLORIDE SOLUTION
M. DTPA (0.05 M SOLUTION OF DIETHYLENETRIAMINE PENTAACETIC ACID)
N. GAMMA RAY DETECTOR

..... 2. STERILE, PYROGEN-FREE INDIUM IN 111 CHLORIDE SOLUTION MUST BE UTILIZED IN THE PREPARATION OF THE INDIUM IN 111 PROSTASCINT™. THE USE OF HIGH PURITY INDIUM IN 111 CHLORIDE MANUFACTURED BY AMERSHAM, INC. OR BY MALLINCKRODT, INC. IS REQUIRED. THE INDIUM IN 111 CHLORIDE SHOULD BE USED ONLY TO RADIOLABEL THE PROSTASCINT™ AND SHOULD NOT BE INJECTED DIRECTLY INTO THE PATIENT. THE INDIUM IN 111 CHLORIDE SHOULD NOT BE UTILIZED AFTER ITS EXPIRATION DATE.

..... 3. BEFORE RADIOLABELING BRING THE REFRIGERATED PROSTASCINT™ TO ROOM TEMPERATURE. NOTE: PROSTASCINT™ IS A PROTEIN SOLUTION WHICH MAY DEVELOP TRANSLUCENT PARTICULATES. THESE PARTICULATES WILL BE REMOVED BY FILTRATION.

..... 4. CLEAN THE RUBBER STOPPER OF EACH VIAL WITH AN ALCOHOL WIPE. WITH A STERILE 1 ML SYRINGE ADD 0.1 ML OF SODIUM ACETATE SOLUTION TO THE SHIELDED VIAL OF INDIUM IN 111 CHLORIDE AND MIX. RETAIN REMAINING SODIUM ACETATE FOR USE IN STEP 7.

..... 5. WITH THE SAME 1 ML SYRINGE, WITHDRAW BETWEEN 6 & 7 MCI OF THE BUFFERED INDIUM IN 111 CHLORIDE AND ADD TO THE PROSTASCINT™ VIAL. FLUSH THE SYRINGE TO MIX THE PREPARATION. TO NORMALIZE PRESSURE, WITHDRAW AN EQUAL VOLUME OF AIR. SWIRL GENTLY TO MIX, AND ASSAY CONTENTS IN A DOSE CALIBRATOR. ON ONE OF THE LABELS PROVIDED, RECORD THE PATIENT'S IDENTIFICATION, THE DATE, TIME OF PREPARATION, AND ACTIVITY IN THE VIAL. AFFIX THE LABEL TO THE VIAL SHIELD.

..... 6. ALLOW THE LABELING REACTION TO PROCEED AT ROOM TEMPERATURE FOR 30 MINUTES.

..... 7. WITH A SYRINGE, ADD THE REMAINING SODIUM ACETATE TO THE PROSTASCINT™ REACTION VIAL. TO NORMALIZE PRESSURE, WITHDRAW AN EQUAL VOLUME OF AIR.

Samsung Bioepis Exhibit No. 1002
Page 2798

..... 8. Aseptically attach the 0.22 µm Millex® GV sterile filter (provided) and a sterile hypodermic needle to a 10 mL sterile disposable syringe and withdraw the contents of the reaction vial through the filter into the syringe. Keep the needle immersed in the solution to avoid creating an air-lock in the filter.

..... 9. Remove the filter and needle. Aseptically attach a fresh sterile hypodermic needle to the syringe. Assay syringe and contents in a dose calibrator. The syringe should contain not less than 4 mCi (148 MBq) of Indium In-111.

..... 10. Radiochemical purity (RCP) by Instant Thin Layer Chromatography (ITLC) can be determined by the following procedure:

A. Mix equal parts (several drops of each) of Indium In 111 ProstaScint™ with DTPA solution. Allow the mixture to stand at room temperature for one minute. Spot a small drop of the mixture onto an ITLC strip at its origin.

B. Place the strip in a chromatography chamber with the origin at the bottom and allow the solvent to migrate 6 cm from the origin of the strip. Remove and cut the strip in half and measure the counts per minute (CPM) of both halves with a gamma ray detector.

C. Calculate the percent RCP as follows:

$$\%RCP = \frac{\text{CPM bottom half}}{\text{CPM bottom half} + \text{CPM top half}} \times 100$$

D. If the radiochemical purity is <90%, the ITLC procedure should be repeated. If repeat testing remains <90%, the preparation should not be administered.

..... 11. On the second label provided in the kit, record the patient's identification, the date, time of assay, and activity in the syringe. Affix this label to the syringe shield.

..... 12. Indium In 111 ProstaScint™ should be used within 8 hours of radiolabeling.

..... 13. Discard vials, needles, and syringes in accordance with local, state, and federal regulations governing radioactive and biohazardous waste.

Image Acquisition and Interpretation

Images should be acquired using a large field of view gamma camera equipped with a parallel hole medium energy collimator. The gamma camera should be calibrated using the 172 and 247 keV photopeaks for Indium In 111 with a 20% symmetric window.

Whole body or spot planar views of the pelvis, abdomen, and thorax should be performed between 72 and 120 hours following Indium In 111 ProstaScint™ infusion. A cathartic is required the evening before imaging and a cleansing enema should be administered within an hour prior to each 72-120 hour imaging session. In addition, the bladder should be catheterized and irrigated.

Whole body acquisition should be carried out from skull through mid-femur. The total scan time over this area should be no less than 35 minutes using a 128x512 or 256x1024 matrix.

PLANAR IMAGES SHOULD BE ACQUIRED IN ANTERIOR AND POSTERIOR VIEWS FOR 7.5 MINUTES PER VIEW USING A 128X128 OR 256X256 MATRIX. DUE TO UPTAKE OF INDIUM IN 111 PROSTASCINT™ BY THE LIVER, PLANAR IMAGES OBTAINED WITH THE LIVER IN THE FIELD OF VIEW MUST BE ACQUIRED WITH ADEQUATE COUNTS TO ALLOW THE DETECTION OF LESIONS IN THE ADJACENT EXTRAHEPATIC ABDOMEN AND PELVIS. THIS MAY RESULT IN PIXEL OVERFLOW WITH IMAGE DEGRADATION IN THE REGION OF THE LIVER.

TWO SPECT IMAGING SESSIONS ARE NECESSARY. THE FIRST SPECT SESSION SHOULD BE OF THE PELVIS AND BE PERFORMED APPROXIMATELY 30 MINUTES AFTER INFUSION TO OBTAIN A BLOOD POOL IMAGE. THE SECOND SPECT SESSION SHOULD INCLUDE BOTH THE PELVIS AND ABDOMEN, INCLUDING THE LOWER LIVER MARGIN THROUGH THE PROSTATIC FOSSA AND BE PERFORMED BETWEEN 72 AND 120 HOURS AFTER INFUSION FOR DETECTION OF BENIGN AND MALIGNANT PROSTATE TISSUE SITES. DEPENDING UPON THE CAPABILITY OF THE CAMERA FIELD OF VIEW TO INCLUDE BOTH PELVIS AND ABDOMEN, EITHER ONE OR TWO SEPARATE ACQUISITIONS MAY BE NECESSARY DURING THE SECOND SESSION.

TO RESOLVE IMAGING AMBIGUITIES POSSIBLY RESULTING FROM ACTIVITY IN BLOOD POOL, STOOL OR URINARY BLADDER, FOLLOW-UP IMAGING SESSIONS WITH FULL PATIENT PREPARATION SHOULD BE PERFORMED.

THE SPECT IMAGES SHOULD BE ACQUIRED USING A 64X64 OR 128X128 MATRIX FOR A MINIMUM OF 60 OR 120 STOPS, RESPECTIVELY, OVER 360 DEGREES ROTATION FOR APPROXIMATELY 25 SECONDS PER VIEW AT THE FIRST SESSION AND 50 SECONDS PER VIEW AT THE SECOND SESSION. RECONSTRUCTION SHOULD BE PERFORMED USING A BUTTERWORTH FILTER OR EQUIVALENT IN THE TRANSVERSE, CORONAL AND SAGITTAL VIEWS. AN ORDER OF 5 AND CUT OFF OF 0.5 MAY BE USED AS A STARTING POINT. SLICE THICKNESS SHOULD BE IN THE RANGE OF 6 TO 12 MM.

FOLLOWING INDIUM IN 111 PROSTASCINT™ ADMINISTRATION, SOME OF THE RADIOLABEL LOCALIZES IN NORMAL LIVER, SPLEEN, BONE MARROW AND GENITALIA.

IT HAS BEEN REPORTED THAT INDIUM IN 111 LABELED ANTIBODIES MAY LOCALIZE NON-SPECIFICALLY IN COLOSTOMY SITES, DEGENERATIVE JOINT DISEASE, ABDOMINAL ANEURYSMS, POST-OPERATIVE BOWEL ADHESIONS, AND LOCAL INFLAMMATORY LESIONS, INCLUDING THOSE TYPICALLY ASSOCIATED WITH INFLAMMATORY BOWEL DISEASE OR SECONDARY TO SURGERY OR RADIATION. INDIUM IN 111 PROSTASCINT™ CAN DEMONSTRATE APPARENT LOCALIZATION TO SITES OF TORTUOUS BLOOD VESSELS. CAREFUL REVIEW OF THE PATIENT'S MEDICAL HISTORY AND OTHER DIAGNOSTIC INFORMATION SHOULD AID IN THE INTERPRETATION OF THE IMAGES.

THE DIAGNOSTIC IMAGES ACQUIRED WITH INDIUM IN 111 PROSTASCINT™ SHOULD BE INTERPRETED IN CONJUNCTION WITH OTHER APPROPRIATE DIAGNOSTIC TESTS.

HOW SUPPLIED

THE PROSTASCINT™ KIT (NDC No. 57902-817-01) FOR THE PREPARATION OF INDIUM IN 111 LABELED CAPROMAB PENDETIDE INCLUDES ONE VIAL CONTAINING 0.5 MG OF PROSTASCINT™ PER 1 ML OF SODIUM PHOSPHATE BUFFERED SALINE AND ONE 2 ML VIAL OF SODIUM ACETATE SOLUTION, 0.5 M. THESE SOLUTIONS ARE STERILE AND PYROGEN FREE AND CONTAIN NO PRESERVATIVES. EACH

Samsung Bioepis Exhibit No. 1002
Page 2800

KIT ALSO INCLUDES ONE STERILE 0.22 μM MILLEX® GV FILTER, PRESCRIBING INFORMATION, AND TWO IDENTIFICATION LABELS.

STORAGE

STORE AT 2° TO 8°C (36° TO 46°F). DO NOT FREEZE. STORE UPRIGHT.

REFERENCES

1. KOCHER, DC: RADIOACTIVE DECAY DATA TABLES. DOE/TIC 115:11026, 1981.

2. DATA SUPPLIED BY OAK RIDGE ASSOCIATED UNIVERSITIES. RADIOPHARMACEUTICAL INTERNAL DOSE INFORMATION CENTER, 1984.

3. WRIGHT, GL, JR; ET AL. EXPRESSION OF PROSTATE-SPECIFIC MEMBRANE ANTIGEN IN NORMAL, BENIGN, AND MALIGNANT PROSTATE TISSUES. UROL ONCOL. 1995: 1:18-28.

PROSTASCINT™ (CAPROMAB PENDETIDE) IS COVERED IN WHOLE OR IN PART BY AT LEAST THE FOLLOWING US PATENTS: #4,671,958, #4,741,900, AND #5,162,504.

MANUFACTURED AND DISTRIBUTED BY:
CYTOGEN CORPORATION
PRINCETON, NJ 08540-5308

REVISED 10/28/96

Samsung Bioepis Exhibit No. 1002
Page 2801

TABLES#

TABLE 1 - INDIUM IN 111 PRINCIPAL RADIATION EMISSION DATA[1]

RADIATION	MEAN % PER DISINTEGRATION	MEAN ENERGY (KEV)
GAMMA 2	90.2	171.3
GAMMA 3	94	245.4

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TABLE 2 - INDIUM IN 111 RADIATION ATTENUATION OF LEAD SHIELDING[2]

SHIELD THICKNESS (PB) CM	ATTENUATION FACTOR
0.023	0.5
0.203	10^{-1}
0.513	10^{-2}
0.834	10^{-3}
1.120	10^{-4}

TO BE INSERTED INTO THE TEXT AT THE MARKED LOCATIONS.

Samsung Bioepis Exhibit No. 1002
Page 2802

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TABLE 3 - INDIUM IN 111 PHYSICAL DECAY CHART. HALF-LIFE 67.2 HOURS (2.8 DAYS)

HOURS	FRACTION REMAINING
-48	1.64
-36	1.44
-24	1.28
-12	1.13
0*	1.00
12	0.88
24	0.78
36	0.69
48	0.61
60	0.54
72	0.48
84	0.42
96	0.37
108	0.33
120	0.29
132	0.26
144	0.23

* CALIBRATION TIME

15

Samsung Bioepis Exhibit No. 1002
Page 2803

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TABLE 4 - COMPARISON OF INDIUM IN 111 PROSTASCINT™ AND HISTOPATHOLOGIC RESULTS FOR PRESURGICAL PATIENTS

	Number of Patients		
	Indium In 111 ProstaScint™		
	+	-	
Biopsy +	40	24	*Sensitivity 62%*
Biopsy -	25	63	*Specificity 72%*
	Positive Predictive Value 62%	*Negative Predictive Value 72%*	*Overall Accuracy 68%*

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TABLE 5 - INDIUM IN 111 PROSTASCINT™ AND HISTOPATHOLOGIC RESULTS FOR RECURRENT OR RESIDUAL DISEASE PATIENTS

	Number of Patients		
	Indium In 111 ProstaScint™		
	+	-	
Biopsy +	29	30	*Sensitivity 49%*
Biopsy -	29	70	*Specificity 71%*
	Positive Predictive Value 50%	*Negative Predictive Value 70%*	*Overall Accuracy 63%*

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Samsung Bioepis Exhibit No. 1002
Page 2804

TABLE 6 - ESTIMATED AVERAGE ABSORBED RADIATION DOSE IN ADULT PATIENTS FROM INTRAVENOUS ADMINISTRATION OF PROSTASCINT® LABELED WITH 5 MCI (185 MBQ) OF INDIUM IN 111 CHLORIDE

ORGAN	AVERAGE DOSE (RAD/5 MCI)	AVERAGE DOSE (MGY/ 185MBQ)
TOTAL BODY	2.7	27
BRAIN	1.1	11
LIVER	18.5	185
SPLEEN	16.3	163
KIDNEYS	12.4	124
LUNGS	5.6	56
HEART WALL	7.8	78
RED MARROW	4.3	43
ADRENALS	5.2	52
URINE BLADDER WALL	2.2	22
BONE SURFACES	4.0	40
STOMACH	3.1	31
GALL BLADDER WALL	7.3	73
SMALL INTESTINE	3.3	33
UPPER LARGE INTESTINE WALL	5.0	50
LOWER LARGE INTESTINE WALL	7.6	76
PANCREAS	5.1	51
SKIN	1.1	11
TESTES	5.6	56
PROSTATE	8.2	82
THYMUS	2.6	26
THYROID	1.4	14
OTHER TISSUES	2.0	20

*BASED ON DATA FROM 21 PATIENTS WHO RECEIVED DOSES OF PROSTASCINT® LABELED WITH A MEAN (± SD) INDIUM IN 111 DOSE OF 4.6 ± 1.0 MCI.

Samsung Bioepis Exhibit No. 1002
Page 2805

Opposition against EP 2 359 834 B1
AMG17203OP

Declaration by Dr. Ronald Taylor

I, Ronald Taylor, Ph.D., declare that:

1. I was employed as a Professor of Biochemistry and Molecular Genetics at the University of Virginia from 1973 to 2016. I retired in 2016 and I am now Professor Emeritus and I still have an active work schedule based on submitting papers for publication, giving invited talks, serving on a study section, and reviewing papers. For almost 30 years, my laboratory has had a productive record in the study and characterization of monoclonal antibodies (mAbs), including humanized mAbs, and their use in clinical applications. A major focus of my research is anti-CD20 mAbs. In collaboration with investigators at the NIH and the Mayo Clinic, we have participated in numerous pilot clinical trials testing the feasibility of changing amount and frequency of dosing for CD20 mAb therapies for CLL. My work has resulted in 199 publications in peer-reviewed journals. A current copy of my CV is provided as **Appendix A.**

2. I have reviewed U.S. Patent Application Publication No. 2005/0191298 ("**D1**"), which is titled "Method of Treating Hemolytic Disease" and published on September 1, 2005. **D1** discloses that patients with paroxysmal nocturnal hemoglobinuria (PNH) were treated by administering eculizumab in four weekly 600 mg intravenous infusions, followed one week later by a dose of 900 mg, and then 900 mg on a bi-weekly basis (**D1**, [0082]). **D1** further discloses that anti-C5 mAbs such as eculizumab "can be administered in a variety of unit dosage forms" (**D1**, [0058]-[0060], [0062]).

3. The skilled artisan would have understood that pharmaceutical unit dosage forms containing a mAb should not be shared among patients or returned to storage after opening, in order to prevent contamination of the dosage form and any unnecessary health risks to the patient being treated with the dosage form. The skilled artisan also would have understood that

wasting mAb increases medical costs to the patient without any commensurate benefit. Thus, the skilled artisan would have had reason to prepare a single-use dosage form of eculizumab that could be used to administer both of the doses disclosed by **D1** (600 mg and 900 mg) without leaving any leftover drug as unusable waste. The skilled artisan would have understood that the most efficient way to meet that goal would be to prepare a 300 mg single-use dosage form because that dosage form could be used to administer both of the doses disclosed by **D1**—*i.e.*, two, 300 mg single-use dosage forms to administer the 600 mg dose and three, 300 mg single-use dosage forms to administer the 900 mg dose. Therefore, a 300 mg single-use dosage form would have been the most obvious and convenient single-use dosage form for preparing both the 600 mg and 900 mg doses of eculizumab for treating PNH.

4. The skilled artisan also would have recognized that preparing a single dosage form that could flexibly be used to administer either 600 mg or 900 mg offered advantages over developing a separate dosage form for each of the 600 mg and 900 mg doses. Developing any given dosage form requires testing, validating, manufacturing, storing, and transporting that dosage form. The skilled artisan would have known that it would be more efficient to avoid the redundancy and simply find a dosage form that could be used to administer both desired doses rather than creating multiple different dosage forms that would each require individual testing and validation, and may each require different storage or transport set-ups. Accordingly, efficiency would have provided the skilled artisan with further reason to use a 300 mg single-use dosage form.

5. One example of aligning antibody dosage form and administered doses known as of 2006 is the administration of the therapeutic antibody rituximab. Rituximab was administered in doses of 500 or 1000 mg to treat rheumatoid arthritis (e.g., Emery *et al.*, *Arthritis & Rheumatism* 2006, *54(5)*, 1390-1400 ("**D43**") at p. 1390). Consistent with maximizing efficiency and minimizing waste, the dosage forms of rituximab available as of 2006 were supplied as 100 mg and 500 mg sterile, preservative-free, single-use vials—both of which could be used to administer either a 500 or 1000 mg dose without waste and with maximal efficiency (e.g., Rituximab 1997 Product Data Sheet ("**D44**") at p. 2, right column, "How Supplied" section).

6. In preparing the 300 mg eculizumab single-use dosage form, the skilled artisan also would have considered the appropriate concentration at which to formulate the mAb. Eculizumab is a humanized mAb with a hybrid IgG2/IgG4 constant domain. U.S. Patent No. 6,407,213 ("**D42**") is titled "Method for Making Humanized Antibodies" and issued on June 18, 2002. **D42** relates to methods for making and using humanized antibodies, including antibodies with hybrid constant domains (**D42**, col. 8, ll. 47-51). **D42** states that such humanized "antibodies will typically be formulated … at concentrations of about 1 mg/ml to 10 mg/ml" (**D42**, col. 45, li. 57, to col. 46, li. 2). Thus, selection of 10 mg/ml as a concentration for a humanized mAb with a hybrid IgG2/IgG4 constant domain, such as eculizumab, would have been a typical, arbitrary choice among known concentrations. Similarly, U.S. Patent Application Publication No. 2005/0271660 ("**D22**") discloses using eculizumab at a concentration between 1 and 30 mg/ml in nebulized formulations (**D22**, Fig. 10, [0170]-[0172]). From this, the skilled artisan would have understood that eculizumab is sufficiently stable and biologically active to be used as a drug at concentrations including 10 mg/ml. Thus, **D22** reinforces that selection of 10 mg/ml as a concentration for eculizumab would have been a typical, arbitrary choice among known concentrations, regardless of the route of administration. **D44** reinforces the typical nature of a 10 mg/ml concentration because it discloses that rituximab was supplied at that concentration (e.g., **D44** at p. 2, right column, "How Supplied" section).

7. Additionally, in choosing among these *typical* concentrations, the skilled artisan would have had a reason to prepare the 300 mg single-use dosage form using a 10 mg/ml concentration of eculizumab in order to minimize the volume of the 600 mg and 900 mg doses required for treating PNH. For example, preparing the 600 mg and 900 mg doses taught by **D1** would require 60 ml and 90 ml, respectively, of a 300 mg single-use dosage form having 10 mg/ml of the mAb. But preparing the same 600 mg and 900 mg doses would require 600 ml and 900 ml, respectively, of a 300 mg single-use dosage form having 1 mg/ml of the antibody. The skilled artisan would have understood that smaller volumes would be more convenient for storage, transportation, and administration of the mAb. Further, the skilled artisan would have been reluctant to use concentrations higher than the 10 mg/ml taught by **D42** because increased aggregation was known to result from increased concentration of antibodies (e.g., Wang *et al.*, *J. Pharm. Sci*. 2007, *96(1)*, 1-26 ("**D20**") at p. 9, left column, 2nd full paragraph).

8. Given that the skilled artisan would have had reason to use a 300 mg single-use dosage form with a concentration of 10 mg/mL, the skilled artisan necessarily would have arrived at a 30 mL volume. The volume of a 10 mg/mL solution needed to prepare 300 mg is, and can only be, 30 mL.

I certify that the above statements are correct to the best of my knowledge and belief.

Keswick, Virginia Nov 20, 2018
Place and Date

Dr. Ronald Taylor

Appendix A

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Ronald P. Taylor

eRA COMMONS USER NAME (credential, e.g., agency login): RPTNIH

POSITION TITLE: Emeritus Professor of Biochemistry and Molecular Genetics, University of Virginia Health Science Center

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
City College of New York, New York, NY	B.S.	1966	Chemistry
Princeton University, Princeton, NJ	Ph.D.	1970	Physical Chemistry
University of Minnesota, Mpls., MN	Post-doc	1971-1973	Physical Chemistry

A. Personal Statement

For almost 30 years my laboratory has had a long and productive record in the study and characterization of monoclonal antibodies and their use in several different clinical applications. We have a considerable level of experience in studying complement activation by, as well as effector cell-mediated killing of, tumor cells opsonized with monoclonal antibodies. Our laboratory discovered and has substantially characterized the shaving/trogocytosis reaction: that is, the loss of CD20 that occurs when CLL patients are treated with the usual (high) doses of rituximab or of ofatumumab. We have demonstrated, in several pilot clinical studies as well as in vitro investigations that much lower doses of CD20 mAbs, given more frequently, may provide far more effective therapies for CLL. We have also now described, in some detail at the cellular level, the complex series of events that occur when mAb-opsonized nucleated cells are killed by complement. Our laboratory also first described the exhaustion of complement which follows high dose rituximab therapy in CLL treatment.

I was appointed Professor Emeritus as of July 1, 2016. Since then I have maintained on-going collaborations with investigators in the US: Dr. D. Portilla (UVA), Dr. C. Zent (Rochester), Dr. A. Wiestner (NIH), Dr. J. Lambris (Penn), Dr. J. Thurman (U Colorado), Dr. S. Ram (U Mass); and in Europe: Dr. T. Mollnes (Norway) Dr. M. Huber-Lang (Germany), Dr. A. Verschoor (Germany) and the Genmab Biotech Company (The Netherlands).

B. Positions and Honors

Professional Positions:

1973-1978, Assistant Professor, Dept. of Biochemistry, University of Virginia.
1978-1983, Associate Professor, Dept. of Biochemistry, University of Virginia.
1983-present, Professor, Dept. of Biochemistry and Molecular Genetics, University of Virginia.
1991-present, Member, Beirne B. Carter Center for Immunology Research, University of Virginia.
1997-present, Member, NCI Sponsored Cancer Center, University of Virginia.

Awards and Other Professional Activities:

1965, Phi Beta Kappa; 1966, ECAC Scholar-Athlete Award; 1966-1969, NDEA Graduate Fellow; 1970, NSF Graduate Fellow; 1972-1973, NIH Fellow; 1975-1980, NIH Research Career Development Award; 1992, Ad

Hoc Study Section, Immunological Sciences, NIH; 1991-1993, Shannon Award, NIH; 1990-1994, Editorial Board, Arthritis and Rheumatism; 1990, Fellowship Subcommittee, Arthritis Fdn; 2008, DARPA Significant Technical Achievement Award; 2010-2015, Editorial Board, Haematologica; 2010-2014, Associate Editor, Journal of Immunology; Ad hoc reviewer: J. Immunology, J. Immunological Methods, J. Clin Invest, Blood, Mol. Immunology, Immunology, Science Translational Medicine, and many Cancer Journals: Reviewer, NIH: 2003-2004, Exptl. Immunol; 2004,Tumor Immunol./Immunotherapy; 2007-2018 (ongoing), Bench to Bedside NIH Study Section.

C. Contributions to Science (total publications: 199)

1. Our laboratory has pioneered the detailed and comprehensive evaluation of blood samples taken from patients before, during, and after CD20 therapy for CLL. This work led to our discovery of the shaving/trogocytosis reaction, as well as to the finding that immune effector functions in the body, such as complement, can be saturated/exhausted when CLL patients receive the usual high CD20 mAb doses (300 mg to 2 grams). This work also directly led to our in vitro analyses and mechanistic descriptions of trogocytosis, in which we demonstrated that it is mediated by Fcγ receptors on effector cells including monocyte/macrophages, neutrophils, and NK cells. We have also described the process in a mouse model and demonstrated that the reaction can be generalized to include other FDA-approved immunotherapeutic mAbs including cetuximab and traustuzumab. Finally, in collaboration with investigators at the NIH and the Mayo Clinic, we have been able to participate in numerous pilot clinical trials that have indicated the potential feasibility of changing CD20 mAb therapies for CLL to low doses (~35 mg) three times a week, which ultimately can be based on subcutaneous infusions. If this approach can be clearly validated and generalized, it would provide a very important and revolutionary paradigm shift that could benefit both patients and health-care providers, based on efficacy, costs, and convenience.

Most recently, in collaboration with several investigators we have also been studying how complement plays a role in the pathogenesis of several diseases and syndromes associated with inflammation and tissue damage, including malaria and kidney injury due to tubulointerstitial fibrosis. This work has the potential to lead to new and innovative strategies for disease treatment, based on selective inhibition of key steps in the complement cascade(s).

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2. Beum, P.V., Kennedy, A.D., Williams, M.E., Lindorfer, M.A., **Taylor, R.P.** The shaving reaction: Rituximab/CD20 complexes are removed from mantle cell lymphoma and chronic lymphocytic leukemia cells by THP-1 monocytes. *J. Immunology* 176:2600-2609, 2006. PMID: 16456022
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4. Glennie, M.J. ,French, R., Cragg, M.S., **Taylor, R.P.** Mechanisms of killing by anti-CD20 monoclonal antibodies. *Molecular Immunology* 44: 3823-3837, 2007. PMID: 17768100
5. Li, Y., Williams, M.E., Cousar, J.B., Pawluczkowycz, A.W. Lindorfer, M.A., **Taylor, R.P.** Rituximab/CD20 Complexes Are Shaved from Z138 Mantle Cell Lymphoma Cells in Intravenous and Subcutaneous SCID Mouse Models. *J. Immunology* 179: 4263-4271, 2007. PMID: 17785867
6. Beum, PV, Lindorfer, MA, **Taylor, R.P.** Within Peripheral Blood Mononuclear Cells, Antibody-Dependent Cellular Cytotoxicity of Rituximab-opsonized Daudi cells is Promoted by Natural Killer Cells and Inhibited by Monocytes Due to Shaving. *J. Immunology* 181: 2916-2924, 2008. PMID: 18684983
7. Aue, G, Lindorfer, MA, Beum, PV, Pawluczkowycz, AW, Vire, B, Hughes, T, **Taylor, R.P.**, Wiestner, A Fractionated Subcutaneous Rituximab Preserves CD20 Expression on Tumor Cells in Patients with Chronic Lymphocytic Leukemia. *Haematolgica* 95:329-332, 2010. PMCID: PMC2817038
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far more effective than wild-type mAbs in promoting CDC. It is very likely that these next generation mAbs (for CD20 as well as other targets) will find clinical applications in the not-too-distant future.

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IN THE MATTER OF
European Patent No. EP 2 359 834

DECLARATION OF PROFESSOR ALEXANDER M. KLIBANOV

I, Dr. Alexander M. Klibanov, hereby declare as follows:

1. I am a Professor of Chemistry and Bioengineering at the Massachusetts Institute of Technology ("M.I.T."), where I have been teaching and conducting research for more than 39 years. I currently hold the Novartis Endowed Chair Professorship. I obtained an M.S. degree in Chemistry in 1971 and a Ph.D. in Chemical Enzymology in 1974, both from Moscow University in Russia.

2. Throughout my career as a practicing chemist, I have extensively researched, published, taught, and lectured in many areas of biological, medicinal, protein, and pharmaceutical formulation chemistry. For my professional accomplishments, I have received numerous honors. For example, I was elected to the U.S. National Academy of Sciences and the U.S. National Academy of Engineering; I have also received various awards and prizes from the American Chemical Society, as well as other national and international organizations. I serve on the Editorial Boards of over a dozen scientific journals, including the "Journal of Biological Chemistry and Molecular Pharmacology" and the "International Journal of Drug Design, Delivery and Safety." I have published some 315 scientific papers in various chemical disciplines, including protein stability and stabilization, as well as drug formulation and delivery. I have also founded six pharmaceutical companies and have been on the scientific advisory boards and/or boards of directors of those companies, as well as of many others. A number of these advisory and directorship activities have involved the formulation, stability, stabilization, administration, and pharmacological evaluation of protein-based therapeutic agents, including antibodies. A copy of my curriculum vitae is attached as Exhibit 1.

3. I have been asked by Alexion to provide information regarding the field of protein, including antibody, pharmaceutical formulations as of 2007 and what understanding and expectations a person skilled in that field would have had concerning their stability at that time.

4. One of the major challenges in developing a successful pharmaceutical formulation of a therapeutic antibody (and of any therapeutic protein) around 2007 was, and still is today, being able to create a composition that is stable enough for sufficiently long periods of time such that the antibody does not undergo deleterious physical or chemical degradation reactions or form aggregates prior to administration. If the antibody is not adequately stable in the composition, serious problems can arise, such as drastically diminished efficacy and adverse immune responses in the patient. Moreover, when developing an antibody for commercial therapeutic use, it is often critical to be able to formulate the antibody at a rather high concentration (thus allowing a low-volume dosage).

5. Based on my professional experience, in the 2007 time-frame (or even today) there was no assurance that a skilled person, when provided with a new therapeutic protein, including an antibody, would have been able to develop a suitably stable pharmaceutical composition to make it commercially viable. My experience in this regard is confirmed by relevant scientific publications around that time period, as discussed below.

6. For example, a 2002 book entitled "Rational Design of Stable Protein Formulations. Theory and Practice" (the "2002 book;" Ex. 2) describes the uncertainty that existed in the protein formulation field regarding the ability to develop a suitably stable liquid pharmaceutical composition for a new protein. The authors of the 2002 book state that "protein drugs may not be stable enough to be handled as a liquid formulation" and concede that "with many proteins, it is not possible – especially considering the time constraints with product development – to develop sufficiently stable aqueous formulations." (Ex. 2 at 10 and 109.) The authors suggested that there was no reasonable expectation at the time of being able to develop such a composition, especially a liquid one, *e.g.*, stating that "[i]t can be *assumed* that most proteins will not exhibit sufficient stability in aqueous solution to allow a liquid formulation to be developed." (Ex. 2 at 188.) Consequently, most pharmaceutical proteins, such as antibodies, commercially available even today are in a lyophilized form, which requires their reconstitution prior to administration, thereby making it far less convenient and more expensive than aqueous solutions.

7. As the 2002 book recognized and was understood both then and now, numerous factors influence the stability of an antibody (or any protein), thus making the development of a suitable pharmaceutical composition in each new specific case a unique challenge. The book explains that "the exquisite sensitivity of protein structure, function, and stability to the primary sequence does not readily lend itself to a generic approach for protein formulation" and that "[e]ven for closely related proteins, the relative stability and major pathways for degradation might be quite different." (Ex. 2 at 185-186.) In the 2007 time-frame, researchers like myself understood that therapeutic antibody (or protein in general) formulations, especially liquid ones, are ***not*** amenable to a "one size fits all" approach. Therefore, when given a new antibody and tasked with creating its suitable pharmaceutical composition, a person skilled in the art would have had little guidance in that endeavor. Indeed, the 2002 book recognized that developing such a stable composition "is not a simple task" and that "for most proteins, maintaining physical and chemical stabilities in aqueous solution for an extended period of time is extremely difficult" (Ex. 2 at 10-11 and 184).

8. A review article entitled "Formulation and delivery issues for monoclonal antibody therapeutics" published in 2006 in a peer-reviewed international journal had recognized the uniqueness of each therapeutic antibody's challenge when developing a suitably robust formulation: "A priori, one might assume that by finding a stable formulation for one of these antibody drugs, that such a formulation would be good for most[,] if not all, similar antibodies. If this were borne out by experience, there would be no need for a review such as this. Instead, each antibody seems to have a unique personality related to its requirements for

Samsung Bioepis Exhibit No. 1002
Page 2816

stability." (D21 at 690). For this reason, proteins typically have to be evaluated individually and stabilized on an empirical basis, which inevitably leads to unpredictability.

9. Another, 2007 review paper entitled "Antibody structure, instability, and formulation" points out that "antibodies, like other proteins, may generate a variety of degradants during production, processing, and storage both in liquid and solid states. Their tendency to generate such degradants depends very much on individual sequence, pI, hydrophobicity, and carbohydrate content." (D20 at 14). The review noted numerous variables affecting each of the many causes of antibody instability; because they were, in turn, influenced by a number of intertwining factors, as well as by the structure of the protein molecule itself, a person skilled in the art in 2007 would have recognized that predicting in advance whether or how a new antibody could be stabilized under relevant conditions at a particular concentration in a pharmaceutical composition was not possible: "It should be stressed that one formulation excipient stabilizing a specific antibody may not be suitable for another because of the difference in their sequence." (D20 at 14).

10. Given the complexity and the sheer number of the various modes of protein instability and the diverse mechanisms underlying each of them, there was no universal approach to successfully formulating an antibody in 2007 (nor is there one now). My both academic and industrial experience at the time was that the field of antibody formulation was difficult, case-specific, and uncertain. For these reasons, a person skilled in the art aiming to develop a liquid formulation of a new antibody with a sufficient storage stability at a high concentration would ***not*** have been able to predict which, if any, such formulation would meet the necessary requirements.

I do hereby declare that the facts stated above are true to the best of my knowledge.



Dr. Alexander M. Klibanov

November 16, 2018

Date

Samsung Bioepis Exhibit No. 1002
Page 2817

Exhibit 1: Dr Klibanov Curriculum Vitae

Exhibit 2: Rational Design of Stable Protein Formulations, Pharmaceutical Biotechnology Volume 13, 2002 Kluwer Academic/Plenium Publishers; Chapters 1, 5 and 8 (D40).

Curriculum vitae

ALEXANDER M. KLIBANOV

Date and Place of Birth: July 15, 1949, in Moscow (Russia)

Nationality: Naturalized U.S. Citizen (1983)

Education:

1974	Ph.D. in Chemical Enzymology, Moscow University
1971	M.S. in Chemistry, Moscow University

Honors:

2015	Tau Beta Pi's Leonardo Da Vinci Lecturer, MIT
2007-11 and 2014-	Novartis Chair Endowed Professorship, MIT
2012-13	Roger and Georges Firmenich Endowed Professorship, MIT
2011	MIT Biological Engineering Senior Class Faculty Award
2006	Distinguished GRUM Lecturer in Drug Discovery & Development, University of Montreal (Canada)
2004	UNAM Distinguished Lecturer, National University of Mexico (Mexico City)
2001	Walter Enz Lecturer in Pharmaceutical Chemistry, University of Kansas
2001	Elected a Corresponding Fellow of the Royal Society of Edinburgh (Scotland's National Academy of Science and Letters)
2000	Merck Distinguished Lecturer, Rutgers University
2000	Top 20 *Biotechnology & Bioengineering* Papers of the Last Forty Years
1998	Robert Lutz Lecturer, University of Virginia
1996	Perkin-Elmer Distinguished Lecturer, University of Pittsburgh
1995	Elected to the National Academy of Sciences of the U.S.A.
1995	Nathan O. Kaplan Memorial Lecturer in Biological Chemistry, University of California at San Diego
1994	R.W. Johnson PRI Lecturer, Pharmaceutical Research Institute
1994	Warren McCabe Lecturer, North Carolina State University
1993	Elected to the National Academy of Engineering of the U.S.A.
1993	Arthur C. Cope Scholar Award of the American Chemical Society
1993	Biotechnology Divisional Lectureship Award of the Institute of Food Technologists
1992	Charles Sabat Lecturer, Rutgers University
1992	Elected a Founding Fellow of the American Institute for Medical and Biological Engineering
1992	Louis C. Jordy Research Scholar Lecturer, Drew University
1991	International Enzyme Engineering Award
1991	Marvin J. Johnson Award of the American Chemical Society
1990	Monsanto Lecturer, Ohio State University
1990	NRC Distinguished Lecturer, Academia Sinica (Taiwan)
1989	Ipatieff Prize of the American Chemical Society
1989	Backer Lecturer, Groningen University (Holland)
1988	Dow Lecturer, University of Ottawa (Canada)
1987	Distinguished Scholar Lecturer, Hope College
1986	Leo Friend Award of the American Chemical Society
1984	Who's Who in Frontier Science and Technology

Momenta Pharm. v. BMS, IPR215-01537
1/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2819

1984	Sohio Lecturer, Case Western Reserve University
1982	American Men and Women of Science
1981-1983	Henry L. Doherty Career Development Professorship, MIT
1975	U.S.S.R. Ministry of Higher Education Prize

Professional Experience:

2007-11 and 2014-	Novartis Chair Endowed Professor of Chemistry and Bioengineering, MIT
2012-2013	Roger and Georges Firmenich Professor of Natural Products Chemistry Department of Chemistry, MIT
2000-present	Professor of Bioengineering Department of Biological Engineering, MIT
1988-present	Professor of Chemistry Department of Chemistry, MIT
1987-1988	Professor of Applied Biochemistry Department of Applied Biological Sciences, MIT
1983-1987	Associate Professor of Applied Biochemistry Department of Applied Biological Sciences (formerly Department of Nutrition and Food Science), MIT
1979-1983	Assistant Professor of Applied Biochemistry Department of Nutrition and Food Science, MIT
1977-1979	Postdoctoral Associate, Department of Chemistry University of California at San Diego
1974-1977	Research Chemist Department of Chemistry, Moscow University

Current Journal Editorial/Advisory Boards:

"Biocatalysis and Biotransformation", "Applied Biochemistry and Biotechnology", "Central European Journal of Chemistry", "Biotechnology Progress", "Biotechnology & Bioengineering", "Microbial Biotechnology", "Open Journal of Pharmacology", "Nanocarriers", "Journal of Antivirals and Antiretrovirals", "Open Access Academic Books in Chemistry", "Journal of Biological Chemistry and Molecular Pharmacology", "Recent Patents in Biotechnology", "Current Pharmaceutical Biotechnology"

Professional Societies:

U.S. National Academy of Sciences, U.S. National Academy of Engineering, American Chemical Society, Society for Applied Microbiology

Current Research Interests:

Enzyme chemistry and biotechnology
Medicinal and formulation chemistry
Protein drug delivery
Antimicrobial polymers
Enzymes as stereoselective catalysts in organic syntheses
Stabilization and formulation of macromolecular pharmaceuticals

Momenta Pharm. v. BMS, IPR215-01537
2/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2820

Publications:

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2. Varfolomeyev, S.D., Klibanov, A.M., Martinek, K., Berezin, I.V. 1972. Light-sensitive catalysts. 4-Nitrocinnamoyl residue as a chromophoric reporter group in the α-chymotrypsin active center. Dokl. Acad. Nauk SSSR 203: 616-619.

3. Klibanov, A.M., Samokhin, G.P., Martinek, K., Berezin, I.V. 1974. Mechanochemistry of catalytic systems. Regulation by a mechanical action of the enzymatic properties of α-chymotrypsin covalently attached to a nylon fiber. Dokl. Acad. Nauk SSSR 218: 715-718.

4. Berezin, I.V., Varfolomeyev, S.D., Klibanov, A.M., Martinek, K. 1974. Light and ultrasonic regulation of α-chymotrypsin catalytic activity. Proflavin as a light- and sound- sensitive competitive inhibitor. FEBS Lett. 39: 329-331.

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6. Berezin, I.V., Klibanov, A.M., Goldmacher, V.S., Martinek, K. 1974. Mechanochemistry of catalytic systems. Regulation by a mechanical action of the enzymatic activity of trypsin entrapped in polyacrylamide gel. Dokl. Acad. Nauk SSSR 218: 367-370.

7. Klibanov, A.M., Martinek, K., Berezin, I.V. 1974. The effect of ultrasound on α-chymotrypsin. A novel approach to studying conformational transitions in active centers of enzymes. Biochemistry SSSR 39: 878-887.

8. Berezin, I.V., Klibanov, A.M., Klyosov, A.A., Martinek, K., Svedas, V.K. 1975. The effect of ultrasound as a new method of studying conformational transitions in enzyme active centers. pH- and temperature-induced conformational transitions in the active center of penicillin amidase. FEBS Lett. 49: 325-328.

9. Berezin, I.V., Klibanov, A.M., Martinek, K. 1975. Kinetic and thermodynamic aspects of catalysis by immobilized enzymes. Russ. Chem. Revs. 44: 17-47.

10. Martinek, K., Goldmacher, V.S., Klibanov, A.M., Berezin, I.V. 1975. Denaturing agents (urea, acrylamide) protect enzymes against irreversible thermoinactivation: a study with native and immobilized α-chymotrypsin and trypsin. FEBS Lett. 51: 152-155.

11. Martinek, K., Klibanov, A.M., Tchernysheva, A.V., Berezin, I.V. 1975. The stabilization of α-chymotrypsin by entrapment in polymethacrylate gels. Dokl. Acad. Nauk SSSR 223: 233-236.

12. Tchernysheva, A.V., Goldmacher, V.S., Klibanov, A.M., Martinek, K., Berezin, I.V. 1975. The catalytic activity and thermostability of α-chymotrypsin oligomers entrapped in cross-linked polymeric gels. Bull. Moscow Univ. 19: 428-431.

Momenta Pharm. v. BMS, IPR215-01537
3/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2821

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17. Klibanov, A.M., Kazanskaya, N.F., Larionova, N.I., Martinek, K., Berezin, I.V. 1976. A comparative study of the dynamic structures of the active centers of proteolytic enzymes by the ultrasonic method. The effect of ultrasound on α-trypsin, ß-trypsin, and trypsinogen. J. Bioorg. Chem. (Russ.) 2: 828-836.

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Momenta Pharm. v. BMS, IPR215-01537
4/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2822

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6/41 BMS EXHIBIT 2016

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8/41 BMS EXHIBIT 2016

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11/41 BMS EXHIBIT 2016

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12/41 BMS EXHIBIT 2016

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16/41 BMS EXHIBIT 2016

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19/41 BMS EXHIBIT 2016

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Page 2837

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Page 2838

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Momenta Pharm. v. BMS, IPR215-01537
21/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2839

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Momenta Pharm. v. BMS, IPR215-01537
22/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2840

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Momenta Pharm. v. BMS, IPR215-01537
23/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2841

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Patents:

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2. Klibanov, A.M., Kirchner, G. Enzymatic production of optical isomers of 2-halopropionic acids. U.S. Patent No. 4,601,987; July 22, 1986.

3. Klibanov, A.M. Removal of combined organic substances from aqueous solutions. U.S. Patent No. 4,623,465; November 18, 1986.

4. Klibanov, A.M. Enzymatic resolution of racemic mixtures of hydroxy compounds. U.S. Patent No. 4,659,671; 1987.

5. Klibanov, A.M., Dordick, J.S. Enzymatic temperature change indicator. U.S. Patent No. 4,826,762; May 2, 1989.

6. Dorval, B.L., Denham, L., Klibanov, A.M. Radial flow assay, delivering membrane, test kit, and methods. U.S. Patent No. 5,547,833; August 20, 1996.

7. Dorval, B.L., Denham, L., Klibanov, A.M. Detection reagent, particle, and immunoassay method. U.S. Patent No. 5,561,045; October 1, 1996.

8. Dorval, B.L., Chow, M., Klibanov, A.M. Stabilized vaccine compositions. U.S. Patent No. 5,618,539; April 8, 1997. European Patent No. 0 487 632; March 4, 1998.

9. Klibanov, A.M., Lewis, K., Ferrante, A.A., Coyle, C.L., Zylstra, G., Logan, M.S.P., Grossman, M.J. Solvent-resistant microorganisms. U.S. Patent No. 5,807,735; September 15, 1998.

10. Gresser, J.D., Trantolo, D.J., Langer, R., Klibanov, A.M., Wise, D.L. Material for buffered resorbable internal fixation devices and method for making the same. U.S. Patent No. 5,817,328; October 6, 1998.

11. Hekal, I.M., Langer, R.S., Klibanov, A.M., Mathiowitz, E. Monolithic polymer composition having a water absorption material. U.S. Patent No. 6,174,952; January 16, 2001.

Momenta Pharm. v. BMS, IPR215-01537
24/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2842

12. Gresser, J.D., Trantolo, D.J., Langer, R., Lewandrowski, K.-U., Klibanov, A.M., Wise, D.L. Resorbable interbody spinal fusion devices. U.S. Patent No. 6,241,771; June 5, 2001.

13. Trantolo, D.J., Langer, R., Klibanov, A.M., Wise, D.L. Buffered resorbable internal fixation devices and methods for making material therefor. U.S. Patent No. 6,419,945; July 16, 2002.

14. Gresser, J.D., Trantolo, D.J., Langer, R., Lewandrowski, K.-U., Klibanov, A.M., Wise, D.L. Method of making biodegradable interbody spinal fusion devices. U.S. Patent No. 6,548,002; April 15, 2003.

15. Klibanov, A.M. Nonaqueous solutions and suspensions of macromolecules for pulmonary delivery. U.S. Patent No. 6,660,715; December 9, 2003.

16. Tiller, J.C., Lewis, K., Liao, C.-J., Klibanov, A.M. Antimicrobial polymeric surfaces. U.S. Patent No. 7,151,139; December 19, 2006.

17. Hirsh, J.C., Fleming, A.B., Rariy, R.V., Klibanov, A.M. Abuse-deterrent pharmaceutical formulations. U.S. Patent No. 8,449,909; May 28, 2013.

18. Rariy, R.V., Fleming, A.B., Hirsh, J.C., Klibanov, A.M. Abuse-deterrent pharmaceutical compositions of opioids and other drugs. U.S. Patent No. 8,557,291; October 15, 2013.

19. Hirsh, J.C., Klibanov, A.M., Swager, T.M., Buchwald, S.L., Lo, W.Y., Fleming, Rariy, R.V. Abuse-deterrent pharmaceutical compositions of opioids and other drugs. U.S. Patent No. 9,044,398; June 2, 2015.

20. Schaer, T.P., Stewart, S., Klibanov, A.M. Antibacterial coatings that inhibit biofilm formation on implants. U.S. Patent No. 9,089,407; July 28, 2015.

21. Schaer, P.T., Hsu, B.B., Stewart, S., Klibanov, A.M. Hydrophobic polycationic coatings that inhibit biofilm formation on orthopedic implants and support healing during infection, pending.

22. Haldar, J., Chen, J., Klibanov, A.M. Non-leaching virucidal coatings, pending.

23. Haldar, J., An, D., Álvarez de Cienfuegos, L., Chen, J., Klibanov, A.M. Polymeric coatings that inactivate viruses and bacteria. U.S. Patent Appl. Publ. No. 2010/0136072; June 3, 2010.

24. Pedersen, M., Stanley, M., Gouin, S., Klibanov, A.M, Hsu, B.B. Antimicrobial quat coating on fabrics. PCT Intl. Appl. WO 2012/06561, 2012.

25. Haldar, J., Alvarez de Cienfuegos, L., Klibanov, A.M., Chen, J. Bi-functional polymer-attached inhibitors of influenza virus. PCT Intl. Appl. WO 2009/032605, 2012.

Invited Lectures:

1. Gordon Conference on Immobilized Enzymes, Complexes, and Cells (Plymouth, NH). August 1978.

2. 5th International Conference on Enzyme Engineering (Henniker, NH). August 1978.

Momenta Pharm. v. BMS, IPR215-01537
25/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2843

3. Miles Laboratories (Elkhart, IN). February 1980.
4. Gordon Conference on Immobilized Species (Henniker, NH). August 1980.
5. Novo Laboratories (Wilton, CT). October 1980.
6. Corning Glass Works Co. (Corning, NY). October 1980.
7. Stauffer Chemical Co. (Dobbs Ferry, NY). December 1980.
8. DuPont Experimental Station (Wilmington, DE). January 1981.
9. Dow Chemical Co. (Wayland, MA). April 1981.
10. Cetus Corp. (Berkeley, CA). April 1981.
11. Exxon Research & Engineering Co. (Linden, NJ). April 1981.
12. Meeting of the American Solar Energy Society (Philadelphia, PA). May 1981.
13. 6th International Conference on Enzyme Engineering (Kashikojima, Japan). September 1981.
14. Genex Corp. (Rockville, MD). October 1981.
15. ILP Symposium for Senior Executives (London, England). November 1981.
16. California Institute of Technology (Pasadena, CA). December 1981.
17. Symposium of the American Society for Microbiology (Atlanta, GA). March 1982.
18. Kodak Research Center (Rochester, NY). March 1982.
19. Repligen Corp. (Cambridge, MA). April 1982.
20. Symposium on the Biological Basis of New Developments in Biotechnology (Minneapolis, MN). May 1982.
21. Meeting of the Biochemical Society (Oxford, England). July 1982.
22. Meeting of the American Chemical Society (Kansas City, MO). September 1982.
23. Rotenburger Symposium on Enzyme Technology (Kassel, Germany). September 1982.
24. Genetics Institute (Boston, MA). September 1982.
25. Meeting of the American Institute of Chemical Engineers (Los Angeles, CA). November 1982.
26. G.D. Searle Co. (Chicago, IL). October 1982.
27. Halcon International Research Center (Montvale, NJ). October 1982.

Momenta Pharm. v. BMS, IPR215-01537
26/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2844

28. International Workshop on Immobilized Enzymes and Cells (Bangkok, Thailand). December 1982.

29. Amoco Research Center (Naperville, IL). January 1983.

30. University College London (London, England). May 1983.

31. International Conference on Biotechnology Biotech '83 (London, England). May 1983.

32. Tufts University Workshop on Immobilized Enzymes and Proteins (Medford, MA). June 1983.

33. Gordon Conference on Organic Reactions and Processes (New Hampton, NH). July 1983.

34. Symposium "Biocatalysis: New Discoveries and Uses", Meeting of the American Society for Industrial Microbiology (Sarasota, FL). August 1983.

35. Monsanto Co. (St. Louis, MO). September 1983.

36. Pasteur Biosciences '83 Conference (Paris, France). September 1983.

37. 7th International Conference on Enzyme Engineering (White Haven, PA). September 1983.

38. University of Iowa (Iowa City, IA). October 1983.

39. CPC Moffett Technical Center (Summitt-Argo, IL). December 1983.

40. General Foods Co. (Dobbs Ferry, NY). December 1983.

41. U.S. National Bureau of Standards (Gaithersburg, MD). April 1984.

42. Millipore Corp. (Bedford, MA). April 1984.

43. Technical University of Munich (Munich, F.R.G.). May 1984.

44. Danish Academy of Technical Sciences (Copenhagen, Denmark). May 1984.

45. Novo International Biotechnology Symposium (Copenhagen, Denmark). May 1984.

46. CRA Scientific Conference (St. Charles, IL). June 1984.

47. Gordon Conference on Immobilized Species in Biotechnology (Plymouth, NH). August 1984.

48. Meeting of the American Chemical Society (Philadelphia, PA). August 1984.

49. International Biotechnology Conference Biotech '84 (Washington, DC). September 1984.

50. Staley Manufacturing Co. (Decatur, IL). October 1984.

51. Case Western Reserve University (Cleveland, OH). October 1984.

Momenta Pharm. v. BMS, IPR215-01537
27/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2845

52. IMC Corp. (Terre Haute, IN). October 1984.
53. Syracuse University (Syracuse, NY). November 1984.
54. National University of Mexico (Mexico City, Mexico). November 1984.
55. National Starch & Chemical Corp. (Bridgewater, NJ). November 1984.
56. Mead Corp. (Chillicothe, OH). November 1984.
57. Hebrew University of Jerusalem (Jerusalem, Israel). November 1984.
58. U.S. Army Chemical R&D Center (Aberdeen Proving Ground, MD). December 1984.
59. Allied Corp. (Morristown, NJ). December 1984.
60. Bristol Laboratories (Syracuse, NY). January 1985.
61. University of Rochester (Rochester, NY). January 1985.
62. G.D. Searle Co. (Chicago, IL). February 1985.
63. Wellcome Research Laboratories (Research Triangle Park, NC). February 1985.
64. UCLA Symposium on Protein Structure, Folding and Design (Keystone, CO). April 1985.
65. International Conference on Application of Biocatalysts in Organic Synthesis (Noordwijkerhout, Holland). April 1985.
66. Guido Donegani Research Institute (Novara, Italy). April 1985.
67. Hoffmann-LaRoche Co. (Basel, Switzerland). April 1985.
68. Pittsburgh-Cleveland Catalysis Society Meeting (Pittsburgh, PA). May 1985.
69. Ethyl Corp. (Baton Rouge, LA). May 1985.
70. Stony Brook Symposium on Protein Engineering (Stony Brook, NY). May 1985.
71. Amgen Corp. (Thousand Oaks, CA). June 1985.
72. Gordon Conference on Applied Microbiology (New London, NH). August 1985.
73. International Congress of Biochemistry (Amsterdam, Holland). August 1985.
74. IUPAC Congress (Manchester, England). September 1985.
75. International Enzyme Engineering Conference (Helsingor, Denmark). September 1985.
76. Biotechnica '85 (Hannover, West Germany). October 1985.

Momenta Pharm. v. BMS, IPR215-01537
28/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2846

77. Biotec 85 (Dusseldorf, West Germany). October 1985.

78. Smith Kline & French Co. (Philadelphia, PA). November 1985.

79. Exxon Corporate Research (Anandale, NJ). December 1985.

80. Pennsylvania State University (University Park, PA). December 1985.

81. Georgetown University (Washington, DC). February 1986.

82. Columbia University (New York, NY). February 1986.

83. Upjohn Co. (Kalamazoo, MI). February 1986.

84. UNIDO Workshop on Biotechnology (Trieste, Italy). March 1986.

85. Enericherche (Monterotondo, Italy). March 1986.

86. EEC Biomolecular Engineering Meeting (Compiegne, France). April 1986.

87. ACS Conference on Chemical Aspects of Biotechnology (Gaithersburg, MD). May 1986.

88. Biotech '86 Europe International Conference (London, England). May 1986.

89. IUPAC International Symposium on Organic Chemistry in Technological Perspective (Jerusalem, Israel). June 1986.

90. University of Tel-Aviv (Ramat-Aviv, Israel). June 1986.

91. Annual Meeting of the Canadian Biochemical Society (Guelph, Canada). June 1986.

92. International Symposium on Biologically Engineered Polymers (Cambridge, England). July 1986.

93. TNO Institute of Applied Chemistry (Zeist, The Netherlands). August 1986.

94. IUPAC International Symposium on the Chemistry of Natural Products (The Hague, The Netherlands). August 1986.

95. American Chemical Society National Meeting (Anaheim, CA). September 1986.

96. Catalytica Annual Science and Technology Symposium (San Jose, CA). September 1986.

97. International Conference on Enzyme Engineering (Cambridge, England). September 1986.

98. Ohio State University (Columbus, OH). October 1986.

99. Michigan State University (Lansing, MI). October 1986.

100. Biotechnology Process Engineering Symposium (Cambridge, MA). October 1986.

Momenta Pharm. v. BMS, IPR215-01537
29/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2847

101. University of Delaware (Newark, DE). November 1986.

102. American Red Cross Blood Research Institute (Bethesda, MD). November 1986.

103. International Symposium on Biocatalysis in Non-Aqueous Solvents (Wageningen, The Netherlands). December 1986.

104. University of Trondheim (Trondheim, Norway). December 1986.

105. Colgate-Palmolive Research Center (Piscataway, NJ). January 1987.

106. International Paper Co. (Tuxedo Park, NY). January 1987.

107. University of Massachusetts (Amherst, MA). February 1987.

108. American Society for Microbiology Biocatalysis Symposium (Atlanta, GA). March 1987.

109. Hope College (Holland, MI). March 1987.

110. Pfizer Corporate Research Center (Groton, CT). April 1987.

111. OBBS Symposium on Protein Engineering (Ottawa, Canada). April 1987.

112. University of Texas (Austin, TX). April 1987.

113. Florida Catalysis Conference (Palm Coast, FL). May 1987.

114. PPG Industries (Barberton, OH). May 1987.

115. Abbott Laboratories (North Chicago, IL). May 1987.

116. American Oil Chemists Society Symposium on Lipases (New Orleans, LA). May 1987.

117. Argonne National Laboratory (Argonne, IL). June 1987.

118. Canadian Chemical Conference (Quebec City, Canada). June 1987.

119. Squibb Co. (New Brunswick, NJ). June 1987.

120. FEBS Annual Meeting (Ljubljana, Yugoslavia). July 1987.

121. L'Oreal Research Institute (Paris, France). July 1987.

122. International Symposium on Biointeractions (Cambridge, England). July 1987.

123. Eli Lilly Co. (Indianapolis, IN). August 1987.

124. Upjohn Co. (Kalamazoo, MI). September 1987.

125. International Enzyme Engineering Conference (Santa Barbara, CA). October 1987.

Momenta Pharm. v. BMS, IPR215-01537
30/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2848

126. Shell Development Co. (Houston, TX). October 1987.

127. DuPont Experimental Station (Wilmington, DE). October 1987.

128. University of Helsinki (Helsinki, Finland). November 1987.

129. International Conference on Bioreactors and Biotransformations (Gleneagles, Scotland). November 1987.

130. Sandoz Co. (Basel, Switzerland). November 1987.

131. University of Waterloo (Waterloo, Canada). December 1987.

132. Norwegian Biochemical Society Annual Meeting (Bejito, Norway). January 1988.

133. Rutgers University (New Brunswick, NJ). February 1988.

134. University of Ottawa (Ottawa, Canada). February 1988.

135. Boston Biomedical Research Institute (Boston, MA). March 1988.

136. Princeton University (Princeton, NJ). March 1988.

137. Royal Society of Chemistry Meeting (Kent, England). April 1988.

138. Stanford University (Stanford, CA). May 1988.

139. University of Graz (Graz, Austria). May 1988.

140. Belgian Organic Chemistry Symposium (Ghent, Belgium). May 1988.

141. Dow Chemical Co. (Midland, MI). May 1988.

142. Michigan State University (Lansing, MI). May 1988.

143. American Chemical Society Meeting (Toronto, Canada). June 1988.

144. Proctor & Gamble Co. (Cincinnati, OH). June 1988.

145. International Congress of Biochemistry (Prague, Czechoslovakia). July 1988.

146. International Biotechnology Symposium (Paris, France). July 1988.

147. Gordon Conference on Catalysis (Newport, RI). August 1988.

148. Conference on Biocatalytic Synthesis of Organic Compounds (Saratoga, NY). August 1988.

149. International Symposium on Thermodynamics Applied to Biological Systems (Santa Margherita, Italy). September 1988.

150. Cornell University (Ithaca, NY). September 1988.

Momenta Pharm. v. BMS, IPR215-01537
31/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2849

151. Eastman Kodak Co. (Kingsman, TN). September 1988.

152. Symposium on Biotechnology Challenges in the Food Industry (Williamsburg, VA). October 1988.

153. University of California at Irvine (Irvine, CA). October 1988.

154. UCSF Symposium on Protein and Drug Design (San Francisco, CA). October 1988.

155. Boston University (Boston, MA). October 1988.

156. Wayne State University (Detroit, MI). November 1988.

157. Kyoto Symposium on Frontiers in Biocatalysis (Kyoto, Japan). November 1988.

158. Rohm & Haas Co. (Spring House, PA). January 1989.

159. Block Drug Co. (Jersey City, NJ). January 1989.

160. State University of New York (Albany, NY). March 1989.

161. Scripps Clinic and Research Foundation (La Jolla, CA). April 1989.

162. Texas A&M University (College Station, TX). May 1989.

163. University of Texas (Austin, TX). May 1989.

164. International Congress of Food Engineering (Cologne, F.R.G.). May 1989.

165. University of Groningen (Groningen, The Netherlands). June 1989.

166. Gordon Conference on Enzymes, Coenzymes, and Metabolic Pathways (Meridan, NH). July 1989.

167. American Peptide Symposium (La Jolla, CA). July 1989.

168. International Symposium on Prospects in Protein Engineering (Haren, The Netherlands). August 1989.

169. Connaught Laboratories (Toronto, Canada). September 1989.

170. University of Georgia (Athens, GA). October 1989.

171. Spanish National Congress of Biochemistry (Alicante, Spain). October 1989.

172. Symposium on Receptor- and Enzyme-Based Drug Design (Chapel Hill, NC). October 1989.

173. Wesleyan University (Middletown, CT). November 1989.

174. University of Wisconsin (Madison, WI). February 1990.

175. Merck Sharp & Dohme Research Laboratories (Rahway, NJ). February 1990.

Momenta Pharm. v. BMS, IPR215-01537
32/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2850

176. International Symposium on Biochemical Engineering (Stuttgart, F.R.G.). March 1990.

177. University of Geneva (Geneva, Switzerland). March 1990.

178. University of Lausanne (Lausanne, Switzerland). March 1990.

179. University of Bern (Bern, Switzerland). March 1990.

180. University of Fribourg (Fribourg, Switzerland). March 1990.

181. International Conference on Protein Stability (Cambridge, England). March 1990.

182. AFRC Institute of Food Research (Reading, England). March 1990.

183. Lehigh University (Bethlehem, PA). April 1990.

184. Biotechnology Research Institute (Montreal, Canada). April 1990.

185. Ohio State University (Columbus, OH). April 1990.

186. Pharmaceutical Manufacturers Association Annual Meeting (St. Louis, MO). April 1990.

187. International Conference on Industrial Use of Enzymes (Chicago, IL). May 1990.

188. Korea Advanced Institute of Science and Technology (Seoul, Korea). June 1990.

189. Academia Sinica and National Taiwan University (Taipei, Taiwan). June 1990.

190. Gordon Conference on Biocatalysis (Plymouth, NH). June 1990.

191. IUPAC Conference on Organic Synthesis (Helsinki, Finland). July 1990.

192. International Conference on Solution Chemistry (Ottawa, Canada). August 1990.

193. Gordon Conference on Biomolecular Recognition (Plymouth, NH). August 1990.

194. University of Naples (Naples, Italy). September 1990.

195. International Conference on Water and Life (Crans-sur-Sierre, Switzerland). September 1990.

196. International Symposium on Thermodynamic Basis of Protein Structure and Function (Kansas City, MO). October 1990.

197. Johns Hopkins University (Baltimore, MD). October 1990.

198. Institute of Medicine Workshop on Microheterogeneity of Biological Macromolecules (Arlington, VA). November 1990.

199. U.S.-Japan Biotechnology Conference (Honolulu, HI). January 1991.

Momenta Pharm. v. BMS, IPR215-01537
33/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2851

200. Ciba-Geigy Corp. (Summit, NJ). February 1991.

201. City University of New York (New York, NY). March 1991.

202. Rensselaer Polytechnic Institute (Troy, NY). March 1991.

203. Bristol-Myers Squibb Co. (Wallingford, CT). April 1991.

204. British Biochemical Society Meeting (Reading, England). April 1991.

205. University of California at Berkeley (Berkeley, CA). April 1991.

206. California Institute of Technology (Pasadena, CA). April 1991.

207. Merck, Sharp & Dohme Research Laboratories (West Point, PA). May 1991.

208. Bristol-Myers Squibb Research Institute (Princeton, NJ). May 1991.

209. International Conference on Biocatalysis (Orlando, FL). June 1991.

210. U.S. Army Research, Development and Engineering Center (Natick, MA). July 1991.

211. Gordon Conference on Natural Products (Plymouth, NH). July 1991.

212. Lederle-Praxis Biologicals Co. (Sanford, NC). July 1991.

213. International Biochemistry Congress (Jerusalem, Israel). August 1991.

214. American Chemical Society Meeting (New York, NY). August 1991.

215. International Enzyme Engineering Conference (Kona, HI). September 1991.

216. University of Lowell (Lowell, MA). October 1991.

217. Rutgers University (Piscataway, NJ). November 1991.

218. International Symposium on Enzymes in Organic Synthesis (New Delhi, India). January 1992.

219. International BioSymposium Nagoya '92 (Nagoya, Japan). January 1992.

220. International Symposium on Biomolecules in Organic Solvents (Taxco, Mexico). February 1992.

221. Rockefeller University (New York, NY). March 1992.

222. Symposium on Molecular Chirality of the Pharmaceutical Society of Japan (Tokyo, Japan). April 1992.

223. Conference on Enzymes in Organic Chemistry of the Dutch Chemical Society (Wageningen, The Netherlands). April 1992.

Momenta Pharm. v. BMS, IPR215-01537
34/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2852

224. International Symposium on Bioprocessing of Coal (Clearwater Beach, FL). May 1992.

225. Exxon Workshop on New Leads in Coal Depolymerization (Montgomery, TX). May 1992.

226. Italian Symposium on Biochemical Biotechnology (Capri, Italy). June 1992.

227. Gordon Conference on Biocatalysis (Meriden, NH). July 1992.

228. FEBS Meeting (Dublin, Ireland). August 1992.

229. International Biotechnology Symposium (Washington, DC). August 1992.

230. International Symposium Bio Japan '92 (Yokohama, Japan). August 1992.

231. Texas College of Osteopathic Medicine (Fort Worth, TX). October 1992.

232. Rutgers University (Newark, NJ). October 1992.

233. Schering-Plough Co. (Bloomfield, NJ). November 1992.

234. International Symposium ISOPOW-V: *Properties of Water in Foods* (Peniscola, Spain). November 1992.

235. Carlsberg Laboratory (Copenhagen, Denmark). November 1992.

236. International Food Technology Conference (The Hague, The Netherlands). November 1992.

237. Singapore Institute of Standards and Industrial Research (Singapore). November 1992.

238. Drew University (Madison, NJ). December 1992.

239. Northeastern University (Boston, MA). January 1993.

240. Ciba Corning Diagnostics Corp. (Irvine, CA). January 1993.

241. University of Kansas (Lawrence, KS). January 1993.

242. 6th International Symposium on Recent Advances in Drug Delivery Systems (Salt Lake City, UT). February 1993.

243. Pennsylvania State University (University Park, PA). March 1993.

244. Northwestern University (Evanston, IL). March 1993.

245. International Symposium on Technologies for the Production of Enantiomerically Pure Chemicals (Amelia Island, FL). March 1993.

246. Gordon Conference on Bioanalytical Sensors (Ventura, CA). March 1993.

247. Pharmaceutical Manufacturers Association Analytical R&D/Pharmaceutical Development Subsections Joint Annual Meeting (Lake Buena Vista, FL). April 1993.

Momenta Pharm. v. BMS, IPR215-01537
35/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2853

248. University of Massachusetts Symposium on Breakthrough Technologies: *Paradigms for a New Century* (Amherst, MA). May 1993.

249. Parke-Davis Corp. (Ann Arbor, MI). May 1993.

250. NutraSweet Co. (Mt. Prospect, IL). June 1993.

251. IGT/GRI Workshop on Novel Concepts for the Production of Regio- and Stereospecific Compounds (Chicago, IL). June 1993.

252. Northeast Regional Meeting of the American Chemical Society (Boston, MA). June 1993.

253. Institute of Food Technologists' Biotechnology Meeting (Chicago, IL). July 1993.

254. Annual Meeting of Department of Energy's Biological and Chemical Technology Research Program Principal Investigators (Tiburon, CA). July 1993.

255. Allied-Signal Corp. (Des Plains, IL). July 1993.

256. American Chemical Society Meeting (Chicago, IL). August 1993.

257. University of Illinois at Urbana-Champaign (Urbana, IL). September 1993.

258. International Enzyme Engineering Conference (Deauville, France). September 1993.

259. Queens College (Flushing, NY). November 1993.

260. Exxon Chemical Research Meeting (Galveston, TX). November 1993.

261. Hoffmann-LaRoche Co. (Nutley, NJ). December 1993.

262. U.S.-Japan Symposium on Drug Delivery (Maui, HI). December 1993.

263. North Dakota State University (Fargo, ND). February 1994.

264. Annual Meeting of the American Institute for Medical and Biological Engineering (Washington, DC). March 1994.

265. Rhone-Poulenc Rorer Symposium on Visions in Chemistry (Collegeville, PA). May 1994.

266. International Flavors & Fragrances R&D (Union Beach, NJ). May 1994.

267. Istituto degli Ormoni (Milan, Italy). June 1994.

268. Gordon Conference on Enzymes, Coenzymes, and Metabolic Pathways (Meriden, NH). July 1994.

269. Annual Meeting of Department of Energy's Biological and Chemical Technology Research Program Principal Investigators (Denver, CO). July 1994.

270. William S. Johnson Symposium in Organic Chemistry (Stanford, CA). October 1994.

Momenta Pharm. v. BMS, IPR215-01537
36/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2854

271. R.W. Johnson Pharmaceutical Research Institute (Spring House, PA). October 1994.

272. International Congress of Biochemical Engineering (Mexico City, Mexico). October 1994.

273. National University of Mexico (Mexico City, Mexico). October 1994.

274. DuPont Merck Pharmaceutical Co. (Deepwater, NJ). December 1994.

275. Symposium on Therapeutic Protein Production and Processing (Cambridge, MA). January 1995.

276 International Symposium on Biocatalysts for Flavor Production, BioFlavour '95 (Dijon, France). February 1995.

277. Institut Pasteur (Paris, France). February 1995.

278. Florida State University (Tallahassee, FL). March 1995.

279. Abbott Laboratories (North Chicago, IL). March 1995.

280. University of Maryland, Baltimore County (Baltimore, MD). March 1995.

281 University of California at San Diego (La Jolla, CA). April 1995.

282. Great Lakes Regional Meeting of the American Chemical Society (LaCrosse, WI). June 1995.

283. Gordon Conference on Bioorganic Chemistry (Andover, NH). June 1995.

284. Annual Meeting of Department of Energy's Biochemical and Chemical Technology Research Principal Investigators (Albuquerque, NM). July 1995.

285. International Symposium "Biocatalysis-95" (Suzdal, Russia). August 1995.

286. International Enzyme Engineering Conference (San Diego, CA). October 1995.

287. Symposium on Biomacromolecules: *From 3-D Structure to Applications* (Pasco, WA). October 1995.

288. Enzymol International Inc. (Columbus, OH). December 1995.

289. International Symposium Pacifichem '95 (Honolulu, HI). December 1995.

290. 3rd U.S.-Japan Symposium on Drug Delivery (Maui, HI). December 1995.

291. New England Section of the Society for Industrial Microbiology (Cambridge, MA). February 1996.

292. Brown University (Providence, RI). February 1996.

293. University of Pittsburgh (Pittsburgh, PA). February 1996.

294. Food Research Institute (Reading, England). March 1996.

Momenta Pharm. v. BMS, IPR215-01537
37/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2855

295. International Symposium Chiral USA '96 (Boston, MA). May 1996.

296. BP Catalyst Colloquium (Uxbridge, England). June 1996.

297. International Symposium on Homogeneous Catalysis (Princeton, NJ). August 1996.

298. Juan March Foundation's International Workshop on Novel Biocatalysts (Madrid, Spain). March 1997.

299. University of Kentucky (Lexington, KY). April 1997.

300. Gordon Conference on Organic Reactions and Processes (Henniker, NH). August 1997.

301. Novo Nordisk Symposium on Protein Stability (Klampenborg, Denmark). August 1997.

302. International Enzyme Engineering Conference (Beijing, China). October 1997.

303. Biogen Inc. (Cambridge, MA). December 1997.

304. INBIO Europe'98 Conference on Advances in Industrial Biocatalysis (Amsterdam, The Netherlands). February 1998.

305. International Symposium on Stability and Stabilization of Biocatalysts (Córdoba, Spain). April 1998.

306. International Symposium on Protein Structure, Stability and Folding: *Fundamental and Medical Aspects* (Moscow, Russia). June 1998.

307. International Conference on Protein Stabilization (Leeds, England). June 1998.

308. University of Virginia (Charlottesville, VA). September 1998.

309. Danish Biotechnology Conference (Vejle, Denmark). May 1999.

310. International Conference on Biocatalysis and Biotransformation Biotrans '99 (Giardini Naxos, Italy). September 1999.

311. Annual Meeting of the American Association of Pharmaceutical Scientists (New Orleans, LA). November 1999.

312. Brandeis University (Waltham, MA). November 1999.

313. Rutgers University (Piscataway, NJ). March 2000.

314. Chiral USA 2000 Conference (Boston, MA). May 2000.

315. Land O'Lakes Conference on Solid-State Reactivity in Pharmaceutical Systems (Merrimac, WI). June 2000.

316. Agouron Pharmaceuticals Inc. (San Diego, CA). July 2000.

Momenta Pharm. v. BMS, IPR215-01537
38/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2856

317. U.S. Patent and Trademark Office Technology Fair (Arlington, VA). July 2000.

318. 23rd Gulf Coast Chemistry Conference (Pensacola, FL). September 2000.

319. Avon Products (Suffern, NY). November 2000.

320. Procter & Gamble Co. (Cincinnati, OH). November 2000.

321. Flamel Technologies (Venissieux, France). December 2000.

322. BioVision 2001 International Conference (Lyon, France). February 2001.

323. 9th Baxter Science and Technology Symposium (Hyland, IL). May 2001.

324. International Symposium on Applications of Enzymes in Chemical and Biological Defense (Orlando, FL). May 2001.

325. International Conference on Applied Biocatalysis (Trondheim, Norway). June 2001.

326. PPG Industries (Allison Park, PA). August 2001.

327 University of Kansas (Lawrence, KS). August 2001.

328. Alkermes Inc. (Cambridge, MA). October 2001.

329. 6th U.S.-Japan Symposium on Drug Delivery Systems (Maui, HI). December 2001.

330. Acambis Inc. (Cambridge, MA). January 2002.

331. Baxter BioScience Co. (Duarte, CA). January 2002.

332. International Symposium on Challenges in Drug Discovery and Development in the 21st Century (Kolkata, India). March 2002.

333. National Institute of Standards and Technology (Gaithersburg, MD). April 2002.

334. Albany Molecular Research, Inc. (Mt. Prospect, IL). April 2002.

335. Institute for International Research Conference on Protein and Peptide Formulation Strategies for Drug Development and Delivery (San Francisco, CA). August 2002.

336. R&D Management Council Meeting of the Technical Association of the Pulp and Paper Industry (Cambridge, MA). November 2002.

337. DuPont Central R&D Center (Wilmington, DE). December 2002.

338. *ACTIVE*Pack 2003 Conference (Ponte Verde Beach, FL). February 2003.

339. International Symposium on Industrial Applications of Biocatalysis: *Chemical Development Issues* (Boston, MA). September 2003.

Momenta Pharm. v. BMS, IPR215-01537
39/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2857

340. U.S. Army Workshop on Self-Decontaminating Materials and Multifunctional Coatings (Arlington, VA). October 2003.

341. British Royal Society Meeting on Biocatalysis in Alternative Media (London, England). December 2003.

342. National Autonomous University of Mexico (Mexico City, Mexico). March 2004.

343. Healthcare Research Initiative Workshop on the Effect of Radiofrequency Radiation on Pharmaceutical Product Quality (Cambridge, MA). June 2004.

344. Rensselaer Biotechnology Symposium on Biological Discoveries That Will Change the World (Troy, NY). September 2004.

345. Barnett International Conference: *The Consequences of Aggregation in Drug Development, Immunology, Quality Control, and Disease Genesis* (Philadelphia, PA). September 2004.

346. 4th Annual IBC Conference on Formulation Strategies for Protein Therapeutics (Boston, MA). October 2004.

347. U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). August 2005.

348. World Congress on Synthetic Receptors (Salzburg, Austria). September 2005.

349. International Conference "Celebrating 30 Years of Robert Langer's Science" (Cambridge, MA). July 2006.

350. University of Montreal (Montréal, Québec, Canada). October 2006.

351. The Boeing Company Research Center (Everett, WA). December 2006.

352. Instrumentation Laboratory (Lexington, MA). December 2006.

353. Acambis Inc. (Cambridge, MA). January 2007.

354. 6th International Symposium on Industrial Microbiology and Biotechnology (Cambridge, MA). August 2007.

355. Scientific Conference on Chemical & Biological Defense Research (Timonium, MD). November 2007.

356. MIT Research and Development Conference (Cambridge, MA). November 2007.

357. Nanotech '2008 International Conference (Boston, MA). June 2008.

358. Materials Research Society Symposium (Boston, MA). December 2008.

359. Johnson & Johnson Research Briefing at MIT (Cambridge, MA). January 2009.

360. 3rd Annual Lyophilization Conference: *Ensuring Optimum Formulations for Pharmaceuticals*

Momenta Pharm. v. BMS, IPR215-01537
40/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2858

and Biologics (Boston, MA). February 2010.

361. DSM Corporate Research Center (Geleen, The Netherlands). June 2010.

362. Merck Serono Pharmaceutical Co. (Darmstadt, Germany). June 2010.

363. NanoFormulations '2010 International Conference (Stockholm, Sweden). June 2010.

364. Covidien Surgical Devices and Vascular Therapies Divisions (Cambridge, MA). October 2010.

365. World Lyophilization Summit (Cambridge, MA). May 2011.

366. Alvogen (Cambridge, MA). March 2012.

367. Colgate-Palmolive Co. (Cambridge, MA). April 2012.

368. FDA Parenteral Drug Association Glass Quality Conference (Washington, DC). June 2012.

369. University of Puerto Rico (Humacao, Puerto Rico). January 2013.

370. International Conference on Creating and Leveraging Intellectual Property in Developing Countries (Durban, South Africa – *via teleconference*). November 2013.

371. Northwestern University (Evanston, IL). April 2015.

Momenta Pharm. v. BMS, IPR215-01537
41/41 BMS EXHIBIT 2016

Samsung Bioepis Exhibit No. 1002
Page 2859

EP 16 19 5355.9
Alexion Pharmaceuticals, Inc.
Our Ref.: P2913 EP/3

DECLARATION REGARDING DETERMINING THE STARTING RESIDUE OF THE ACTIVE LIGHT CHAIN SEQUENCE OF ECULIZUMAB

I, Bruce Andrien, Director, Early Assay Development, of Alexion Pharmaceuticals, Inc., of 100 College Street, New Haven, CT 06510, United States of America, make the following factual declaration concerning the signal peptide and n-terminal amino acid residue of the light chain of eculizumab:

1) I am Director, Early Assay Development, of Alexion Pharmaceuticals, Inc. ("Alexion"), as shown in my curriculum vitae, a copy of which is **attached** herewith and marked Exhibit A.

2) As also shown in my curriculum vitae, I have a Ph.D. from the University of California, San Diego in Engineering Physics. My Ph.D. training focused on designing materials and optimizing scientific instrumentation including mass spectrometers for use in determining accurate molecular weights of materials. Since my Ph.D. was completed in 1991, my career has been devoted to application of increasingly more precise methods for determining the molecular weight, protein sequence and chemical signature information of large molecular weight proteins in the biotechnology industry. In my 10 year role at Analytica of Branford, I developed electrospray sources and mass spectrometers for analyzing peptides, proteins and monoclonal antibodies under the supervision of John B. Fenn, the recipient of the 2002 Nobel Prize in Chemistry for the development of electrospray for the analysis of biological macromolecules.

3) Through my work at Alexion over the past 16 years, I have become very familiar with the protein sequences and structures of antibodies.

4) I understand that EP 16 17 2335.8 is a European patent application whose parent international application was filed on March 16, 2007.

5) I understand the pending claim 1 of EP 16 17 2335.8 reads as follows:

Samsung Bioepis Exhibit No. 1002
Page 2860

Claim 1. A pharmaceutical composition comprising an antibody that binds C5 or an active antibody fragment thereof for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH), wherein the antibody binds C5 or an active fragment thereof comprises a heavy chain and a light chain, wherein the heavy chain consists of SEQ ID NO:2 and the light chain consists of SEQ ID NO:4.

6) I understand that SEQ ID NO: 4 in EP 16 17 2335.8 reads as follows:

MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDRVTITCGASENIYGALNWYQQKPGK
APKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQNVLNTPLTFGQGTKVEIKRT
VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLS
STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

7) I was asked to determine whether one skilled in the art of antibody structure would have immediately recognized that the light chain identified by SEQ ID NO:4 includes the leader peptide of the light chain, and whether one of skill in the art could have easily determined where the leader peptide ends and the active fragment begins in SEQ ID NO:4.

8) Upon being shown the light chain of SEQ ID NO:4, I immediately recognized three features that indicated the leader peptide was included. The first two features were that the first residue was a methionine (M) and that this first methionine residue was followed by 21 amino acid residues, most of which are hydrophobic. Methionine is encoded in DNA as the ATG start codon and first amino acid in eukaryotic proteins. Methionine, is therefore, also the starting residue of signal peptides expressing the light chain of antibodies, but is cleaved off with the entire signal peptide in the cell from light chains as the multi-chain protein folds and is assembled into a fully formed antibody. The presence of Met as the first amino acid present in SEQ ID NO:4, combined with the length and amino acid content of this peptide, is characteristic of signal (leader) peptides of immature proteins and indicated the signal peptide was still present.

The third feature was that the entire peptide sequence of the light chain was 236 residues, which is longer than the average light chain of a mature antibody which ranges from about 211-217 amino acids. See Janeway *et al.*, Immunobiology, 5th

Samsung Bioepis Exhibit No. 1002
Page 2861

Edition New York, Garland Science 2001. Again, this factor reinforced to me that the signal peptide was present in SEQ ID NO:4. The signal peptide is naturally present on the N-terminus of immature antibody proteins and this fact is well-known and established amongst scientists who work with antibodies.

9) People familiar with the field of antibody sequences know that antibody sequences are usually numbered according to the "Kabat" numbering scheme. This is because the Kabat's database, which began in 1969, but was first published in 1976, was one of the oldest biological sequence databases maintained and free to the public. Kabat's database was also unique because it contained protein sequence alignments. Kabat's database was published in printed form in five growing editions between 1976 and 1991. Kabat's database continued online for a while after the last printed version in 1991 at http/kabatdatabase.com, but because the information contained in the database has grown with our ability to sequence more easily, it has increased to be more than five times larger than the last printed version containing 2387 pages. The database is still used today by practitioners in the art, but contains a convenient user interface. See Kabatman at www.bioinf.org.uk. To answer this question, I consulted an in-house copy of the iconic 2387 page, last printed version of "Sequences of Proteins of Immunological Interest" Volume 1, 5th Edition, published in 1991 by the U.S. Department of Health and Human Services and compiled by Elvin A. Kabat *et al.*, which is also known as NIH Publication No. 91-3242. See attached Exhibit B.

10) In the Table of Contents on page (iii) for "Sequences of Proteins of Immunological Interest" Volume 1, 5th Edition, the page indicated to show "Signal Sequences of Light Chains" for "Human Kappa Light Chains" is page 1. See Exhibit C. The active fragment sequences of "Human Kappa Chains Subgroup I" are indicated to be shown on page 103. See Exhibit C.

11) As directed by the Table of Contents in Exhibit C, on page 1, top table, **for 22-residue signal peptides** (1, 2, 5-11, 15, 16, & 18 sequences) of human kappa light chains the first three residues (sequences in columns) are predominantly Met-Asp-Met or MDM in single letter code, just as in the beginning of SEQ ID NO:4. See Exhibit D. The numbering system on the left column of this page in the top table uses a negative sign

Samsung Bioepis Exhibit No. 1002
Page 2862

in front of the signal peptide residue numbers; therefore Met-Asp-Met corresponds to -22 to -20. See Exhibit D. The final residues in **signal peptides having a 22 amino acid length** as shown in the top table on page 1 are predominantly Arg-Cys (RC), numbered as -2 and -1, just as in SEQ ID NO:4. Although there are three occurrences of Lys-Cys (KC) residues as -2 and -1 in **the 22 residue signal peptides** in the top table on page 1 of Exhibit D, the pattern is always a positively-charged residue followed by Cys (C) at the -1 position, just as in position 22 of SEQ ID NO:4.

12) As described above, the active peptide sequences for human kappa light chains begin on page 103 of "Sequences of Proteins of Immunological Interest" Volume 1, 5th Edition, published in 1991. Pages 103-108 are included as Exhibit E. The numbering system of Framework 1 is again on the left side of the page and is numbered downward from 0 to 23, for example, for Framework 1 (FR1). It is well-known to scientists working in the field of antibodies that Framework 1 is the N-terminus of the active antibody light chain. Here, even in 1991, it was evident that Asp-Ile-Gln-Met (DIQM) were the first 4 residues of an active human kappa light chain subgroup 1. See Exhibit E. Human kappa light chain subgroup I was examined first because it was known that subgroup 1 outnumbers each of the other subgroups in usage. See Solomon et al., American Journal of Pathology 137(4) 855-862 (1990).

I found that in SEQ ID NO:4, immediately following the 22-residue signal peptide, is the clear and unambiguous start site to the active antibody fragment, i.e., DIQM at positions 23-26. The vast majority of human kappa light chains catalogued in 1991 started with this sequence. The statistics on page 108 of Exhibit E show the number of full and/or partial sequences of kappa light chains known in 1991 that began with D (118/125), followed by I (120/123), followed by Q (112/123) and followed by M (109/124). Therefore, for all of the reasons above, I conclude that one skilled in the art in 2007 would have immediately recognized that the signal peptide is included in SEQ ID NO:4 and would have also found it straightforward and simple to delineate the signal peptide from the active N-terminus of the light chain in SEQ ID NO:4, as starting with (DIQM).

Samsung Bioepis Exhibit No. 1002
Page 2863

DECLARED at New Haven, CT
United States of America

This 23 Day of May 2017

Bruce A. Andrien Jr.

Bruce A. Andrien Jr., Ph.D.

Witness signature

J. Darrell Fontenot Ph.D., J.D.,
Sr. Dir., Patent Counsel,
Alexion Pharmaceuticals, Inc.
100 College Street, New Haven, CT, 06510,
United States of America

Witness Name, Capacity and Address

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: January 19, 2017
Electronic Signature for Jill Gorny Sloper, Esq.: /Jill Gorny Sloper, Esq./

EP 16 19 5355.9
Alexion Pharmaceuticals, Inc.
Our Ref.: P2913 EP/3

Docket No.: AXJ-114BUSCN3
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Leonard Bell *et al.*

Application No.: 15/260,888

Filed: September 9, 2016

For: TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

Confirmation No.: 2558

Art Unit: 1644

Examiner: J. L. Rogers

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION PURSUANT TO 37 CFR 1.132 BY DR. LEONARD BELL

I, Leonard Bell, M.D., a co-inventor of the subject invention, declare that:

1. I founded Alexion Pharmaceuticals, Inc. and served as its Chief Executive Officer from 1992 until 2015. In that role, I was closely involved in the development of eculizumab and in the administration and monitoring of all clinical trials involving eculizumab.

2. Prior to March 15, 2007, which is the date the subject patent application was filed, eculizumab was not available to the public. In order to be approved for marketing, eculizumab was used in clinical trials before March 15, 2007, but only under strict control and conditions of confidentiality. Patients in clinical trials of eculizumab received intravenous infusions of eculizumab at hospitals and infusion clinics and never themselves possessed samples of eculizumab. Researchers and staff involved in clinical trials of eculizumab were not permitted to use or distribute eculizumab for

purposes other than conducting the clinical trials, and were strictly accountable to Alexion for the inventory of eculizumab and for information about eculizumab.

3. Moreover, prior to March 15, 2007, the complete structure of eculizumab was not disclosed to the public. For example, the Hillmen reference (Hillmen et al., N. ENGL. J. MED., 350, pp. 552-59 (2004)) does not disclose any parts of the sequence of eculizumab and the '245 patent (U.S. Pat. No. 6,355,245) does not disclose or even hint at the unique non-naturally occurring, protein-engineered heavy chain (including the CH1-hinge-CH2-CH3 regions) of eculizumab.

4. Nor does the Thomas reference (Thomas et al., MOLECULAR IMMUNOLOGY, 33 (17/18), pp. 1389-1401 (1996)) describe eculizumab. The Thomas reference describes an antibody comprising the humanized variable region of eculizumab, but does not disclose or hint at the unique non-naturally occurring, protein-engineered heavy chain of eculizumab, which confers important functional effects (as discussed below). Rather, the antibody described in the Thomas reference contains a *naturally-occurring* IgG4 heavy chain. The antibody described in the Thomas reference therefore is not eculizumab. It is both structurally and functionally different from eculizumab.

5. As shown below, a sequence alignment of the heavy chain constant regions and hinge of eculizumab (shown in bold on the top line below) and that of the antibody described in the Thomas reference (shown on the bottom line below) demonstrates that the two antibodies are structurally very different, with fourteen different amino acid residues in the constant regions (underlined and highlighted) (four different residues in the CH1 domain, six different residues in the hinge region and four different residues in the CH2 domain):

```
-----------------------------CH1-------------------
ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
```

```
------------CH1-------------------------------|Hinge-
FPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVERKCCV
FPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGP

-Hinge--|-----------------CH2------------------
ECPPCPAPP-VAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDP
PCPSCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDP

---------CH2-----------------------------------------
EVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKC
EVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKC

--------CH2-------|-------------CH3---------------
KVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKG
KVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKG

---------------------------CH3---------------------
FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNV
FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNV

---------CH3-------------
FSCSVMHEALHNHYTQKSLSLSLGK
FSCSVMHEALHNHYTQKSLSLSLGK
```

6. Also, the functional properties of eculizumab and the antibody described in the Thomas reference are very different. For example, eculizumab does not bind as strongly to Fcγ receptors as the Thomas antibody. Binding to the Fcγ receptors initiates and amplifies immune responses that are undesirable for an antibody intended to shut down the terminal complement pathway. Thus, engineering eculizumab to minimize binding to these receptors provided important advantages over the antibody described in the Thomas reference.

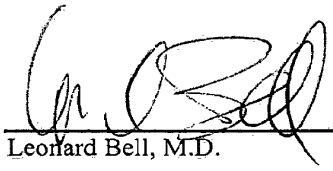
7. Eculizumab also has a much longer plasma half-life than the antibody described in the Thomas reference. It is advantageous for an antibody like eculizumab to remain in the circulation longer so that it does not need to be administered to patients as frequently.

Samsung Bioepis Exhibit No. 1002
Page 2867

8. Accordingly, as demonstrated by comparative data between eculizumab and the antibody described in the Thomas reference, the non-natural, protein-engineered, heavy chain of eculizumab reduces effector function activation, minimizes immunogenicity, and contributes to a longer half-life of eculizumab compared to the antibody described in the Thomas reference. These properties were surprising and unpredictable, and made eculizumab a better drug candidate than the antibody described in the Thomas reference.

9. In sum: the complete structure of eculizumab was not available to the public on or before March 15, 2007; eculizumab was not available to the public or being used by the public prior to that time; the unique protein-engineered heavy chain of eculizumab was not described in the Hillmen reference, the Thomas reference or the '245 patent; and the unique protein-engineered heavy chain of eculizumab could not be derived from the combined disclosures of the Hillmen reference, the Thomas reference and/or the '245 patent.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or of any patent issued thereon.



Leonard Bell, M.D. 1/18/2017 Date

SEQUENCE LISTING

<110> ALEXION PHARMACEUTICALS, INC.

<120> TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

<130> P2913 EP/3

<150> 60/783,070
<151> 2006-03-15

<160> 10

<170> BiSSAP 1.3.6

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<211> 123
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic polypeptide

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20 25 30
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35 40 45
Gly Glu Ile Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
50 55 60
Lys Asp Arg Val Thr Met Thr Arg Asp Thr Ser Thr Ser Thr Val Tyr
65 70 75 80
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95
Ala Arg Tyr Phe Phe Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val Trp
100 105 110
Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala
115 120

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<211> 448
<212> PRT
<213> Artificial Sequence

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Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1 5 10 15
Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn Tyr
20 25 30
Trp Ile Gln Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
35 40 45
Gly Glu Ile Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
50 55 60
Lys Asp Arg Val Thr Met Thr Arg Asp Thr Ser Thr Ser Thr Val Tyr
65 70 75 80

```
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95
Ala Arg Tyr Phe Phe Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val Trp
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Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125
Ser Val Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr
    130                 135                 140
Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160
Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175
Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190
Val Pro Ser Ser Asn Phe Gly Thr Gln Thr Tyr Thr Cys Asn Val Asp
        195                 200                 205
His Lys Pro Ser Asn Thr Lys Val Asp Lys Thr Val Glu Arg Lys Cys
    210                 215                 220
Cys Val Glu Cys Pro Pro Cys Pro Ala Pro Pro Val Ala Gly Pro Ser
225                 230                 235                 240
Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg
                245                 250                 255
Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser Gln Glu Asp Pro
            260                 265                 270
Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala
        275                 280                 285
Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr Tyr Arg Val Val
    290                 295                 300
Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr
305                 310                 315                 320
Lys Cys Lys Val Ser Asn Lys Gly Leu Pro Ser Ser Ile Glu Lys Thr
                325                 330                 335
Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu
            340                 345                 350
Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys
        355                 360                 365
Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
    370                 375                 380
Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp
385                 390                 395                 400
Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr Val Asp Lys Ser
                405                 410                 415
Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala
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<210> 3
<211> 109
<212> PRT
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<220>
<223> Description of Artificial Sequence: Synthetic polypeptide

<400> 3

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Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80
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<211> 214
<212> PRT
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<223> Description of Artificial Sequence: Synthetic polypeptide

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Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
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Asp Arg Val Thr Ile Thr Cys Gly Ala Ser Glu Asn Ile Tyr Gly Ala
            20                  25                  30
Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80
Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn Thr Pro Leu
                85                  90                  95
Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
            100                 105                 110
Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
        115                 120                 125
Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
    130                 135                 140
Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
145                 150                 155                 160
Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
                165                 170                 175
Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
            180                 185                 190
Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
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Phe Asn Arg Gly Glu Cys
    210

<210> 5
<211> 5
<212> PRT
<213> Artificial Sequence


<220>
<223> Description of Artificial Sequence: Synthetic peptide

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1               5
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<210> 6
<211> 17
<212> PRT
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Glu Ile Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe Lys
1 5 10 15
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<210> 7
<211> 13
<212> PRT
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<400> 7
Tyr Phe Phe Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
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<210> 8
<211> 11
<212> PRT
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Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu Asn
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Gly Ala Thr Asn Leu Ala Asp
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<223> Description of Artificial Sequence: Synthetic peptide

<400> 10
Gln Asn Val Leu Asn Thr Pro Leu Thr
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TABLE OF CONTENTS

INTRODUCTION....xiii
REFERENCES TO INTRODUCTION....lxxiii

SIGNAL SEQUENCES OF LIGHT CHAINS

HUMAN KAPPA CHAINS....1
HUMAN LAMBDA CHAINS....5

MOUSE KAPPA CHAINS....7
MOUSE LAMBDA CHAINS....17

MISCELLANEOUS KAPPA CHAINS....19
MISCELLANEOUS LAMBDA CHAINS....21

SIGNAL SEQUENCES OF HEAVY CHAINS

HUMAN....23

MOUSE....29

MISCELLANEOUS....40

SIGNAL SEQUENCES OF T-LYMPHOCYTE RECEPTOR

HUMAN ALPHA CHAINS....45
HUMAN BETA CHAINS....49
HUMAN GAMMA CHAINS....53
HUMAN DELTA CHAINS....55

MOUSE ALPHA CHAINS....57
MOUSE BETA CHAINS....61
MOUSE GAMMA CHAINS....65
MOUSE DELTA CHAINS....67

MISCELLANEOUS ALPHA CHAINS....69
MISCELLANEOUS BETA CHAINS....71

SIGNAL SEQUENCES OF RELATED PROTEINS

BETA-2-MICROGLOBULINS....73

MAJOR HISTOCOMPATIBILITY ANTIGENS CLASS I....75

I REGION ANTIGENS CLASS II....82

COMPLEMENT....89

T-CELL SURFACE ANTIGENS....91

INTEGRINS....94

MISCELLANEOUS PROTEINS....96

VARIABLE REGION LIGHT CHAIN SEQUENCES

HUMAN KAPPA CHAINS SUBGROUP I....103
HUMAN KAPPA CHAINS SUBGROUP II....113
HUMAN KAPPA CHAINS SUBGROUP III....118
HUMAN KAPPA CHAINS SUBGROUP IV....128

HUMAN LAMBDA CHAINS SUBGROUP I....131
HUMAN LAMBDA CHAINS SUBGROUP II....135
HUMAN LAMBDA CHAINS SUBGROUP III....139
HUMAN LAMBDA CHAINS SUBGROUP IV....144
HUMAN LAMBDA CHAINS SUBGROUP V....146
HUMAN LAMBDA CHAINS SUBGROUP VI....148

MOUSE KAPPA CHAINS SUBGROUP I....151
MOUSE KAPPA CHAINS SUBGROUP II....163
MOUSE KAPPA CHAINS SUBGROUP III....190
MOUSE KAPPA CHAINS SUBGROUP IV....201
MOUSE KAPPA CHAINS SUBGROUP V....208
MOUSE KAPPA CHAINS SUBGROUP VI....239
MOUSE KAPPA CHAINS SUBGROUP VII....257

MISCELLANEOUS MOUSE KAPPA CHAINS....259

MOUSE LAMBDA CHAINS....263

RAT KAPPA CHAINS....271

RAT LAMBDA CHAINS....274

RABBIT KAPPA CHAINS....276

RABBIT LAMBDA CHAINS....290

CHICKEN LAMBDA CHAINS....293

MISCELLANEOUS KAPPA CHAINS....303

MISCELLANEOUS LAMBDA CHAINS....306

MISCELLANEOUS LIGHT CHAINS....308

EP 16 19 5355.9
Alexion Pharmaceuticals, Inc.
Our Ref.: P2913 EP/3

Exhibit A

BRUCE A. ANDRIEN, Jr., Ph.D.

7 Finch Lane, Guilford, CT 06437, (203) 457-0067
E-Mail: bldandrien@comcast.net

CAREER EXPERIENCE:

Alexion Pharmaceuticals, Incorporated, Cheshire, Connecticut; Director Early Assay Development; July 2015 - Present.

Development responsibilities

- Developed processes to support Research's ever expanding needs including testing of proteins within the following areas: Biacore, HPLC, LOC, IEF, Mass Spectrometry, and biological assays.
- Lead the Process Development and Analytical Sciences Quality Improvement Team
- Lead the Process Development and Analytical Sciences Bi-Paratopic team
- Supported four diligences teams
- Supported three early research teams
- Provide response to regulatory agency questions

Alexion Pharmaceuticals, Incorporated, Cheshire, Connecticut; Director Analytical Sciences; July 2014 – July 2015.

GMP Compliance responsibilities

- Provide analytical support of CMC submissions, address comments from regulatory agencies regarding test methods and validation
- Direct and coordinate the transfer of release and stability test methods into Quality Control or to contract laboratories worldwide to support release testing of commercial drug product
- Provide support and troubleshooting to Quality Control groups for analytical test methods. Support includes assisting in assay invalids and/or OOS investigations, aberrant trends or special studies, etc.
- Manage, delegate, and prioritize GMP release and stability testing for all new pre-clinical and early stage (Phase 1 and Phase 2) clinical products.
- Direct formulation development activities, oversee design, execution, and analysis of formulation development studies.
- Manage qualification and validation of analytical methods for early stage clinical products in addition to existing commercial products.
- Manage in-process testing and coordinate outsource testing for new pre-clinical and early stage (Phase 1 and Phase 2) clinical products.
- Coordinate quarterly review meetings and provide updates, and generate reports for early stage (Phase 1 and Phase 2) clinical products.
- Generate or revise specifications for early stage (Phase 1 and Phase 2) clinical products
- Maintain appropriate compliance within area of responsibility with regards to current SOPs, validation / qualifications, and training. Represent the area functions as required during audits by external agencies and auditors, as well as by internal auditors
- Establish systems within area of responsibility to ensure compliance, good lab practices and efficiency

Development responsibilities

- Direct the development, improvement, qualification, and validation of analytical testing of proteins. Tests within area are: HPLC, SDS-PAGE, Western blots, IEF, ELISAs, biological assays and protein determinations.
- Generate and oversee product comparability protocols for early stage (Phase 1 and Phase 2) clinical products.
- Oversee the analytical support of cell culture and purification development groups in support of new clone or media optimizations and improvements to purification processes.
- Work with Purification Development to plan and coordinate formulation development studies, including generating and executing formulation stability protocols, compiling data, trending and presenting to all facets of the organization.

Other Responsibilities:

- Train, coach, and mentor Analytical Sciences laboratory personnel by working with employees to develop performance objectives and to monitor performance against the objectives.
- Interact with Alexion Pharmaceuticals personnel within Development, and other departments as required to meet objectives; interact with Alexion Pharmaceuticals business partners and technical representatives as required to meet objectives
- Represent Analytical services on Task Force or Planning Committees as assigned

BRUCE A. ANDRIEN, Jr., Ph.D.

7 Finch Lane, Guilford, CT 06437, (203) 457-0067

E-Mail: bldandrien@comcast.net

Alexion Pharmaceuticals, Incorporated, Cheshire, Connecticut; Director Protein Characterization; Sep. 2001 – Jul. 2014.

Manage the Protein Characterization group for characterizing proteins to support IND and BLA filings along with drug product understanding for FDA, EMEA, TGA, PMDA, and Health Canada. These assays include SDS-Page, vIEF, hIEF, 2D-Page, enzyme digestions, HPLC-UV (peptide maps, oligosaccharide, monosaccharide, sialic acid), CD, fluorometry, ligand/antigen kinetics by surface plasma resonance, N-terminal, flow field fractionation multi angel light scattering, ion mobility, mass spectrometry (small molecule contamination identification, intact molecular weight, peptide maps by µLC/nLC ion trap/TOF/Q-TOF, MALDI peptide mass fingerprints, disulfide mapping, identification of unknown proteins, glycan characterization, peptide confirmation by LC-MS/MS and MALDI-PSD, and in-house bioinformatics and proprietary Alexion database). Method robustness and method transfer. Review, write and oversee SOP's, GTR's, and protocols.

Managed more than $1,000,000 budget/year along with FTE's including Associate Directors, Ph.D., Masters, and Bachelors level as direct reports at Alexion.

Managed the staff meeting commutations between VP and Fermentation, Purification, Analytical Sciences, and Protein Characterization Groups.

Managed CMC documentation personal, review and write CMC sections for IND, NDA, BLA, updates and responses for drug product and drug substance for FDA, EMEA and other countries.

Support Alexion's Research, Fermentation, Purification, Quality Control/Quality Assurance and Clinical with techniques available in Protein Characterizations core facility.

Analytica of Branford, Inc., Branford, Connecticut; Senior Research Scientist; 1991 - 2001.

Conducted research and development on time-of-flight mass spectrometers, ion guides, ion optics, electrospray, nanoelectrospray and atmospheric pressure chemical ionization ion sources. Developed automation techniques using flow injection analysis and liquid chromatography for proteins, peptides, and small molecules. Generated data to support marketing, client needs, competitive demos, product development, research, and conference presentations. Author of patents, grants, manuals for various hardware, conference presentations, articles, and internal reports.

Principle Investigator on phase I and phase II NIH grants. Project manager of nanospray, dual electrospray, APCI, and parts of a TOF-MS project, taking these from the initial research and development phase though prototype design and finally to production and qualification testing. Responsible for installations, client training, and trouble shooting for electrospray, nanospray, dual electrospray, APCI, and TOF-MS products.

Conducted computer modeling scenarios on various parts of time-of-flight mass spectrometers, ion guides, and ion optics. Designed, developed, and coded software packages including: (1) "MoleculeMaker™" for generating theoretical isotope profiles and electrospray spectra from elemental formulas or amino acid sequences, (2) "Balancer" for calculating sample concentrations and dilution sequences, and (3) numerous other small programs and spread sheets. Project manager, which included supervising internal and external computer programmers, for a commercialized software package, "MacroMass™," used for deconvoluting multiply-charged electrospray mass spectra.

University of California, San Diego, California; Graduate Research Assistant; 1984 - 1991.

Ph.D. dissertation on metal cluster beams and magnetic thin films. Designed and constructed the following instruments: (1) Ultra High Vacuum (UHV) system, (2) 2200°C nozzle for metal beam film growth, and (3) UHV M-H Magnetometer. Analyzed thin films by Auger Electron Spectroscopy (AES), Low Energy Electron Diffraction (LEED), Temperature Program Mass Desorption (TPD), M-H Magnetometry, Electron Spectroscopy for Chemical Analysis (ESCA), and Transmission Electron Microscopy (TEM). Interfaced an Auger Electron Spectrometer, a M-H Magnetometer, and a Mass Spectrometer for temperature program mass desorption, to an IBM PC controller.

Lycoming College, Williamsport, Pennsylvania; Computer Assistant Manager; 1981 - 1984.

Responsibilities included tutoring, programming, systems operations, and supervision of assistants.

Samsung Bioepis Exhibit No. 1002
Page 2876

BRUCE A. ANDRIEN, Jr., Ph.D.

7 Finch Lane, Guilford, CT 06437, (203) 457-0067
E-Mail: bldandrien@comcast.net

EDUCATION:

Ph.D., University of California, San Diego, 1991. Engineering Physics.
M.S., University of California, San Diego, 1986. Engineering Physics, (GPA) 3.54.
B.A., Lycoming College, Pennsylvania, 1984. Computer Science (GPA) 4.00, Physics (GPA) 3.88, Magna Cum Laude (GPA) 3.69.

FELLOWSHIPS AND HONORS:

Dissertation Research Fellowship, 1991.
University of California Regents Fellowship, 1984-1985.
Charles J. Kocian Computer Science Award, 1984.
Ferree Mathematical Sciences Award, 1984.
Phi Kappa Phi National Honor Society, 1984.
Lycoming Scholar, 1980-1984.
Dean's List, 1980-1981, 1983-1984.
Sigma Pi Sigma National Physics Honor Society, 1983.
Blue Key Superior Academic Freshman Award, 1981.

RELATED ACTIVITIES:

Society of Physics Students, Lycoming College, 1980-1984.
Student Advisory Committee for Mathematical Sciences, Lycoming College, 1983-1984.
Student Judiciary Appointment Board, Lycoming College, 1983-1984.
ACM Allegheny Region Programming Contest, 1982 and 1983.
SHARE program, observed engineering and computer departments at AVCO Lycoming, 1983.

PROFESSIONAL SOCIETIES:

American Society for Mass Spectrometry, American Chemical Society, American Vacuum Society, Materials Research Society, Phi Kappa Phi.

SPECIAL SKILLS:

Proteomics, Electrospray, Atmospheric Pressure Chemical Ionization (APCI), Time-of-Flight Mass Spectrometry (TOF-MS), Mass Spectrometry (MS), Molecular Beams, CAD, SPR BIACORE, Ion Mobility, AFFF/MALS, Visual Basic, computer programming.

Samsung Bioepis Exhibit No. 1002
Page 2877

BRUCE A. ANDRIEN, Jr., Ph.D.

7 Finch Lane, Guilford, CT 06437, (203) 457-0067

E-Mail: bldandrien@comcast.net

SEMINARS:

Invited guest speaker at WATERS Japanese Executive Technology Forum 2009
Connecticut Separation Science Council (CSSC) Monthly Meetings (2001-present)
CT Mass Spectrometry Monthly Discussion Group (1991-2001)
Fenn 90 Symposium 2007
Discovery Proteomics, New Mass Spectrometry, Biomarker, Quantitation and PTM Analysis, Applied Biosystems, May 4, 2004
Invited Speaker for Amersham Biosciences European Proteomics Tour, (Summer 2001)

SHORT COURSES:

Practical Mass Spectrometric Characterization of Protein Therapeutics, (ASMS 2012)
Proteins Structural Analysis By Mass Spectrometry, (ASMS 2011)
MALDI Imaging Mass Spectrometry: Basic Tools and Techniques, (ASMS 2010)
Metabolomics (ASMS 2009)
Wyatt Light Scattering University (2007)
Quantitative MS, (ASMS 2007)
Proteins and Peptides by Mass Spectrometry, (ASMS 2005)
Biacore University SPR Basics Customer Training (April 2005)
Mass Spectrometry in Drug Discovery (ASMS 2004)
Applied BioSystems Voyager De-Pro Customer Training, (December 2002)
Bruker Esquire Ion Trap Customer Training, (April 2002)
Agilent 1100 HPLC ChemStation Customer Training, (Spring 2002)
Well Characterized Biologicals, (IBC's 5th International Conference 2001)
Characterization of Proteins and Peptides by Mass Spectrometry, (ASMS 2001)
Advanced SIMION 7.0 (ASMS 2000).
Fundamentals and Applications of MALDI-TOF-MS (ASMS 1999).
High Performance Liquid Chromatography, HPLC, and LC/MS (EAS 1998)
Quadrupole Ion Trap and Tandem Mass Spectrometry (ASMS 1998).
PHI Surface Analysis and Instrumentation Course on ESCA, (1987).

CONFERENCES ATTENDED:

American Society of Mass Spectrometry Conference, (1992-2012)
Well Characterized Biologicals, (IBC's Conference 2001 and 2009)
Next Generation Biologics, (IBC's Conference 2009)
Csaba Horvath Memorial Symposium (2005, 2007, 2009)
18th Annual International Light Scattering Colloquium(2007)
DiPIA 2007 Developments in Protein Interaction Analysis Biacore 2007
Pittsburgh Conference, (1993-2001)

Samsung Bioepis Exhibit No. 1002
Page 2878

BRUCE A. ANDRIEN, Jr., Ph.D.

7 Finch Lane, Guilford, CT 06437, (203) 457-0067

E-Mail: bldandrien@comcast.net

ALEXION TRAINING:

GMP Training (2001-2015)
Presentation Skills (30Jul08)
Intercultural Training for Filing in Japan (29Jul08)
21 CFR Part 11 (General Awareness Training) (14Jun07)
Tips, Techniques & Shortcuts for Organizing & Maintaining Files & Records (Skillpath) (17Oct06)
Change Control Training (SOP-QA-0038), (11Jul06)
SAP Training, (07Jul06)
Performance Management Process Appraisal Writing & Delivery Training, (30Jun06)
SharePoint Portal Server 2003 Training, (16Jun06)
On-Site Regulatory Inspections (SOP-RA-1003.00), (April 3, 2006)
Change Control Program (SOP-QA-0038.03), (10Mar06)
cGMP Briefing Presentation for Pharmaceutical Stakeholders, (02Mar06)
CP-0001.00, CP-0002.00, CP-0003.00 (16Nov05)
WPD-TS-001-00, WPD-TS-002-00, WPD-TS-003.01 (16Nov05)
Coaching and Teambuilding Skills for Managers and Supervisors, SkillPath (June 15, 2005)
Goal Setting for Groups, SkillPath (May 18, 2005)
Excelling as a First-Time Manager, SkillPath (January 21, 2005)
VBA for MS Excel, (November 10 and 19, December 15, 2004)
Fundamentals of Successful Project Management, SkillPath, (November 3, 2004)
SOP-QA-0001.05, 0002.04, 0003.05, 0017.05 0022.05 Doc Control (04Jun04)
Excel Microsoft XP- Pivot Tables, (April 2004)
Excel Microsoft XP- Formulas, (March 2004)
RDM-0033.00, Determination of Eculizumab Purity by SEC-HPLC for In Process Testing (09Feb04)
SOP-CAL-0029.00 Standardization of the AND Electronic Balance, Model #FD-6000 (09Feb04)
RDM-0012.00 Quantification of Protein Concentration in Pexelizumab Samples by UV-Vis Spectrophotometry (03Feb04)

Sexual Harassment Prevention Training, (2003)
SOP-QA-0055.00 Labaratory Notebook Requisition, Issuance, and Tracking (22Dec03)
Laboratory Notebook Documentation, Jeff Gassman, (09Dec03)
PowerPoint Microsoft, Key-Logic, Dynamic Slide Enhancements, (28Oct03)
SOP-QCG-0025.03 Quality Control Sample Submission and Handling (20Oct03)
SOP-QA-0017.04 Document Control System, (04Aug03)
Intro to Good Scientific Practices (GSP), (23May03)
SOP-QA-0043.01, (21Jan03)

SOP-QA-0001.03, 0002.02, 0003.02, 0004.02, 0017.03, and 0022.03, (10Nov02)
Document Control System, Controlled Copy of SOP, and SOP Training (11Oct02)
21 CFR Compliance Training Session II; cGMP, GLP, GCP, and QA, (24Jun02)
Pipettes Training, Alpha-Omega, (29May02)
Basic Good Laboratory Practices (21 CFR Part 58), (28May02)

cGMP's and Compliance Document Control System, (17Sep01)
Annual OSHA Training/Safety Training, (Mar 2006, 2005, 2004, 2003, 2002, 2001)

Samsung Bioepis Exhibit No. 1002
Page 2879

BRUCE A. ANDRIEN, Jr., Ph.D.

7 Finch Lane, Guilford, CT 06437, (203) 457-0067

E-Mail: bldandrien@comcast.net

PUBLICATIONS AND PRESENTATIONS:

"Kinetic analysis of a monoclonal therapeutic antibody and its single-chain homolog by surface plasmon resonance", Rekha Patel *, Bruce A. Andrien Jr., Anal. Biochem. 396 (2010) 59–68.

"Kinetic Analysis of Anti-C5 Subclasses to Gamma Receptors by Surface Plasmon Resonance", Rekha Patel, Bruce Andrien, Krista Johnson, Jeff Hunter, Pat Bento, Michelle Giannoni and Paul Tamburini, Developments in Protein Interaction Conference 2009

"The Role of LC/MS to Support Biotechnological/Biological Product Regulatory Submissions", Invited guest speaker at WATERS Japanese Executive Technology Forum 2009

"AFFF/MALS and Ion Mobility for Analysis of Antibodies and Non-Covalent Complexes", Bruce Andrien, Csaba Horvath Symposium 2009

"Integration of Waters BiopharmaLynx Application Manager into a Monoclonal Antibody Characterization Strategy", Adam Lucka, Rekha Patel, Christine Nowak, and Bruce Andrien, WATERS users meeting at the American Society of Mass Spectrometry Conference 2009

"Ion Mobility Spectrometry for Determining Binding, Bridging and the Formation of Non-Covalent Complexes", Bruce Andrien, Rekha Patel, Christine Nowak, and Adam Lucka, American Society of Mass Spectrometry Conference 2009

"Characterizing Five Deamidation Products from a Single Asparagine Residue in Recombinant mAb's Through LC-MS Peptide Mapping", Adam Lucka, Christine Nowak, Rekha Patel, and Bruce Andrien, American Society of Mass Spectrometry Conference 2009

"Analysis of Antibodies, Aggregates and Non-Covalent Complexes by IMS, SEC/MALS/IMS and AFFF/MALS/IMS", Bruce Andrien, Christine Nowak, and Adam Lucka, Invited Guest Speaker at TSI User Meeting at ASMS Conference, (2008).

"Coupling AFFF/MALS or SEC/MALS to Ion Mobility Spectrometry for Analysis of Antibodies, Fragments, Aggregates and Non-Covalent Complexes", Bruce Andrien, Christine Nowak, and Adam Lucka, 56th ASMS Conference, (2008).

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"An Investigation of Protein Therapeutics Including Antibodies and Antibody Conjugates with Ion Mobility Spectrometry", Bruce A. Andrien, Rekha Patel, Lynellen Willard, and Rachael Alford, 55th ASMS Conference, (2007).

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"Kinetic Analysis of a Monoclonal Antibody (eculizumab) and its Single Chain Homolog (pexelizumab) by Surface Plasmon Resonance", Rekha Patel and Bruce Andrien, DiPIA Conference 2007.

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Samsung Bioepis Exhibit No. 1002
Page 2880

BRUCE A. ANDRIEN, Jr., Ph.D.
7 Finch Lane, Guilford, CT 06437, (203) 457-0067
E-Mail: bldandrien@comcast.net

"Characterization of a Monoclonal Antibody and its Single Chain Therapeutic", Rekha Patel, Adam Lucka, and Bruce Andrien, Invited Guest Speaker at Agilent World Tour 2006, Cambridge, MA, (16May06).

"Workflow for the Characterization of Proteins Therapeutics", Bruce A. Andrien, Jr., Adam Lucka, Rekha Patel, Invited Guest Speaker at Agilent Lunch and Learn during 53th ASMS Conference, (2005).

"A Bioinformatics Strategy for the Characterization of Proteins versus Protein Identification", Bruce A. Andrien, Jr., Adam Lucka, Rekha Patel, 53th ASMS Conference, (2005).

"Improving Protein Coverage in nanoLC-MS Peptide Mapping by using a Sub-Ambient Enrichment Column to Bind Hydrophilic Peptides", Adam Lucka, Rekha Patel, Bruce A. Andrien, Jr., 53th ASMS Conference, (2005).

"Protein Identification after Two-Dimensional Gel Electrophoresis by MALDI ToF MS and LC ESI ToF MS", Staffan Renlund; Ylva Laurin; Maria Liminga; Henrik Wadensten; Bruce A. Andrien, Jr.; Marketa Berkova; Erol E. Gulcicek, 49th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, (2001).

"Confirmation Analysis of Protein Purification using Electrospray Time of Flight Mass Spectrometry", Henrik Wadensten; Ninni Salomonsson; Christel Ellström; Staffan Lindqvist; Staffan Renlund; Bruce A. Andrien, Jr.; Marketa Berkova; Erol E. Gulcicek, 49th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, (2001).

"Evaluation of Rugged Microelectrospray Iinterfaces for Identification of Peptides and Proteins Wiith APII-TOF-MS", Erol E. Gulcicek; Bruce A. Andrien, Jr; Marketa Berkova; Craig M. Whitehouse; Staffan Renlund, 49th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, (2001).

"Investigating the Trap-Pulse Mode of Operation to Improve the Sensitivity of Electrospray Time-Of-Flight Mass Spectrometer", Marketa Berkova; Craig Whitehouse; Erol Gulcicek; Bruce Andrien, 49th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, (2001).

"Identification of Post-Translational Modifications by Fast LC-MS and In-Source CID Using a New API TOF MS", Staffan Renlund, Henrik Wadensten, Lars Rönnstrand, Rannar Sillard, Bruce A . Andrien, Jr., Marketa Berkova, and Erol E. Gulcicek, Montreux (2000).

"Optimization of Rapid State Switching API TOF Operation for Fast LC-MS", Erol E. Gulcicek, Bruce A. Andrien, Marketa Berkova, James G. Boyle, Shida Shen, and Craig M. Whitehouse, and Staffan Renlund, Montreux (2000).

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"Identification of Proteins and Post Translational Modifications Using New MALDI ToF and ESI ToF Mass Spectrometer Systems", Staffan Renlund, Jonas Åström, Henrik Wadensten, Rannar Sillard, Lars Rönnstrand, Bruce A. Andrien, Jr., Marketa Berkova, and Erol E. Gulcicek, 4th Int Seminar on Biomolecule Identification and Function (2000).

"Fast LC-MS for Peptide Mass Mapping, Protein Analysis and Identification of Post-Translational Modification Using API TOF", S. Renlund, H. Wadensten, J. Astrom, J. Ericsson, S. Ekman, U. Engstrom, L. Ronnstrand, B. A. Andrien, E. E. Gulcicek, 48th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Long Beach, CA, (2000).

Samsung Bioepis Exhibit No. 1002
Page 2881

BRUCE A. ANDRIEN, Jr., Ph.D.
7 Finch Lane, Guilford, CT 06437, (203) 457-0067
E-Mail: bldandrien@comcast.net

"Analysis of Proteins from 2D-Gels by MALDI-TOF MS and LC-ESI-TOF MS", M. Raida, G. Reil, A. Gorg, J. Astrom, H. Wadensten, S. Renlund, and B. A. Andrien, 48th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Long Beach, CA, (2000).

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"Multiple Inlet Probes for Electrospray and APCI Sources", B. A. Andrien, M.A. Sansone, and C. M. Whitehouse, 46th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Orlando, FL, (1998).

"Minimizing Chemical Noise through Rational Design of a "Universal" API Source", S. Shen, B. Andrien Jr., M. Sansone, and C. M. Whitehouse, 46th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Orlando, FL, (1998).

"A Two Dimensional Ion Trap API TOF Mass Spectrometer", C. M. Whitehouse, E. E. Gulcicek, J.F. Banks, B. Andrien, 46th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Orlando, FL, (1998).

"Performance of High Throughput API TOF MS Detector", E. E. Gulcicek, B. A. Andrien, J.F. Banks, J.G. Boyle, and C. M. Whitehouse, 10th Sanibel Conference on Mass Spectrometry, Sanibel, FL, (1998).

"Atmospheric Pressure Chemical Ionization and Electron Capture Negative Chemical Ionization Mass Spectrometry in Studying ß-Carotene Conversion to Retinol in Humans", Guangwen Tang, Bruce A. Andrien, Gregory G. Dolnikowski, and R. M. Russell, Methods in Enzymology, **282**, 140-154, (1998).

Samsung Bioepis Exhibit No. 1002
Page 2882

BRUCE A. ANDRIEN, Jr., Ph.D.
7 Finch Lane, Guilford, CT 06437, (203) 457-0067
E-Mail: bldandrien@comcast.net

"A Disposable μ-Tip Probe for Low Flow Electrospray", B. A. Andrien, D. M. Sauro, M.A. Sansone, G.P Whitehouse, C. M. Whitehouse, and A.G. Burt, 45th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Palm Springs, CA (1997).

"Performance of a Hexapole Ion Guide on HP5988", B. A. Andrien, M.A. Sansone, B.L. Mancini, C. M. Whitehouse, A.G. Burt, Guangwen Tang, and Gregory G. Dolnikowski 45th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Palm Springs, CA (1997).

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"Detecting the *in vivo* Conversion of b-Carotene-d_8 to Retinol-d_4 by APCI-MS and GC/ECNCI-MS", Guangwen Tang, Bruce A. Andrien, and Gregory G. Dolnikowski, 44th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Portland OR (1996).
"Atmospheric Pressure Ionization Flow Injection Analysis by Orthogonal Time-of-Flight Mass Spectrometry", B.A. Andrien, T. Dresch, P.J. Haren, and C. M. Whitehouse, 44th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Portland OR (1996).

"On-Line Detection of Fast Chromatographic Events by Electrospray Ionization Time-of-Flight Mass Spectrometry", Erol E. Gulcicek, B. Andrien, J.F. Banks, J.G.Boyle, T.Dresch, P.Haren,and C.Whitehouse, 44th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Portland OR (1996).

"Micro and Nano Flow Atmospheric Pressure Chemical Ionization", B. A. Andrien and C. M. Whitehouse, Pittsburgh Conference Proceedings, (1996).

"API Time of Flight Mass Spectrometry as a Chromatography Detector", B. Andrien, J. F. Banks, T.Dresch, E.Gulcicek, P.Haren, J.Quinn, and C.Whitehouse, Pittsburgh Conference Proceedings, (1996).

"Electrospray and APCI Interface for Mass Spectrometry", B. A. Andrien and J. G. Boyle, *Spectroscopy*, **10**, 2, 42-44, (1995).

"Improvements to Electrospray and APCI Ion Sources for CE/MS and LC/MS Applications with TOF, Quadrupoles, FTMS, Ion Traps and Magnetic Sector Spectrometers" C. M. Whitehouse, J. F. Banks, E.Gulcicek, T.Dresch, B. Andrien, and S. Shen, 37th Rocky Mountain Conference on Analytical Chemistry, Denver, CO, (1995).

"Atmospheric Pressure Chemical Ionization by Corona Discharge", B. A. Andrien, T. Dresch, P.J. Haren, C. M. Whitehouse, and S. Shen, 43th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Atlanta GA (1995).

"A Mass Analyzer for High Speed Liquid Chromatography", B. A. Andrien, J. F. Banks, J. G. Bolye, T. Dresch, P.J. Haren, and C. M. Whitehouse, 43th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Atlanta GA (1995).

"Performance of an Atmospheric Pressure Corona Discharge Ion Source with Time of Flight Mass Spectrometry", B. A. Andrien, T. Dresch, P.J. Haren, and C. M. Whitehouse, Pittsburgh Conference Proceedings, (1995).

"Atmospheric Pressure Chemical Ionization by Corona Discharge with Pneumatic and Ultrasonic Nebulization", B. A. Andrien, J. P. Quinn, and C. M. Whitehouse, 42th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Chicago (1994).

Samsung Bioepis Exhibit No. 1002
Page 2883

BRUCE A. ANDRIEN, Jr., Ph.D.
7 Finch Lane, Guilford, CT 06437, (203) 457-0067
E-Mail: bldandrien@comcast.net

"Theoretical and Practical Considerations for Determining Resolution and Conducting Mass Calibration with Multiply Charged Ions", B. A. Andrien, P.J. Haren, and C. M. Whitehouse, 42th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Chicago (1994).

"Improved Performance of a New Electrospray and APCI source Interfaced to a ZAB or 7070 Series Magnetic Sector Mass Spectrometer", S. Shen, C. M. Whitehouse, B. A. Andrien, and Walter McMurrary, 42th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Chicago (1994).

"Performance of an Atmospheric Pressure Corona Discharge Ion Source with Ultrasonic Nebulization", B. A. Andrien, J. P. Quinn, and C. M. Whitehouse, Pittsburgh Conference Proceedings, Chicago (1994).
"Performance of an Atmospheric Pressure Ion Source with Corona Discharge and Ultrasonic Nebulization", B. A. Andrien, C. M. Whitehouse, and J. P. Quinn, 41th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, San Francisco (1993).

"Deconvolution of Electrospray Generated Multiply Charged Ion Spectra Using a Three Dimensional Technique", B. A. Andrien, M. Labowski, and C. M. Whitehouse, The Pittsburgh Conference Proceedings (1993).

"A Comparison of Magnetic Properties of Thin Films of Fe/GaAs(100) and Ni/Mica", B. Andrien and D.R. Miller, Materials Research Society Symposia Proceedings (submitted Dec. 1992). Fall Meeting, Boston, MA, Dec. 1992.

"Deconvolution of Electrospray Generated Multiply Charged Spectra of Mixtures Using a Three Dimensional Technique", B. A. Andrien, M. Labowski, and C. M. Whitehouse, 40th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, (1992). Washington, DC (1992).

"Thin Film Growth and Magnetic Properties for Fe/GaAs(100)", B. Andrien and D.R. Miller, Materials Research Society Symposia Proceedings, **202**, 193 (1991). Fall Meeting, Boston, MA, Dec. 1990.

"Magnetization Loop Tracer for Superconductors", B. Andrien and J. Haugdahl, Review of Scientific Instruments **61(8)**, 2260 (1990).

"Chemisorption on Ferromagnetic Metal Films", B. Andrien and D.R. Miller, Materials Research Society, Spring Meeting, San Diego, CA, Apr. 1989.

"Thin Ferromagnetic Metal Cluster Films", B. Andrien and D.R. Miller, American Vacuum Society Vacuum Metallurgy Division, 15th International Conference on Metallurgical Coatings, San Diego, CA, Apr. 1988.

"Magnetic Properties of Thin Metal Cluster Films", B. Andrien, J. Haugdahl, and D.R. Miller, Journal of Vacuum Science and Technology A **6(3)**, 1865 (May 1988). American Vacuum Society, 34th National Symposium, Anaheim, CA, Nov. 1987.

"A Spectroscopic Investigation of Van Der Waals Complexes", B. Andrien, 38th Eastern Colleges Science Conference, Providence College, RI, Apr. 1984.

Samsung Bioepis Exhibit No. 1002
Page 2884

BRUCE A. ANDRIEN, Jr., Ph.D.

7 Finch Lane, Guilford, CT 06437, (203) 457-0067
E-Mail: bldandrien@comcast.net

AWARDED PATENTS:

Title: "Nucleic Acid Encoding Anti-C5 Antibodies having Improved Pharmacokinetics"
Inventors: Bruce A. Andrien, Jr.; Douglas L. Sheridan; and Paul P. Tamburini
Patent No.: 9,206,251 B2
Date of Patent: Dec. 8, 2015

Title: "Methods of Treating C5 Mediated Complement-Associated Conditions with Anti-C5 Antibodies having Improved Pharmacokinetics"
Inventors: Bruce A. Andrien, Jr.; Douglas L. Sheridan; and Paul P. Tamburini
Patent No.: 9,107,861 B1
Date of Patent: Aug. 18, 2015

Title: "Anti-C5 Antibodies having Improved Pharmacokinetics"
Inventors: Bruce A. Andrien, Jr.; Douglas L. Sheridan; and Paul P. Tamburini
Patent No.: 9,079,949 B1; WO 2015/134894 A1; New Zealand 631007; European 15 710 396.1
Date of Patent: Jul. 14, 2015; 06Mar. 2015; 02Feb2016; 06.03.2015

Title: "Mass Spectrometry with Multipole Ion Guides"
Inventors: Craig M. Whitehouse, Bruce A. Andrien, Jr.; and Gulcicek; Erol E.
Patent No.: 6,753,523; 6,987,264; and 7,189,967
Date of Patent: June 22, 2004

Title: "Atmospheric Pressure Ion Source"
Inventors: Craig M. Whitehouse, Bruce A. Andrien Jr., Michael A. Sansone, Clement Catalano
Patent No.: 6,593,568 B1
Date of Patent: July 15, 2003

Title: "Direct Flow Injection Analysis Nebulization Electrospray and APCI Mass Spectrometry"
Inventors: Bruce A. Andrien Jr., Fred Banks, James Boyle
Patent No.: 6,458,597 B1; 6,858,437 B2; and 7,364,913
Date of Patent: October 1, 2002; and Feb 22, 2005

Title: "Curved Introduction for Mass Spectrometry"
Inventors: Bruce A. Andrien Jr., Michael A. Sansone, Allan G. Burt, Craig M. Whitehouse
Patent No.: 6,326,616 B1 and 6,573,494 B1
Date of Patent: December 4, 2001 and June 3, 2003

Title: "Multiple Sample Introduction Mass Spectrometry"
Inventors: Bruce A. Andrien Jr., Craig M. Whitehouse, Shida Shen, Michael A. Sansone,
Patent No.: 6,207,954 B1 and 6,541,768 B2
Date of Patent: Mar 27, 2001 and April 1, 2003

Title: "Disposable Microtip Probe for Low Flow Electrospray"
Inventors: Bruce A. Andrien Jr., Craig M. Whitehouse, Michael A. Sansone, Denise M. Sauro, Glenn P. Whitehouse
Patent No.: 6,127,680 and 6,337,480 B1
Date of Patent: October 3, 2000 and January 8, 2002

Title: "Time-Of-Flight Mass Spectrometry from Surfaces"
Inventors: Craig M. Whitehouse, Bruce A. Andrien Jr.
Patent No.: 6,040,575 and 6,204,500 B1 and 6,600,155 B1
Date of Patent: March 21, 2000 and Jul 29 ,2003

Title: "Multipole Ion Guide Trap Time-of-Flight Mass Spectrometry with MS/MS^n Analysis Capability"
Inventors: Craig M. Whitehouse, Thomas Dresch, and Bruce A. Andrien Jr.
Patent No.: 6,011,259
Date of Patent: January 4, 2000

Samsung Bioepis Exhibit No. 1002
Page 2885

BRUCE A. ANDRIEN, Jr., Ph.D.
7 Finch Lane, Guilford, CT 06437, (203) 457-0067
E-Mail: bldandrien@comcast.net

PRINCIPLE INVESTIGATOR for the FOLLOWING GRANTS:
Title: "Microflow APCI Method for High Throughput LC-MS"
Funding: NIH Phase I (National Center for Research Resources)
ID Grant #: 1 R 43 GMS6604-01; Awarded: $90,000.00
Support Period: 9/1/97-3/1/98
Description: To Develop a microflow APCI interface for mass spectrometry to improve the limits of detection for trace non-polar species in complex mixtures, while at the same time making on-line coupling to microbore chromatographic methods easier, more reproducible and robust.

Title: "MSD Mass Detector with Atmospheric Pressure Inlet"
Funding: NIH Phase I (National Center for Research Resources)
ID Grant #: 1 R 43 RR12058-01; Awarded: $90,000.00
Support Period: 9/1/97-9/1/98
Description: Develop a platform which will provide a high productivity, low cost entry point for both LC and CE users, as well as for GC-MS labs needing the ability to handle non-volatile samples without requiring time consuming derivitization procedures.

Title: "Mass Spectrometer Detector for Liquid Chromatography"
Funding NIH Phase I and II (national Center for Research Resources)
ID Grant #: 1 R 43 RR08538-01, 2 R 44 RR092252-02; Awarded: $50,000, $500,000
Support Period: 3/1/94-8/31/94, 4/1/95-3/31/97
Description: Develop a pre-production prototype API-TOF-MS bench top system for non mass spectrometrist. It should connect to any LC or CE system and ionizes the sample by APCI and ESI to analyze polar and non-polar samples.

Title: "Deconvolution of Mass Spectra for Multiply Charged Ions"
Funding: NIH Phase I (National Center for Research Resources)
ID Grant #: 1 R 43 RR08538-01; Awarded: $50,000.00
Support Period: 9/30/92-3/29/93
Description: To Develop a 3D software package that will take full advantage of the coherent pattern for determining the mass of higher molecular weight compounds from a series of multiply charged peaks produced by any multiply charged ionization process.

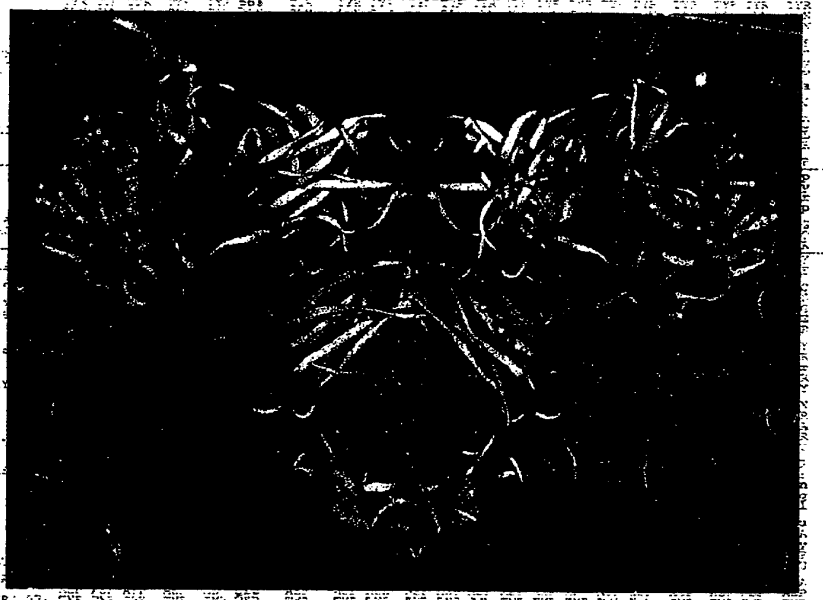
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Exhibit B

SEQUENCES OF PROTEINS OF IMMUNOLOGICAL INTEREST

VOL I

FIFTH EDITION



1991

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
National Institutes of Health

SEQUENCES OF PROTEINS OF IMMUNOLOGICAL INTEREST

FIFTH EDITION

Tabulation and Analysis of
Amino Acid and Nucleic Acid Sequences of Precursors,
V-Regions, C-Regions, J-Chain, T-Cell Receptors for Antigen,
T-Cell Surface Antigens, β_2-Microglobulins,
Major Histocompatibility Antigens, Thy-1, Complement,
C-Reactive Protein, Thymopoietin, Integrins, Post-gamma Globulin,
α_2-Macroglobulins, and Other Related Proteins

1991

Elvin A. Kabat, Tai Te Wu*, Harold M. Perry†,*
Kay S. Gottesman‡, and Carl Foeller†

*Depts. of Microbiology, Genetics and Development, and Neurology, Cancer Center/Institute of Cancer Research, College of Physicians and Surgeons, Columbia University, New York, NY 10032 and the National Institute of Allergy and Infectious Diseases, and the Office of the Director National Institutes of Health, Bethesda, MD 20892.

*Depts. of Biochemistry, Molecular Biology, and Cell Biology, and Engineering Sciences and Applied Mathematics and Biomedical Engineering, Northwestern University, Evanston, IL 60208 and the Cancer Center, Northwestern University Medical School, Chicago, IL 60611

†BBN Systems and Technologies, 10 Moulton Street, Cambridge, MA 02138

‡Formerly with BBN. Present address Laboratory for Applied Research in Academic Information, William H. Welch Medical Library, The Johns Hopkins University, Baltimore, Md 21205

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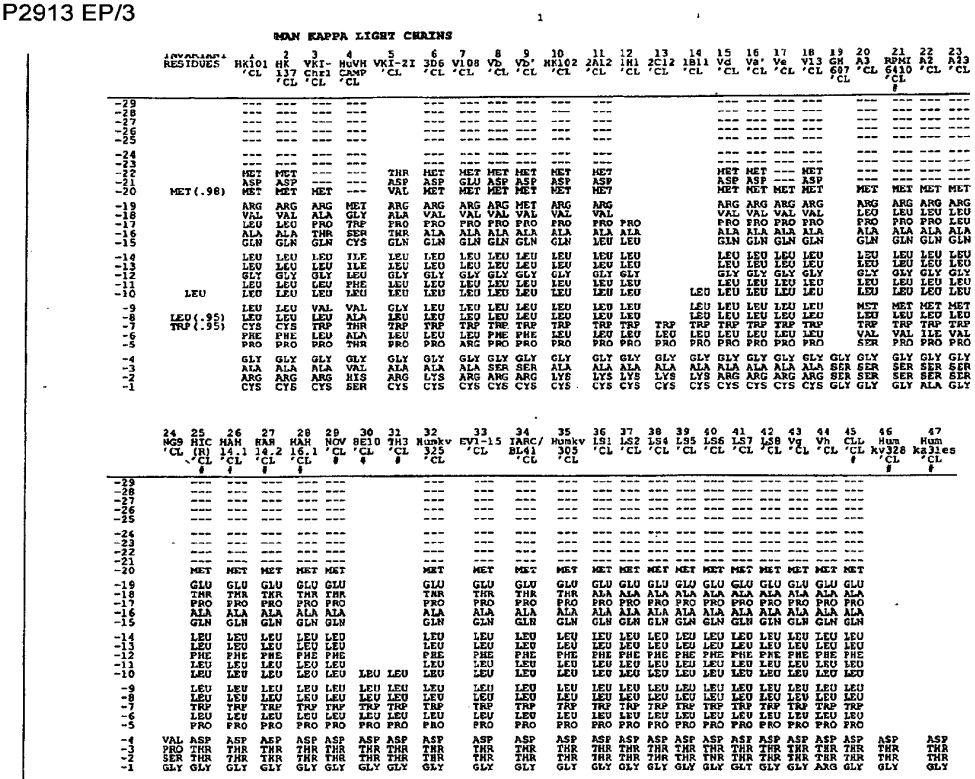
U.S. DEPARTMENT OF HEALTH
AND HUMAN SERVICES

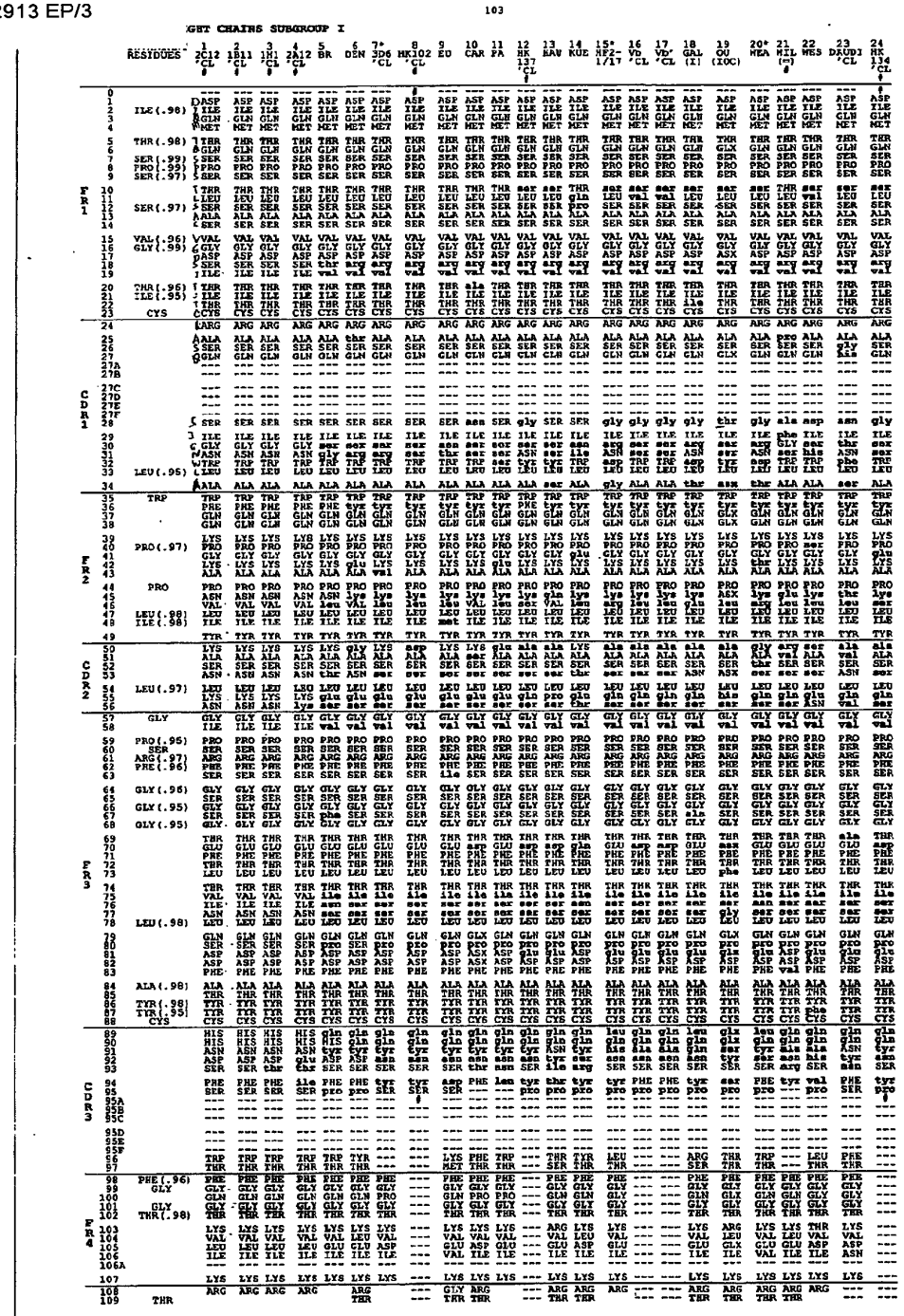
Public Health Service
National Institutes of Health

NIH Publication No. 91-3242

Samsung Bioepis Exhibit No. 1002
Page 2888

Exhibit D




GET CHAINS SUBGROUP I
RESIDUES
1 2C12 'CL
2 1B11 'CL
3 1H1 'CL
4 2A12 'CL
5 BR
6 DEN
7* 3D6 'CL
8 HK102 'CL
9 EU
10 CAR
11 FA
12 HK 137 'CL
13 RAV
14 KUE
15* MF2-1/17
16 VD 'CL
17 VD' 'CL
18 GAL (I)
19 OU (IOC)
20* MEA
21 MIL (=7)
22 WE3
23 DAUD 'CL
24 HK 134 'CL
FR1
CDR1
FR2
CDR2
FR3
CDR3
FR4

HUMAN KAPPA LIGHT CHAINS SUBGROUP I (cont'd)

		25 Va 'CL	26 WALKER 'CL	27 HIL01 'CL	28 Va 'CL	29 VRI-CHEJ 'CL	30 AMYLOID BAN	31 Va 'CL	32 VJ1 'CL	33 DEE	34 WIL (-)	35 AMO	36 V108 'CL	37 VRI-II 'CL	38 REI	39 AU	40 ROY	41 SI	42 HOVH CAMP 'CL	43 AG	44 SCW	45 REV	46 KA	47 LAY	48 SEL	49 NI
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	1	ASP	ASP	ASP	ala	ASP	ASP	ala	val	ASX	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP
	2	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	val	ILE	ILE	ILE	ILE	ILE
	3	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	4	leu	MET	MET	leu	MET	leu	MET	MET	MET	MET	MET	val	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	leu	MET
	5	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR
	6	GLN	GLU	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	7	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	8	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
	9	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
FR1	10	the	ser	ser	ser	ser	ser	ser	leu	ser	THR	ser	ser	ser	ser	ser	ser	pro	ser	ser	ser	ser	THR	ser	ser	ser	ser
	11	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU
	12	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	13	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	val	ALA	ALA	ALA
	14	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	thr
	15	VAL	VAL	VAL	VAL	VAL	VAL	thr	thr	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL
	16	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY
	17	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	gly	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP
	18	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	SER	arg	arg	arg	arg	arg	arg	arg	arg	arg
	19	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val
	20	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	ile	ile	THR	THR	THR	THR	THR
	21	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	phe	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	leu
	22	THR	THR	THR	THR	THR	THR	THR	ser	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	leu
	23	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS
	24	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	gln	gln	gln	gln	lys	gln	gln	ARG	glu	gln	glu	gln	glu
	25	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ser	ALA	val	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA
	26	SER	SER	asp	SER	SER	SER	SER	SER	gly	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	27	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	27A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	ser
	27B	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	val
CDR1	27C	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	leu
	27D	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	gln
	27E	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	ile
	27F	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	ala
	28	gly	SER	gly	gly	gly	SER	gly	gly	SER	SER	SER	gly	gly	asp	asp	asp	asp	asn	asp	asp	SER	thr	asn	asn	SER
	29	ILE	ILE	ILE	ILE	ILE	val	ILE	ILE	val	val	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ser	val	ILE	gly
	30	ser	ser	ser	ser	ser	ser	ser	ser	asn	ser	GLY	ser	ser	ile	ser	ser	asn	asp	asn	val	leu	asn	ser	ser
	31	ser	ASN	ser	ser	ASN	ASN	ser	ser	lys	tyr	gly	ASN	ASN	lys	asp	ile	ASN	lys	asn	val	ser	ser	gln	lys	ASN
	32	tyr	tyr	TRP	ala	ser	tyr	tyr	tyr	TRP	ser	gly	tyr	ser	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	ser	ile	phe	thr
	33	LEU	LEU	LEU	LEU	LEU	val	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU
	34	ALA	asn	ALA	ALA	asn	ALA	ALA	asn	ALA	asn	asn	asn	asn	asn	asn	asn	asn	asn	asn	asn	asn	asn	asn	asn	asn	ALA
	35	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP
	36	tyr	tyr	tyr	tyr	tyr	PHE	tyr	tyr	tyr	tyr	tyr	tyr	ser	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr
	37	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	38	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	39	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	the	LYS	LYS	LYS	LYS	LYS	$	LYS	LYS	LYS	LYS	LYS	LYS
	40	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	$	PRO	PRO	PRO	PRO	PRO	PRO
	41	GLY	GLY	glu	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	$	GLY	GLY	GLY	GLY	GLY	lys
	42	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	$	LYS	LYS	LYS	LYS	lys	LYS
	43	ALA	ALA	ALA	ALA	ALA	the	ALA	ALA	ALA	ALA	ALA	ALA	ALA	the	ALA	ALA	ALA	ALA	ALA	$	ALA	ALA	ALA	ALA	pro	ALA
	44	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
	45	lys	lys	lys	leu	lys	lys	lys	glu	lys	ASN	ser	thr	lys	lys	lys	lys	lys	lys	ser	lys	lys	lys	lys	glu	lys	glu
	46	leu	leu	ser	leu	leu	ser	leu	leu	VAL	leu	leu	leu	leu	leu	leu	leu	leu	phe	leu	leu	leu	leu	leu	leu	leu	leu
	47	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU
	48	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE
	49	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR		TYR	phe	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	phe	TYR	TYR	TYR	TYR
	50	ala	ala	ala	asp	ala	asp	ala	ala	ala	glu	gly	ala	ala	glu	asp	asp	asp	asn	asp	asp	ala	ala	gly	asp	asp	asp
	51	ASN	ASN	ASN	ASN	ASN	ASN	ASN	ASN	ASN	ASN	ASN	ASN	ASN	ASN	ASN	ASN	ASN	thr	ASN	ASN	ASN	ASN	ASN	ASN	ASN	ASN
CDR2	52	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	glu	SER	SER	SER	SER	SER	SER	SER	SER
	53	thr	ser	ser	ser	ser	thr	thr	thr	ser	ile	ser	ser	ser	ASN	ASN	lys	ASN	ASN	ASN	chr	ASN	ser	thr	thr	thr	ASN
	54	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	asp	LEU	LEU	LEU
	55	gln	gln	gln	gln	gln	gln	gln	gln	lys	asp	arg	gln	gln	gln	glu	glu	glu	gln	glu	glu	gln	gln	glu	lys	glu	glu
	56	ser	ser	ser	ser	ser	ser	ser	ser	ser	ile	ser	ser	ser	ser	ala	ile	phe	ser	ile	phe	ser	ser	thr	thr	thr	phe
	57	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY
	58	PRO	thr	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
	59	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	60	ARG	ARG	ARG	ARG	ARG	asn	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG
	61	PHE	PHE	PHE	SER	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE
	62	SER	SER	SER	SER	SER	thr	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	63	GLY	GLY	GLY	GLY	asp	GLY	GLY	GLY	GLY	GLY	GLY	GLY	asp	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY
	64	SER	SER	SER	SER	SER	SER	SER	SER	SER	ago	SER	SER	SER	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY
	65	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLN	GLY	ile	GLY	GLY	GLY	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	66	SER	SER	SER	SER	SER	SER	SER	SER	GLY	SER	SER	SER	SER	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY
	67	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	68	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLN	GLN
	69	THR	THR	THR	THR	ala	THR	THR	THR	THR	THR	THR	THR	ala	THR	ala	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR
	70	GLU	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	bis	asp	asp	asp	asp	asp	asn	asp	bis	asp	asp	asp
	71	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	tyr	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE
	72	THR	THR	THR	THR	ile	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	ala	THR	THR	THR	THR	THR	THR	THR	THR
FR3	73	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	phe	phe	phe	LEU	phe	phe	LEU	LEU	phe	phe	LEU	phe
	74	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	ser	THR	THR	THR	THR	THR	THR	THR	THR
	75	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ser	ile	ile	ile	ile	ile	ile	ile	ile
	76	ser	ser	ser	ser	arg	ser	ser	ser	ser	ser	thr	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser
	77	ser	ser	ser	ser	ser	ser	cys	cys	gly	ser	ser	ser	ser	ser	ser	gly	ser	gly	thr	ser	ser	ser	ser	ser	ser	gly
	78	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU
	79	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	leu	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	80	pro	pro	pro	pro	pro	pro	SER	SER	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro
	81	glu	glu	glu	glu	glu	glu	glu	glu	glu	ASP	glu	glu	glu	glu	glu	glu	glu	glu	glu	glu	ASP	glu	glu	ala	glu	glu
	82	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	asn	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASX	ASP	ASP	ASP	ASP	ASN
	83	PHE	ser	PHE	PHE	PHE	PHE	PHE	PHE	PHE	ser	PHE	val	PHE	ile	ile	ile	PHE	ile	ile	ile	PHE	PHE	ile	PHE	PHE	PHE
	84	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	gly	ALA	ALA	ALA	ALA	ALA	ALA
	85	THR	THR	THR	THR	THR	THR	THR	THR	THR	asp	ala	THR	THR	THR	THR	THR	THR	THR	THR	ser	THR	THR	THR	THR	THR	val
	86	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR
	87	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	phe	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR
	88	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS
	89	gln	gln	gln	gln	gln	gln	gln	gln	glx	gln	gln	leu	gln	gln	gln	gln	gln	leu	gln	gln	gln	gln	gln	gln	gln	gln
	90	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln
	91	leu	ser	tyr	phe	ser	tyr	tyr	tyr	ser	tyr	ser	asp	ser	tyr	tyr	phe	tyr	ile	tyr	tyr	ser	tyr	tyr	tyr	tyr	tyr
	92	ASN	tyr	ASN	ASN	ASP	ASN	ASN	ASN	ASN	ASN	ASN	tyr	ASN	ASP	ASP	ASP	tyr	ile	ASP	ASP	tyr	leu	asn	ASX	ASP	ASP
	93	SER	tyr	SER	SER	SER	SER	SER	SER	SER	SER	SER	tyr	SER	SER	SER	SER	SER	SER	SER	SER	tyr	leu	asn	SER	thr	asp
CDR3	94	tyr	thr	tyr	tyr	thr	tyr	tyr	PHE	thr	tyr	ser	thr	thr	leu	len	len	len	arg	leu	val	asn	leu	trp	PHE	leu	
	95	pro	leu	pro	pro	pro	pro	pro	pro	pro	---	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	
	95A	---	---		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	GLU	---	---	---	---	
	95B	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
	95C	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
	95D	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
	95E	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
	95F	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
	96	---	ILE	---	---	---	TYR	---	---	TYR	---	TRP	---	---	TYR	TRP	LEU	TYR	ARG	ARG	ILE	VAL	ARG	PRO	LEU	SER	
	97	---	THR	---	---	---	THR	---	---	THR	---	THR	---	---	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	
	98	---	PRO	---	---	---	PRO	---	---	PRO	PRO	PRO	---	---	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	
	99	---	GLY	---	---	---	GLY	---	---	GLY	GLY	GLY	---	---	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	
	100	---	GLN	---	---	---	GLN	---	---	PRO	GLN	GLN	---	---	GLN	GLN	GLY	GLN	GLN	GLN	GLN	GLY	GLN	GLN	GLY	VAL	
	101	---	GLY	---	---	---	GLY	---	---	GLY	GLY	GLY	---	---	GLY	GLY	SER	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	
	102	---	THR	---	---	---	THR	---	---	THR	THR	THR	---	---	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	
FR4	103	---	ARG	---	---	---	LYS	---	---	LYS	LYS	LYS	---	---	LYS	LYS	LYS	LYS	LYS	LYS	ARG	THR	LYS	LYS	GLU	LYS	
	104	---	LEU	---	---	---	$	---	---	VAL	LEU	VAL	---	---	LEU	VAL	VAL	LEU	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	
	105	---	ILE	---	---	---	$	---	---	GLU	GLU	GLU	---	---	GLN	GLU	ASP	GLU	GLU	GLU	GLU	ASP	ASP	GLU	GLU	GLU	
	106	---	ILE	---	---	---	ILE	---	---	MET	ILE	ILE	---	---	ILE	ILE	PHE	ILE	ILE	ILE	ASN	ILE	LEU	VAL	VAL	SER	
	106A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
	107	---	LYS	---	---	---	LYS	---	---	THR	LYS	ARG	---	---	THR	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	
	108	---	---	---	---	---	ARG	---	---	ARG	ARG		---	---	ARG	ARG	ARG	ARG	ARG	ARG	GLY	ARG	ARG	ARG		ARG	
	109	---	---	---	---	---	---	---	---	THR	THR		---	---	THR	THR	THR	THR		IRR	THR		THR				THR

HUMAN KAPPA LIGHT CHAINS SUBGROUP I (cont'd)

		50 RI	51* Ku RSV19 VK #	52 HBJ 4	53 BJ 26 #	54 BJ 19 #	55 RFZ	56 HA	57 PAU	58 PSM	59 JBL #	60 NE	61 FRA	62 BJ 40	63 MF3- 1C/6	64 MF2, 1/13B	65 MF2, 18/2	66 GR'	67 GI 'CL #	68 NOM	69 KER	70 ESM IGG	71 ESM IGM	72* DAV	73* FIN	74 PW #	75 SHE	76 ADA
	0	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---		---	---	---	---	---	---	---	---	---
	1	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP		ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP
	2	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE		ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE
	3	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN		GLN	GLN	GLN	GLN	GLN	GLN	his	GLN	GLN
	4	MET	leu	MET	MET	leu	MET	MET	MET	MET	MET	leu	MET	MET	MET	MET	MET	MET		MET	MET	MET	MET	MET	MET	leu	val	val
	5	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR		THR	THR	THR	THR	THR	THR	THR	THR	THR
	6	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLN	GLN	GLN	GLN		GLX	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	7	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	SER	SER	SER	SER	SER	SER	SER
	8	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO		PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
	9	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	SER	SER	SER	SER	SER	thr	SER
F	10	ser	ser	THR	ser	ser	ser	ser	THR	ser	ser	ser	THR	ser	ser	ser	ser	THR		ser	ser	ser	ser	ser	ser	ser	ser	ser
R	11	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU
1	12	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	SER	SER	SER	SER	SER	SER	SER
	13	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA		ALA	ALA	ALA	ALA	thr	thr	ALA	ALA	val
	14	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	tyr	SER	SER	SER	SER	SER	SER		SER	SER	SER	SER	val	val	SER	SER	phe
	15	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL		VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL
	16	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY		GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY
	17	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASX	ASP	ASP	ASP	ASP	ASP	ASP		ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASX	ASP
	18	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg		arg	arg	arg	arg	arg	arg	arg	arg	arg
	19	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val		val	ILE	val	val	val	val	val	val	val
	20	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR		THR	THR	THR	THR	THR	THR	THR	THR	THR
	21	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE		ILE	ILE	ILE	ILE	ILE	ILE	val	phe	ILE
	22	THR	THR	THR	THR	THR	THR	THR	THR	THR	ala	THR	THR	THR	THR	THR	THR	THR		THR	THR	THR	THR	THR	thr	arg	THR	THR
	23	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS		CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS
	24	gln	ARG	ARG	gln	gln	gln	gln	ARG	gln	ARG	glx	ARG		ARG	ARG	ARG	ARG		gln	gln	ARG	ARG	asp	asp	ARG	glx	glx
	25	ALA	ser	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA		ALA	ALA	ALA	ALA		ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA
	26	asn	SER	SER	SER	SER	asn	SER	SER	SER	SER	SER	SER		SER	SER	SER	SER		arg	SER	SER	SER	SER	SER	SER	SER	SER
	27	GLN	GLN	GLN	GLN	GLN	GLN	---	GLN	GLN	GLN	GLX	GLX		GLN	GLN	GLN	arg		his	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	27A	---	thr	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---	---
	27B	---	leu	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---	---
C	27C	---	val	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---	---
D	27D	---	ile	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---	---
R	27E	---	thr	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---	---
1	27F	---	---	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---	---
	28	val	asp	SER	SER	asp	val	---	SER	asp	SER	asn	asn		gly	gly	gly	SER		asp	asp	SER	SER	asn	asn	SER	asn	asn
	29	ILE	gly	val	ILE	ILE	ILE	---	ILE	ILE	ILE	ILE	ILE		ILE	ILE	ILE	ILE		ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE
	30	ser	asn	asn	asn	asn	ser	arg	ser	arg	ser	ser	asn		asn	arg	arg	ser		ser	lys	ser	ser	ser	ser	GLY	ser	
	31	lys	thr	ASN	lys	thr	lys	ASN	ser	ser	thr	ASX	ASX		ASN	ASN	ASN	ser		ASN		ser	ser	ser	ser		ASX	ASX
	32	ser	tyr	TRP	tyr	phe	ser	tyr	ser	ser	tyr	tyr	tyr		asp	asp	asp	phe		tyr		tyr	tyr	TRP	TRP	tyr	tyr	tyr
	33	LEU	LEU	LEU	ala	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU		LEU		LEU	LEU	LEU	LEU	ser	LEU	LEU
	34	asn	glu	ALA	asn	asn	asn	asn	ALA	asn	ser	asx	ALA		gly	gly	gly	ALA		asn		asx	asx	ile	ile	asx	asx	asx
	35	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP		TRP	TRP	TRP	TRP		TRP		TRP	TRP	TRP	TRP	TRP	TRP	TRP
	36	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr		tyr	tyr	tyr	tyr		PHE		tyr	tyr	tyr	tyr		tyr	tyr
	37	GLN	GLN	GLN	GLN	glu	GLN	GLN	GLN	GLX	GLN	GLN	GLX		GLN	GLN	GLN	GLN		GLN		GLX	GLX	GLN	GLN	GLN	Ala	GLY
	38	GLN	GLN	glu	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLX	GLX		GLN	GLN	GLN	gly		GLN				GLN	GLN	GLN	GLX	tyr
	39	arg	LYS	LYS	#	#	arg	LYS	LYS	LYS	LYS	LYS	LYS		LYS	LYS	LYS	arg		LYS				tyr	tyr	LYS	LYS	LYS
	40	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	gln	PRO	PRO	PRO		PRO	PRO	PRO	PRO		PRO				PRO	PRO		PRO	PRO
F	41	GLY	GLY	GLY	#	#	GLY	GLY	GLY	GLY	GLY	GLY	GLY							GLY								
R	42	gln	LYS	glx	LYS	LYS	gln	arg	glx	LYS	LYS	glx																
2	43	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA		ALA														
	44	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO		PRO														
	45	lys	lys	lys	lys	lys	lys	lys	lys	lys	lys	lys		lys														
	46	leu	leu	leu	leu	leu	leu	leu	leu	leu	leu	leu																
	47	LEU	LEU	LEU		LEU	LEU	LEU	LEU	LEU	LEU	LEU		met														
	48	ILE	ILE	ILE		ILE	ILE	ILE	ILE	ILE	ILE			ILE														
	49	TYR	TYR	asx		TYR	TYR	TYR	TYR	TYR	TYR	TYR		gly														
	50	asp	arg	LYS		asp	asp	asp	glx	asp				asp														
C	51	ile	val	thr	asp	ALA	ALA	ALA		SER				ALA														
D	52	SER	SER	SER	SER	SER	SER	phe						SER														
R	53	ASN	ASN	ser	arg	ser	ASX	ASN						asx														
2	54	LEU	arg	LEU	LEU	LEU	LEU	LEU		LEU				LEU														
	55	glu	phe	glu	glu	glu	glu	glu		glu				asx														
	56	ser	ser	arg	thr	thr		ala						ser														
	57	GLY	GLY	GLY	GLY	GLY	GLY	GLY						GLY														
	58	val	val	asx	val	val		val						val					ILE									
	59	PRO	PRO	PRO	PRO	gln	PRO	PRO						val					PRO									
	60	SER	SER	SER	SER	SER		SER						SER					SER									
	61	ARG	ARG	ARG	lys	ARG	ARG	ARG						ARG					ARG									
	62	PHE	PHE	PHE	val	PHE	PHE							gln					PHE									
	63	thr	SER	ala	SER	leu	thr							SER					SER									
	64	GLY	GLY	GLY	GLY	GLY	GLY							GLY					GLY									
	65	SER	SER	SER	SER	SER								SER					SER									
	66	GLY	GLY	GLY	asx	GLY								GLY					GLY									
	67	SER	SER		SER	phe								SER					SER									
	68	GLY	GLY		glu	asx								GLY					GLY									
	69	THR	THR		THR	THR								THR					THR									
	70	asp	asp		asp	lys								asp					GLU									
	71	PHE	PHE		val									pro					PHE									
F	72	phe	THR		THR									ser					THR									
R	73	LEU	phe		val									LEU					LEU									
3	74	THR	THR		asn									gln					THR									
	75	ile	ile		gln									VAL					VAL									
	76	ser	ser		ser									ser					ILE									
	77	ser	ser		ser									arg					ASN									
	78	LEU	LEU		LEU														LEU									
	79	arg	GLN		GLN														GLN									
	80	pro	pro		pro														SER									
	81	gln	glu		glu														ASP									
	82	ASP	ASP		ASP														ASP									
	83	val	ile		ile														PHE									
	84	ALA	ALA		ALA														ALA									
	85	THR	THR		pro														THR									
	86	TYR	TYR		lys														TYR		TYR							
	87	phe	TYR																TYR		TYR							
	88	CYS	CYS																CYS		CYS							
	89	gln	phe		gln														HIS		gln							
	90	GLN	gln		gln														HIS		GLN							
	91	tyr	gly		arg	phe								gly					ASN		tyr							
	92	ile	ser		ASP	ASP								glu					ASP		asp							
	93	asn	ile		asp	asp								SER					thr		asp							
C	94	leu	leu		leu	leu								asp					PHE		leu							
D	95	pro	pro		pro	pro								SER					SER		pro							
R	95A	---	---		---	---								---					---		---							
3	95B	---	---		---	---								---					---		---							
	95C	---	---		---	---								---					---		---							
	95D	---	---		---	---								---					---		---							
	95E	---	---		---	---								---					---		---							
	95F	---	---		---	---								---					---		---							
	96	LEU	ARG		TYR	TYR								ARG					TRP		PRO							
	97	THR	THR		THR	THR								THR					THR		THR							
	98	PHE	PHE		LEU	PHE								PHE					PHE		PHE							
	99	GLY	GLY		GLY	GLY								GLY					GLY		GLY							
	100	GLY	GLY		GLY	PRO								GLN					GLN		PRO							
	101	GLY	GLY		GLY	GLY								GLY					GLY		GLY							
	102	THR	THR		THR	THR								THR					THR		THR							
F	103	LYS	LYS		LYS	LYS								LYS					LYS		LYS							
R	104	VAL	VAL		VAL	VAL								VAL					VAL		VAL							
4	105	GLU	GLU		GLU	GLU								GLU					LEU		LEU							
	106	ILE	ILE		MET	LEU								VAL					ILE		LEU							
	106A	---			---	---								---					---		---							
	107	LYS			LYS	LYS								LYS					LYS		LYS							
	108																		ARG									
	109																											

HUMAN KAPPA LIGHT CHAINS SUBGROUP I (cont'd)

		77 AMYLOID VIII-B	78 PAUL	79 RI #	80 WAT	81* LOW	82 AMYLOID ES305 #	83 OCO	84 BOL	85 AMYLOID X	86 SAC	87 BJ	88 GO	89 F- GUI #	90 S- GUI #	91 HF6- 21/28	92 DIE	93 CAR A	94 TEI	95 MON	96 ALE	97 JOH	98 POT	99 CON	100 TRA	101* R-G2b
	0	---	---	pca	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	1	ASP	ASP	ASP	ASP	ASP	ASP	ASX	ASP	ASP	ASP	ASX	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASX	ASP	ASP	ASP	ASP	ASP
	2	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE		val	ILE	ILE	val	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE
	3	GLN	GLN	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLN		GLN	GLN	GLN	val	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLN	GLN	GLN
	4	MET	MET	MET	MET	MET	leu	MET	ile	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET
	5	THR	THR	THR	THR	THR	THR	THR	met	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR
	6	GLN	GLN	GLN	GLN	GLN	GLN	GLX	GLX	GLN	GLN	GLN	GLX	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLN	GLN	GLN
	7	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	ala	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	8	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	ala	PRO		PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
	9	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	SER	leu	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
F	10	ser	THR	THR	ser	ser	ser	ser	ser	ser	ser		phe	ser	ser	ser	ser	ser	ser	THR		ser	ser	ser	ser	ser
R	11	LEU	LEU	LEU	LEU	LEU	LEU	LEU	val	LEU	LEU		val	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU
1	12	SER	SER	SER	SER	SER	SER	val	SER	SER	SER		SER	SER	SER	pro	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	13	ALA	ALA	ALA	ALA	ALA	ALA	met	ala	ALA	ALA		ALA	ALA	ALA	val	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA
	14	SER	SER	SER	SER	SER	SER	pro	SER	SER	SER		SER	SER	SER	thr	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	15	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL		VAL	VAL	VAL	leu	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL
	16	GLY	GLY	GLY	GLY	GLY	CLY	GLY	GLY	GLY	GLY		GLY	CLY	GLY	GLX	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY
	17	ASP	ASP	ASP	ASP	ASP	ASP	ASX	ASX	ASP	ASP		ASX	ASX	ASX	gly	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP
	18	arg	arg	gly	arg	arg	leu	arg	arg	arg	---	arg	arg	arg	arg	pro	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg
	19	val	val	val	val	val	val	val	val	val	---	val	val	val	val	ala	val	val	val	val	val	val	val	val	val	val
	20	THR	THR	THR	THR	THR	THR	THR	THR	ile	---	THR	THR	THR	THR	ser	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR
	21	ILE	ILE	ILE	ILE	ILE	?	ILE	ILE	ILE	---	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	leu	ILE	ILE	ILE	ILE	ILE
	22	THR	THR	ser	THR	THR	THR	THR	THR		---	THR	THR	THR	THR	ser	THR	THR	THR	THR	ser	THR	THR	THR	ILE	THR
	23	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	---	CYS	CYS	CYS	CYS	CYS	CYS	CY3	CYS		CYS	CYS	CYS			
	24	gln	ARG	ARG	glx	ala	gln	glx	ARG	glx	---	gln	ARG		ARG	ARG					ARG					
	25	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	---	ALA	ALA	ALA	ALA	ser										
	26	SER	SER	SER	SER	SER	SER	SER	SER		---	SER	SER			SER										
	27	GLN	GLN	GLX	GLN	GLN	glu	GLX	GLX	GLX	---	GLN				GLN										
	27A	---	---	---	---	---	---	---	---	---	---	---				ser										
	27B	---	---	---	---	---	---	---	---	---	---	---				leu										
	27C	---	---	---	---	---	---	---	---	---	---	---				val										
C	27D	---	---	---	---	---	---	---	---	---	---	---				leu										
D	27E	---	---	---	---	---	---	---	---	---	---	---				ser										
R	27F	---	---	---	---	---	---	---	---	---	---	---				---										
1	28	asx	SER	SER	asp	asx	ala	asx	gly	asn	---	asp				asp										
	29	ILE	ILE	ILE	ILE	ILE	?	ILE	ILE	ILE	---	ILE				gly										
	30	GLY	ser	ser	ser	ser		ser			---	asn				asp										
	31		asp		tyr		ASN	ASX		pro	---	lys				ser										
	32	tyr	tyr		val		tyr	tyr		tyr	---					tyr										
	33	LEU	val				LEU			LEU	---					LEU										
	34		asn								---					glu										
	35	TRP	TRP		TRP		TRP				---					TRP										
	36						tyr			tyr	---					tyr										
	37										---					GLN										
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	88										CYS		CYS													
	89										glx		gln													
	90										gln		gln													
	91										glx		tyr													
	92										ASX		gln													
	93										ala		asn													
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HUMAN KAPPA LIGHT CHAINS SUBGROUP I (cont'd)

	102* K-G2	103 BRA	104 LUX	105* HEI	106 PAP	107* R-G2s	108 CRA	109 AMYLOID LEP	110* WAG	111 MOE	112 HBJ 1	113 AMYLOID 547	114 WEB	115 HBJ 10	116 LOO	117 BEN	118 MAA	119 GR	120 MUR	121 GM131 'CL 8	122 AMYLOID 394	123* MAR	124 CL*
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1	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP		ASP	ASP	---
2	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE		ILE	ILE	---
3	GLN	GLN	GLN	GLN	GLN		GLN	GLN	GLN	GLN	leu	GLX	GLN	GLN	GLN	GLN	GLN	GLN	GLN		GLN	GLN	---
4	MET	MET	leu	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET		MET	leu	MET	MET		MET	leu	---
5	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR		THR	THR	---
6	GLN	GLX	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLN	GLN	GLN	GLN	GLN		GLN	GLN	---
7	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	---
8	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO		PRO	PRO	---
9	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	thr	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	---
10	SER	SER	pro	THR	SER	SER	SER	SER	SER	THR	SER	SER	ser	SER	THR	ser	THR	ser	ser		SER	SER	---
11	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	val	LEU		LEU	LEU	LEU	LEU		LEU	LEU	---
12	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER				---
13	ALA	val	ALA	ALA	val	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	val					---
14	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER									---
15	VAL	VAL	VAL	VAL	VAL	VAL	leu	VAL	VAL	VAL	VAL	VAL	VAL		VAL								---
16	GLY	GLY	GLY	GLY	GLY	GLY	arg	GLY	GLY		GLY												---
17	ASP	gln	ASP	ASP	ASP	ASP	ASP	ASP	ASP		ASP												---
18	arg	arg	arg	arg	arg	arg	arg	arg															---
19	val	val	val	val	val	val	val	val															---
20	THR	THR	THR	THR	THR		THR	THR															---
21	ILE	ILE	ILE		ILE	ILE	ILE																---
22	THR	THR	THR		ala	THR	THR																---
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88																			CYS				---
89																			gln				---
90																			pro				---
91																			his				---
92																			ASP				ASP
93																			his				ile
94																			leu				PHE
95																			pro				pro
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96																			LEU				GLY
97																			THR				THR
98																			PHE				PHE
99																			GLY				GLY
100																			GLY				GLN
101																			GLY				GLY
102																			THR				THR
103																			THR				LYS
104																			VAL				VAL
105																			GLU				GLU
106																			MET				ILE
106A																			---				---
107																			LYS				LYS
108																			ARG				ARG
109																			THR				THR

HUMAN KAPPA LIGHT CHAINS SUBGROUP I (cont'd)

		125 AMYLOID	126 ABJ .6	127 AMYLOID MS #	128 BEN	# OF SEQUENCES	# OF AMINO ACIDS	OCCURRENCES OF MOST COMMON AMINO ACID	VARIABILITY
	0	---	---	---	---	1	1	1(PCA)	
	1	ASP	ASP	ASP	ASP	125	3,4	122(ASP),118(ASP)	3.1,4.2
	2	ILE	ILE	#	ILE	123	2	120(ILE)	2.
	3	glu	GLN	GLN	GLN	123	7	116(GLN),112(GLN)	7.4,7.7
	4	MET	MET	MET	MET	124	4	109(MET)	4.6
	5	pro	THR	THR		124	3	122(THR)	3.
	6	GLN	GLN			123	1,2	123(GLN),116(GLN)	1.,2.1
	7	SER				121	2	120(SER)	2.
	8	PRO				121	2	120(PRO)	2.
	9	SER				121	4	117(SER)	4.1
F	10	ser				120	5	89(SER)	6.7
FR	11	LEU				119	4	111(LEU)	4.5
1	12					118	3	113(SER)	3.1
	13					117	4	107(ALA)	4.4
	14					113	6	106(SER)	6.4
	15					113	3	109(VAL)	3.1
	16					109	3	108(GLY)	3.
	17					109	3,4	106(ASP),98(ASP)	3.1,4.6
	18					106	6	97(ARG)	6.6
	19					107	3	101(VAL)	3.2
	20					106	4	102(THR)	4.2
	21					104	4	99(ILE)	4.2
	22					104	6	95(THR)	6.6
	23					96	1	96(CYS)	1.
	24					88	6	57(ARG)	9.3
	25					88	6	82(ALA)	6.4
	26					85	4	79(SER)	4.3
	27					84	4	79(GLN),66(GLN)	4.3,5.1
	27A					3	2	2(SER)	
	27B					3	2	2(LEU)	
	27C					3	2	2(VAL)	
C	27D					3	3	1(+)	
DR	27E					2	2	1(+)	
R	27F								
1	28					84	7	29(SER)	20.
	29					83	5	72(ILE)	5.8
	30					81	11	41(SER)	22.
	31					79	12	27(ASN),24(SER)	35.,39.
	32					79	9	34(TYR)	21.
	33					77	4	73(LEU)	4.2
	34					73	7,8	30(ALA)	17.,19.
	35					76	1	76(TRP)	1.
	36					74	2	66(TYR)	2.2
	37					73	4	68(GLN),61(GLN)	4.3,4.8
	38					71	4	68(GLN),63(GLN)	4.2,4.5
	39					68	4	62(LYS)	4.8
	40					70	3	68(PRO)	3.1
	41					58	3	54(GLY)	3.2
F	42					60	6	49(LYS)	7.3
FR	43					61	4	57(ALA)	4.5
2	44					61	1	61(PRO)	1.
	45					61	7,8	46(LYS)	9.3,11.
	46					60	7	42(LEU)	10.
	47					59	2	58(LEU)	2.
	48					57	2	56(ILE)	2.
	49					59	4	55(TYR)	4.3
	50					59	9	12(ALA)	20.
	51					59	5	50(ALA)	5.9
C	52					58	6	53(SER)	6.6
DR	53					57	6,7	26(SER)	13.,15.
2	54					58	2	56(LEU)	2.1
	55					58	8,9	23(GLU)	19.,21.
	56					56	8	34(SER)	13.
	57					57	1	57(GLY)	1.
	58					57	3	50(VAL)	3.5
	59					57	4	54(PRO)	4.2
	60					57	1	57(SER)	1.
	61					58	3	56(ARG)	3.1
	62					57	3	55(PHE)	3.1
	63					57	6	50(SER)	6.8
	64					57	2	55(GLY)	2.1
	65					56	5	51(SER)	5.5
	66					57	4	54(GLY)	4.2
	67					55	3	51(SER)	3.2
	68					55	3	52(GLY)	3.2
	69					55	2	51(THR)	2.2
	70					55	5,6	33(ASP),31(ASP)	8.5,11.
	71					54	4	49(PHE)	4.4
F	72					54	5	50(THR)	5.4
FR	73					54	3	43(LEU)	3.8
3	74					54	4	51(THR)	4.2
	75					54	3	47(ILE)	3.4
	76					53	5	43(SER)	6.2
	77					54	6	40(SER)	8.1
	78					54	2	53(LEU)	2.
	79					54	3,4	52(GLN),48(GLN)	3.1,4.5
	80					54	2	46(PRO)	2.3
	81					54	3,5	37(GLU),34(GLU)	4.4,7.9
	82					54	2	53(ASP),50(ASP)	2.,2.2
	83					54	4	40(PRE)	5.4
	84					54	2	53(ALA)	2.
	85					54	6	48(THR)	6.7
	86					56	2	55(TYR)	2.
	87					55	2	52(TYR)	2.1
	88					56	1	56(CYS)	1.
	89					57	4,5	46(GLN),43(GLN)	5.,6.6
	90					57	2,3	51(GLN),46(GLN)	2.2,3.7
	91					58	11,12	20(TYR)	23.,25.
	92					60	9	20(ASN),19(+)	27.,28.
	93					60	9	29(SER)	19.
	94					60	12	15(LEU)	48.
C	95					56	4	44(PRO)	5.1
DR	95A					1	1	1(GLU)	
3	95B								
	95C								
	95D								
	95E								
	95F								
	96					44	12	10(TRP)	53.
	97					43	4	39(THR)	4.4
	98					46	3	44(PHE)	3.1
	99					46	1	46(GLY)	1.
	100					46	4,5	28(GLN),27(GLN)	6.6,8.5
	101					46	1	46(GLY)	1.
	102					46	2	45(THR)	2.
F	103					46	4	38(LYS)	4.8
FR	104					45	2	36(VAL)	2.5
4	105					45	4,5	29(GLU),28(GLU)	6.2,8.
	106					46	7	30(ILE)	11.
	106A								
	107					45	3	42(LYS)	3.2
	108					33	2	31(ARG)	2.1
	109					22	1	22(THR)	1.

Samsung Bioepis Exhibit No. 1002
Page 2895

IN THE MATTER OF
European Patent No. EP 2 359 834

DECLARATION OF DR. DINO MIANO

I, Dr. Dino Miano, hereby declare as follows:

1. I am Dr. Dino Miano of Alexion Pharmaceuticals, Inc., 100 College St, New Haven, Connecticut, USA. I hold a Ph.D. degree in Chemistry and Biochemistry from the University of Massachusetts, and a Bachelor of Science degree in Chemistry and Biology from the University of Hartford. I have been employed by Alexion Pharmaceuticals for nearly 20 years, and I currently hold the post of Executive Director, Global Analytical & Pharmaceutical Development. Prior to this I was employed by R.W. Johnson Pharmaceutical Research Institute in Raritan, New Jersey for approximately two years. During my employment at Alexion my responsibilities and work have focused on analytical sciences and protein characterization. I have significant expertise in formulation development and analysis, which includes designing, performing and overseeing experiments that assess the impact of protein concentration, formulation, and storage conditions on the integrity and stability of a protein over time. A copy of my resume is attached as Exhibit 1.

2. The commercial formulation of Soliris®(eculizumab) consists of a concentration of 10 mg/mL of eculizumab in a buffer of 10 mM sodium phosphate, 150 mM sodium chloride and 0.02% polysorbate 80 at pH 7.0. The commercial formulation is satisfactorily stable for 18 months when it is stored as drug substance (i.e., in bulk in the commercial formulation) and for an additional 30 months when it is stored as a drug product (i.e., in 30 mL vials as it is supplied to consumers) providing a total of 48 months of stability. (See March 16, 2007 BLA Approval Letter from U.S. FDA, attached as Exhibit 2 (indicating the drug substance is stable for 18 months and the drug product is stable for 24 months at 2-8°C), and May 8, 2009 Letter from U.S. FDA, attached as Exhibit 3 (extending the shelf-life of the drug product from 24 to 30 months at 2-8°C after supplemental stability data was obtained).)

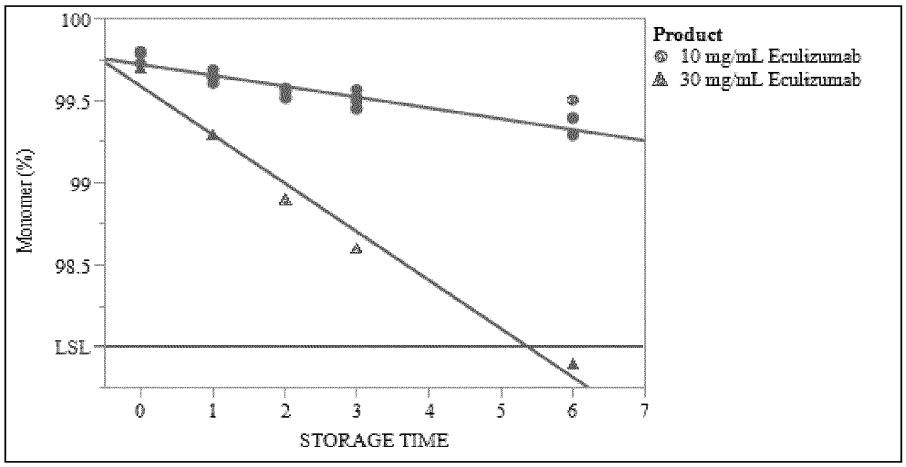
3. One of the stability parameters that the commercial formulation of eculizumab must meet in order to be deemed satisfactorily stable and thus suitable for release into the market is the percent of monomer antibody in the sample must be greater than or equal to 98.0%, which means that there must be less than or equal to 2.0% aggregates of the antibody, as determined via gel permeation high performance liquid chromatography. This release parameter for commercial lots of eculizumab ensures that the drug is satisfactorily stable for the designated storage period.

4. We generated a 30 mg/mL formulation of eculizumab (internally denoted "ALXN5100") that was identical to the commercial formulation of eculizumab, except that ALXN5100 has an eculizumab concentration of 30 mg/mL and the Soliris® commercial formulation has an eculizumab concentration of 10 mg/mL.

5. A batch of ALXN5100 was made under Good Manufacturing Practices (GMP) conditions, and stability studies on the batch were conducted by aliquotting samples into representative HyQ® CX5-14 Hyclone bags to simulate large-scale storage. The Hyclone bags were then stored horizontally at 2 to 8°C for at least 18 months and at 23 to 27°C for at least 6 months, which is how samples were stored for the stability program for the bulk drug substance for the commercial (10 mg/mL) formulation. Although the commercial formulation of eculizumab is to be stored at 2 to 8°C, when performing stability testing, researchers often store test samples at higher temperatures than commercial supplies are typically stored at, such as 23 to 27°C, because storage and stability testing at higher temperatures can provide a good indication of the stability of the formulation in a shorter test period of time. Gel Permeation High Performance Liquid Chromatography (GP-HPLC) was used to determine if there were any changes in monomer and aggregate drug at the two storage temperatures over time.

6. Figures 1 and 2 below demonstrate that the stability of eculizumab at 10 mg/mL is superior to that at 30 mg/mL. Figure 1 compares the percent monomer in the 10 mg/mL eculizumab composition versus the percent monomer in the 30 mg/mL eculizumab composition after storage at 23 to 27°C over six months. Figure 1 clearly shows that the percent monomer of eculizumab decreases (and thus aggregate formation increases) significantly more quickly in the 30 mg/mL eculizumab composition than in the 10 mg/mL eculizumab composition. In addition, whereas the 10 mg/mL eculizumab composition remains sufficiently stable over the specification ("LSL") monomer level of 98.0% over the entire six-month period, the 30 mg/mL eculizumab composition falls below that stability threshold during the same time period.

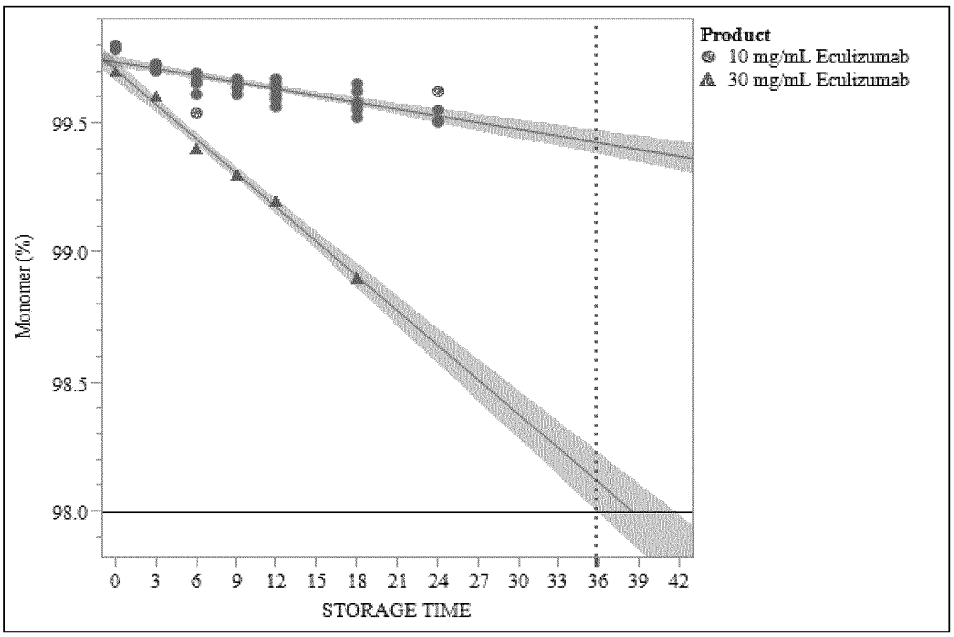
Figure 1. Percent monomer of eculizumab in 10 mg/mL eculizumab composition versus 30 mg/mL eculizumab composition over six months when stored at 23-27°C



Samsung Bioepis Exhibit No. 1002
Page 2897

7. Similar trends are seen between the 10 mg/mL eculizumab composition and the 30 mg/mL eculizumab composition even at storage temperatures of 2 to 8°C. Figure 2 compares the percent monomer in the 10 mg/mL eculizumab composition versus the percent monomer in the 30 mg/mL eculizumab composition after storage at 2 to 8°C over 18 months. Again, as with the 23 to 27°C storage conditions, the percent monomer remaining in the 30 mg/mL eculizumab samples decreased much more rapidly than the percent monomer remaining in the 10 mg/mL eculizumab samples at 2 to 8°C over the 18-month period. In addition, the model generated from this data predicted that the 30 mg/mL eculizumab composition would fall below the acceptable 98.0% monomer level before 36 months of storage.

Figure 2. Percent monomer of eculizumab in 10 mg/mL eculizumab composition versus 30 mg/mL eculizumab composition over 18 months when stored at 2-8°C



8. Thus, these long-term stability studies demonstrate a decrease in monomer and an increase in aggregate formation for ALXN5100, the 30 mg/mL eculizumab concentration, throughout the stability program. In all instances the aggregate formation in the 30 mg/mL concentration was greater than what was observed in the 10 mg/mL concentration. These results are clearly demonstrated through 18 months at 2 to 8°C and are even more pronounced within 6 months at the higher temperature studies. These results suggest that the commercial formulation is optimal for an eculizumab concentration of 10 mg/mL but is not suitable when the eculizumab concentration is increased to 30 mg/mL.

I believe and do hereby declare that the facts stated above are to the best of my knowledge true.

Dr. Dino Miano

17 Mar 2018
Date

Samsung Bioepis Exhibit No. 1002
Page 2899

Exhibit 1

Dino C. Miano, Ph. D.
217 Sunset Ridge
Rocky Hill, CT 06067
(860) 563-5433 (Home)
(860) 983-6178 (Mobile)
dinomiano@sbcglobal.net

QUALIFICATION PROFILE

- Results-oriented professional with eighteen years' successful track record of innovation, management, in depth scientific and regulatory knowledge in analytical and pharmaceutical development in the field of biotherapeutics. Experience encompasses various stages of biologics including early and late phase development, regulatory submission for marketing authorization and life cycle management.

- Well established division-level leader that consistently drives vision toward successful and timely completion of projects, while focusing on translating goals into actionable strategies that support the business

COMPETENCIES

- Pharmaceutical (biological) development
  - Formulation development
  - Fill finish
  - Stability monitoring and trending
  - Device development
  - Analytical support of clinical operations
- Regulatory Compliance
  - CMC development
- Analytical Development
  - Method Validation & Transfer
  - GMP Testing & Compliance
  - Implementing new analytical technologies
  - Analytical support of manufacturing
- Strategic leadership and innovation
  - Cross functional leader

Accomplishments and Qualifications

- Assumed a key analytical role in the clinical development and global commercialization of Soliris® and Strensiq®. Led development and validation activities for commercial release and stability test methods.
- Lead Global Operations efforts in delivering clinical material from early stage through commercial. Lead cross functional teams consisting of manufacturing, supply chain, QA and CMC and working with clinical groups to ensure supply demands are consistently met
- Lead Global Analytical and Pharmaceutical Development groups in providing analytical support to the entire Alexion organization, including analytical development, validation and transfer, GMP testing of clinical products, process development support, formulation and device development, product characterization and stability to allow for expiry determination.
- Implemented GMP quality infrastructure in the Analytical Sciences laboratories to ensure testing compliance with FDA, EU and PMDA regulations.
- Lead formulation development of new, existing and commercial products for IV, IVT and subcutaneous administration.

Samsung Bioepis Exhibit No. 1002
Page 2900

- Implement drug substance and drug product specifications for clinical products
- Generate analytical sections in support of all new and existing CMC submissions
- Lead the drug delivery and device development group in developing a strategy and business process to advance drug and device/combination products for existing commercial and clinical products.

Employment History

Alexion Pharmaceuticals, Inc., New Haven, CT

Executive Director, Global Analytical and Pharmaceutical Development **12/15-present**

- Lead global analytical support function for the entire organization for all commercial and clinical programs.
- Lead the global method technology transfers for Alexion's clinical and commercial products.
- Oversee compatibility assessments for materials used for delivery of product to patients during clinical studies.
- Represent Global Operations as lead for late stage process development, drug substance and drug product manufacturing, supply chain and CMC/IND submissions.
- Lead the device group in collaboration with internal and external partners for the development of delivery devices for Alexion products.

Senior Director, Analytical and Pharmaceutical Development **03/15-12/15**

- Led Early Stage Analytical and Product Characterization groups in supporting Research in efforts to move the best candidates into development.
- Led Analytical Sciences in method development, validation and transfer for new candidates and provide support to process development groups.
- Lead the stability program for all clinical products, including expiry determinations
- Led and aligned formulation development of new candidates with Clinical development and Target product profiles (TPP).
- Led Device Development and Drug Delivery group in identifying and integrating large volume bolus injector options for clinical and commercial development.
- Appointed CMC lead during Regulatory meetings with FDA, Health Canada, EMA and MFDS (F2F) to support CMC section of regulatory filings.

Director, Product Characterization **07/14-03/15**

- Led Global Operations Cross Functional Product Team in supporting second generation development of commercial product.
- Led device teams consisting of Clinical and Commercial Operations, Development, Regulatory and QA in evaluating large volume subcutaneous administration devices. Device included auto-injectors and larger wearables patch devices.
- Managed Mass Spectroscopy Characterization group. Involves the MS characterization of new and existing molecules to support CMC, Research and Development groups.
- Managed the Bio-Physical Characterization group. Involves directing the testing and collaboration of multiple groups to better understand new protein therapeutics
- Provided analytical support of new and existing CMC submissions and address inquiries from agencies

Director, Analytical Sciences **09/08-07/14**

- Managed the Operations group that provided GMP testing of clinical products and analytical testing to all development groups.
- Managed the Analytical Development group that provided method development, qualification, validation and transfer of analytical methods for new clinical products and existing commercial product to both QC and EU laboratories.
- Manage the Formulation Development group that identified stable formulations for new clinical products and determined stable subcutaneous formulations for both clinical and commercials products.
- Managed Stability program of clinical products that generated GMP stability protocols and reports, trended data and provided statistical analysis of trends to support expiry, and led deviations, LIR and OOS for GMP testing.
- Member of Specification committee to direct the generation or revision of specifications for clinical products

Associate Director, Analytical Sciences **05/03-08/08**

- Analytical Group
  - Provided analytical support of new and existing Soliris® CMC submissions
  - Supervised Analytical Operations laboratory in providing method development, qualification, validation, transfer and GMP testing to support clinical products.
- Purification Development group:
  - Managed PD studies in support of Process Validation. Studies included viral clearance, DNA clearance, process limits evaluation (PLE), resin re-use, and UF/DF membrane re-use.

Manager, Quality Control **08/99-05/03**

- Managed QC laboratories; maintained cGMP compliance with regards to current SOPs, validation/qualifications, and training.
- Coordinated studies, supervised investigations, generated reports and implemented systems with QA in response to OOS, invalid assays, aberrant trends/results.
- Represented area functions as required during audits by external agencies and internal QA department.

Senior Scientist, Quality Control **08/98-08/99**

- Developed and optimized RP-HPLC, SEC-HPLC, SDS-PAGE, and IEF methods for all protein based therapeutic product.

R.W. Johnson Pharmaceutical Research Institute, Raritan, NJ
A Johnson & Johnson Company

Scientist, Analytical Development **07/97-07/98**

- Developed HPLC assays for quantitation of becaplermin drug substance in alternative REGRANEX® formulations. Developed HPLC assays for determining content of preservatives in REGRANEX® formulations.

Scientist, Protein Development **01/97-07/97**

- Investigated modified roller bottle processes for the production of erythropoietin (rHuEPO), potentially increasing production/yields by 3-fold.
- Evaluated alternative purification processes for rHuEPO, involving HIC, IEC and Reverse phase chromatography.

Postdoctoral Research Scientist, Protein Development **10/95-01/97**

- Chemically modified rHuEPO using polyethylene glycol (PEG) to enhance in vivo erythropoiesis while prolonging circulatory half-life. Experiments were designed to specifically modify the carbohydrate (NANA) moieties of the glycoprotein with PEG while maintaining the native polypeptide conformation. Animal model analysis revealed successful prolonged in vivo biological activity.

University of Connecticut Health Center, Farmington, CT **01/89-08/90**

Research Scientist, Surgery Department

- Investigated/detected tissue derived inflammatory mediators released from injured corneas, urinary bladders and ischemic hearts. Modified Boyden chambers were employed to evaluate chemotactic response via neutrophil migration through 8 μm membranes.
- Investigated/synthesized cyclocreatine, a creatine analog, to determine inhibitory effects on the production and release of neutrophil chemotactic factors (NCF) in ischemic canines and rabbits myocardium.

EDUCATION

University of Massachusetts, Lowell, MA **09/90-10/95**

Ph. D. Chemistry/Biochemistry
Advisor: Dr. Edwin G.E. Jahngen
Thesis: "The Isolation and characterization of an ubiquitin-histone protein from *Dictyostelium discoideum AX3.*"

University of Hartford, Bloomfield, CT **09/85-05/89**

B.S. Chemistry/Biology
Research: "Corneal derived neutrophil chemotactic factors, intracellular synthesis and release."

PUBLICATIONS

Neill, A., Nowak, C., Patel, R., Ponniah, G., Gonzalez, N., **Miano, D**. and Liu, H., (2015) Characterization of recombinant monoclonal antibody charge variants using OFFGEL fractionator, weak anion exchange chromatography and mass spectrometry, *Analytical Chemistry*, **87**, 6204-6211

Ponniah, G., Nowak, C., Gonzalez, N., **Miano D**., and Liu, H., (2015) Detection and quantitation of low abundance oligosaccharides in recombinant monoclonal antibodies. *Analytical Chemistry*, **87**, 2718-2726

Peckham, N., Neil, F., and **Miano, D.C**., "Quantitative SDS-PAGE Analysis of a Recombinant Protein by Densitometry", IBC's Well Characterized Biologicals Poster Session, Washington D.C. (11/11/04)

Peckham, N., Bosley, K., and **Miano, D.C**., Adaptation of Complexometric Titration Method for Quantitation of EDTA in Biopharmaceutical Formulations to Microplate Format, IBC's Well Characterized Biologicals Poster Session, Washington D.C. (11/11/04).

Samsung Bioepis Exhibit No. 1002
Page 2903

Miano, D.C., Villalobos, A.P., Ghosh-Datidar, P., and Sharma, B. (1997), Chemical Modification of Recombinant Human Erythropoietin by Poly (ethylene glycol). Johnson and Johnson Chromatography Symposium, Princeton, NJ (abstract).

Miano, D.C. (1995) The Isolation and Characterization of a Ubiquitinylated Histone Protein in *Dictyostelium discoideum* AX3. Ph.D. Thesis, University of Massachusetts/Lowell.

Miano, D.C, and Jahngen, G.E. (1994) The Ubiquitinylation of Histones in *Dictyostelium discoideum* AX3 Chromatin May Be Linked to Replication, Transcription and Stress. Keystone Symposium, Tammarron, CO (abstract).

Elgebaly, S.A., **Miano, D.C.**, Ehler, W., Rahha,l R. and Elkerm, A. (1994) The Induction of Corneal Inflammation by Factors Derived From Hydrogen Peroxide Injured Corneas: Effect of Dexamethasone and Indomethacin. *Occ. Pharm.*, **10**, (1), 295-306.

Elgebaly, S.A., Allam, M.E., Houser, S., Hashmi, F., Forouhar, F. and **Miano, D.C.**, (1993) Cyclocreatine Inhibits Neutrophil Accumulation in the Myocardium of Canine Models in Coronary Artery Occlusion and Reperfusion. *J. Pharm. Exp. Ther.*, **266**, (3), 1670-1677.

Elgebaly, S.A., **Miano, D.C.**, Kreutzer, D.L. and Fishman, J.B., (1990) Cornea Derived Neutrophil Chemotactic Factors: Intracellular Synthesis and Release. *Curr. Eye Res.*, **9**, 839-845.

Elgebaly, S.A., **Miano, D.C.**, Ehler, W. and Rahhal, R. (1990) The Induction of Corneal Inflammation by Factors Derived From Hydrogen Peroxide Injured Corneas. ARVO, Sarasota, FL (abstract).

Elgebaly, S.A., **Miano, D.C.**, Kreutzer, D.L. and Fishman, J.B. (1989) Cornea Derived Neutrophil Chemotactic Factors. ARVO, Sarasota, FL (abstract).

Samsung Bioepis Exhibit No. 1002
Page 2904

Exhibit 2

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration
Rockville, MD 20857

Our STN: BL 125166/0

MAR 16 2007

Alexion Pharmaceuticals
Attention: Nancy Motola, Ph.D., RAC
Senior Vice President, Regulatory and Quality
352 Knotter Drive
Cheshire, CT 06410

Dear Dr. Motola:

We are issuing Department of Health and Human Services U.S. License No. 1743 to Alexion Pharmaceuticals, Inc., Cheshire, Connecticut, under the provisions of section 351(a) of the Public Health Service Act controlling the manufacture and sale of biological products. The license authorizes you to introduce or deliver for introduction into interstate commerce, those products for which your company has demonstrated compliance with establishment and product standards.

Under this license, you are approved to manufacture the product eculizumab, which is indicated for the treatment of paroxysmal nocturnal hemoglobinuria to reduce hemolysis.

You are approved to manufacture eculizumab drug substance at Lonza Biologics, PLC in Portsmouth, NH. The final formulated product will be manufactured, filled, labeled, and packaged at Ben Venue Laboratories, Bedford, Ohio. You may label your product with the proprietary name Soliris™ and will market it in 300 mg vials.

The final printed labeling (FPL) must be identical to the enclosed labeling. Marketing product with FPL that is not identical to the approved labeling text may render the product misbranded and an unapproved new drug.

The dating period for eculizumab drug product shall be 24 months from the date of manufacture when stored at 2-8°C. The date of manufacture shall be defined as the date of final sterile filtration of the formulated drug product. The dating period for your drug substance shall be 18 months when stored at 2-8°C.

You currently are not required to submit samples of future lots of eculizumab to the Center for Drug Evaluation and Research (CDER) for release by the Director, CDER, under 21 CFR 610.2. We will continue to monitor compliance with 21 CFR 610.1 requiring completion of tests for conformity with standards applicable to each product prior to release of each lot.

You must submit information to your biologics license application for our review and written approval under 21 CFR 601.12 for any changes in the manufacturing, testing, packaging or labeling of eculizumab, or in the manufacturing facilities.

We acknowledge your written commitments as described in your letter of February 22, 2007, March 12, 2007 and March 12, 2007 as outlined below:

Postmarketing Studies subject to reporting requirements of 21 CFR 601.70.

1. To evaluate long-term safety of eculizumab by analyzing outcomes in the Soliris Safety Registry for a time period of no less than five years. At the end of the five year period, a study report will be submitted to the Biological License Application (BLA) that describes the major safety findings from the registry program, including the specific items listed below and proposing labeling changes as appropriate. Additionally, annual interim reports will be submitted to the BLA, along with expedited reports as specified below. The protocol for addressing this PMC will be submitted to the IND by May 31, 2007, and the five year study report will be submitted by June 30, 2012. All patients within the registry will be followed for the occurrence of:

    a. Serious infections, defined as infections necessitating or prolonging hospitalization or resulting in death. Alexion commits to collecting follow-up information from these patients to assess the nature of the serious infection, the duration of hospitalization, the major features of the clinical course and the survival status. Expedited reporting (15 day telephone or facsimile Medwatch communication) will be provided for the occurrence of these serious infections.

    b. Malignancy, including the nature of the malignancy and the survival status of patients who develop a malignancy;

    c. Use of eculizumab among pediatric patients under 16 years of age, to include collection of eculizumab dosage information, as well as the same information being required for adult patients in the registry;

    d. Pregnancy, including the clinical course of each pregnancy and the detection of congenital abnormalities among babies born to the women exposed to eculizumab during the pregnancy.

    e. Thrombotic events, including the nature of the event, the clinical outcome as well as the anticoagulant management prior to and after the event.

2. To submit a comprehensive description of the Soliris Guardian Program Risk Minimization Action Plan (RiskMAP), including all items listed below. Fulfillment of this post-marketing commitment will be contingent upon FDA concurrence upon the expectations of the Soliris Guardian Program. Submission of all items listed below will occur no later than May 18, 2007. The submitted information will include:

   a. A final version of the Soliris Guardian Program document that, in addition to any other items, provides information fully consistent with the approved prescribing information.

   b. A copy of all educational materials to be provided as part of the program, including but not limited to all components of the Soliris Starter Kit, the Drug Fact Sheet and patient-health care provider documents relating to the Soliris Safety Registry.

   c. A commitment to develop and submit a protocol (with the understanding that this protocol may need modification based upon FDA review findings) that uses surveys of health care providers and patients to assess compliance with the vaccination requirements as well as their knowledge of the risks of eculizumab and the need for vaccination.

   d. A copy of the Soliris Safety Registry protocol and any supportive documents to be provided to health care providers and patients. These documents may need modification based upon FDA review findings. The submitted protocol will indicate that the Registry will continue until Alexion receives written concurrence from FDA to terminate the registry. The protocol will include collection of the occurrence of the following events:

      i. Death;

      ii. Meningococcal vaccination (type and dates of all vaccinations);

      iii. eculizumab administration dates, at designated time points to establish initiation and termination of therapy as well as to correlate eculizumab administration with the fatalities and the events listed below:

         - All meningococcal infections causing sepsis or meningitis
         - Other serious infections
         - Malignancy, including the nature of the malignancy and the survival status of patients who develop a malignancy;
         - Use of eculizumab among pediatric patients under 16 years of age, to include collection of eculizumab dosage information, as well as the required information for adult patients in the registry;
         - Use of eculizumab by indication;

- Pregnancy, including the clinical course of each pregnancy and the detection of congenital abnormalities among off-spring of the women exposed to eculizumab during a pregnancy and;
- Serious hemolysis, as defined by specific criteria.

e. A commitment to submit quarterly RiskMAP reports for the first year and annual reports thereafter (unless FDA provides written request for more frequent submissions) summarizing all information relating to the Soliris Guardian Program. The reports should include the following:

i. An analysis of all cases of the following, including a root cause analysis and factors that might have contributed to serious outcomes:
   - Meningococcal sepsis or meningitis, including the timing of all vaccinations relative to administration of Soliris
   - Other infections (with serious outcomes)
   - All deaths
   - Cases of serious hemolysis and all cases of hemolysis with serious outcomes

ii. Soliris use patterns including indication for use;

iii. Extent of compliance with RiskMAP requirements such as the percentage of patients that were vaccinated prior to receiving eculizumab and the percentage of patients that were re-vaccinated at 3-year or 10-year intervals (as applicable);

iv. Analysis of all cumulative data collected in the Soliris Safety Registry;

v. Results from all health care provider and patient surveys, including:
   - Any known data about patients or physicians who refused to participate in the surveys
   - Any known data about survey participants who are considered "lost" (drop-outs).

3. To conduct a randomized, controlled clinical study to assess the effects of anticoagulant withdrawal among PNH patients receiving eculizumab. This study will randomize at least 100 anticoagulated patients to either continue or discontinue anticoagulation therapy. The major outcomes will assess the safety of discontinuation of anticoagulant therapy while continuing eculizumab, especially with respect to providing important evidence regarding major bleeding and that this discontinuation does not increase the risk for occurrence of thrombotic events in these patients. A full study report and data from this study will be submitted to the BLA and may include a label revision, contingent upon the importance of the study results. The study protocol will be submitted to the investigational new drug application (IND) by June 30, 2007 and patient accrual completed no later than June 1, 2009. A final study report will be submitted no later than March 31, 2014.

4. To develop a validated and quantitative assay for the measurement of human anti-human antibodies (HAHA) for the detection of antibody formation to eculizumab. This assay will assess potential immune responses to the whole eculizumab molecule. Description of the validated assay will be submitted to the BLA as a CBE 30 by July 9, 2008.

5. To develop a validated and sensitive assay for the measurement of neutralizing HAHA to eculizumab. Alternatively, Alexion commits to submit documentation to FDA demonstrating with due diligence that a suitable assay could not be feasibly developed and that the assessment of serum lactate dehydrogenase (LDH) is a sufficiently sensitive indicator of the presence of neutralizing antibodies. This information will be submitted to the BLA by July 9, 2008.

6. To utilize samples from the ongoing E05-001 Phase III extension study (approximately 170 patients for at least 2 years) as test samples for the new validated HAHA assays. Alexion will continue to obtain serum samples from those patients who transition from E05-001 to the Soliris Safety Registry, at intervals of no less than one year, and continue this collection process for an additional three years. Sample collection will cease during this additional three year period for patients who terminate eculizumab administration. Additionally, serum samples will be obtained based upon physician reports of suspected loss of eculizumab bioactivity, based upon unanticipated alterations in serum LDH concentrations. All serum samples will be assayed at least annually and the results provided within an annual report to the BLA. Clinical data, to include the results of serum LDH concentrations, will also be obtained from any patients who show evidence of antibody formation. The protocol describing Alexion's plan for responding to this commitment will be submitted by May 1, 2007 and the final study report submitted by January 31, 2011.

Postmarketing Studies not subject to reporting requirements of 21 CFR 601.70.

7. To revalidate the linearity and accuracy of the Osmolality method across the full specification range using a combination of product samples diluted to lower osmolality and product samples spiked with osmolality standards. The revalidation plan will be submitted in advance to FDA for review and endorsement. This information will be submitted to the BLA as a CBE 30 by August 31, 2007.

8. To revalidate the linearity of the IEF method across a load range of 8 µg to 12 µg. The revalidation plan will be submitted in advance to FDA for review and endorsement. This information will be submitted to the BLA as a CBE 30 supplement by August 31, 2007.

9. To revise the IEF method SOP to specify that the method is validated only for a 10 µg load. This information will be submitted to the BLA as a CBE 30 by August 31, 2007.

10. To improve and revalidate the existing hemolytic assay. Improvements include increasing the number of sample replicates and qualifying the chicken erythrocytes reagent. The revised method SOP and revalidation plan will be submitted in advance to FDA for review and endorsement. This information will be submitted to the BLA as a CBE 30 by August 31, 2007.

11. To develop a new quantitative biological activity assay to replace the existing hemolytic assay, or submit documentation to FDA demonstrating with due diligence that a suitable assay could not be feasibly developed. This information will be submitted to the BLA by February 29, 2008. Validation of the quantitative biological activity assay will be submitted by July 9, 2008.

12. To provide FDA with a completed drug substance and drug product container closure system leachables evaluation using end-of-shelf-life, long-term 2-8°C stability samples. This information will be submitted to the BLA as a CBE 30 by August 31, 2007.

13. To develop a suitable antifoam assay and subsequently confirm the removal of antifoam from the process stream at the Protein A chromatography pool on three drug substance batches. This information will be submitted to the BLA as a CBE 30 by August 31, 2007.

We request that you submit clinical protocols to your IND, with a cross-reference letter to this biologics license application (BLA), STN BL 125166. Submit nonclinical and chemistry, manufacturing, and controls protocols and all study final reports to your BLA, STN BL 125166. Please use the following designators to label prominently all submissions, including supplements, relating to these postmarketing study commitments as appropriate:

- **Postmarketing Study Commitment Protocol**
- **Postmarketing Study Commitment Final Study Report**
- **Postmarketing Study Commitment Correspondence**
- **Annual Status Report of Postmarketing Commitment Studies**

For each postmarketing study subject to the reporting requirements of 21 CFR 601.70, you must describe the status in an annual report on postmarketing studies for this product. The status report for each study should include:

- information to identify and describe the postmarketing commitment,
- the original schedule for the commitment,
- the status of the commitment (i.e. pending, ongoing, delayed, terminated, or submitted),
- an explanation of the status including, for clinical studies, the patient accrual rate (i.e. number enrolled to date and the total planned enrollment), and
- a revised schedule if the study schedule has changed and an explanation of the basis for the revision.

As described in 21 CFR 601.70(e), we may publicly disclose information regarding these postmarketing studies on our Web site (http://www.fda.gov/cder/pmc/default.htm). Please refer to the February 2006 Guidance for Industry: Reports on the Status of Postmarketing Study Commitments - Implementation of Section 130 of the Food and Drug Administration Modernization Act of 1997 (see http://www.fda.gov/cder/guidance/5569fnl.htm) for further information.

Under 21 CFR Part 208, we have determined that this product poses a serious and significant public health concern requiring the distribution of a Medication Guide. Eculizumab is a product for which patient labeling could help prevent serious adverse effects and inform the patient of serious risks relative to benefit that could affect their decisions to use, or continue to use, the product. Therefore, a Medication Guide is necessary for safe and effective use of this product and FDA hereby approves the draft Medication Guide you submitted March 15, 2007. Please note that:

- this Medication Guide must be reprinted at the end of the package insert or accompany it [21 CFR 201.57(c)(18)];
- you are responsible for ensuring that this Medication Guide is available for distribution to every patient who is dispensed a prescription for this product [21 CFR 208];
- the final printed Medication Guide distributed to patients must conform to all conditions described in 21 CFR 208.24, including a minimum of 10 point text; and
- you are responsible for ensuring that the label of each container or package includes a prominent and conspicuous instruction to authorized dispensers to provide a Medication Guide to each patient to whom the drug is dispensed, and states how the Medication Guide is provided.

Please submit within 30 days content of labeling [21 CFR 601.14(b)] in structured product labeling (SPL) format, as described at http://www.fda.gov/oc/datacouncil/spl.html, that is identical in content to the enclosed labeling text dated March 16, 2007. Upon receipt and verification, we will transmit that version to the National Library of Medicine for public dissemination.

You must submit adverse experience reports under the adverse experience reporting requirements for licensed biological products (21 CFR 600.80). You should submit postmarketing adverse experience reports to the Central Document Room, Center for Drug Evaluation and Research, Food and Drug Administration, 5901-B Ammendale Road, Beltsville, MD 20705-1266. Prominently identify all adverse experience reports as described in 21 CFR 600.80.

The MedWatch-to-Manufacturer Program provides manufacturers with copies of serious adverse event reports that are received directly by the FDA. New molecular entities and important new biologics qualify for inclusion for three years after approval. Your firm is eligible to receive copies of reports for this product. To participate in the program, please see the enrollment instructions and program description details at www.fda.gov/medwatch/report/mmp.htm.

You must submit distribution reports under the distribution reporting requirements for licensed biological products (21 CFR 600.81).

You must submit reports of biological product deviations under 21 CFR 600.14. You should promptly identify and investigate all manufacturing deviations, including those associated with processing, testing, packing, labeling, storage, holding and distribution. If the deviation involves a distributed product, may affect the safety, purity, or potency of the product, and meets the other criteria in the regulation, you must submit a report on Form FDA-3486 to the Division of Compliance Risk Management and Surveillance (HFD-330), Center for Drug Evaluation and Research, Food and Drug Administration, 5600 Fishers Lane, Rockville, MD 20857. Biological product deviations sent by courier or overnight mail should be addressed to Food and Drug Administration, CDER, Office of Compliance, Division of Compliance Risk Management and Surveillance, HFD-330, Montrose Metro 2, 11919 Rockville Pike, Rockville, MD 20852.

Please submit all final printed labeling at the time of use and include implementation information on FDA Form 356h. Please provide a PDF-format electronic copy as well as original paper copies (ten for circulars and five for other labels). In addition, you may wish to submit draft copies of the proposed introductory advertising and promotional labeling with a cover letter requesting advisory comments to the Food and Drug Administration, Center for Drug Evaluation and Research, Division of Drug Marketing, Advertising and Communication, 5901-B Ammendale Road, Beltsville, MD 20705-1266. Final printed advertising and promotional labeling should be submitted at the time of initial dissemination, accompanied by a FDA Form 2253.

All promotional claims must be consistent with and not contrary to approved labeling. You should not make a comparative promotional claim or claim of superiority over other products unless you have substantial evidence to support that claim.

Please refer to http://www.fda.gov/cder/biologics/default.htm for information regarding therapeutic biological products, including the addresses for submissions.

Sincerely,

Karen Weiss for

Richard Pazdur, M.D.
Director
Office of Oncology Drug Products
Center for Drug Evaluation and Research

Enclosure: Package Insert
Carton and Vial Labeling
Medication Guide
Patient Safety Card

Soliris ™ (eculizumab)

FULL PRESCRIBING INFORMATION

WARNING: SERIOUS MENINGOCOCCAL INFECTION

Soliris increases the risk of meningococcal infections (5.1)

- **Vaccinate patients with a meningococcal vaccine at least 2 weeks prior to receiving the first dose of Soliris; revaccinate according to current medical guidelines for vaccine use**
- **Monitor patients for early signs of meningococcal infections, evaluate immediately if infection is suspected, and treat with antibiotics if necessary.**

1 INDICATIONS AND USAGE

Soliris is indicated for the treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH) to reduce hemolysis.

2 DOSAGE AND ADMINISTRATION

Patients must be administered a meningococcal vaccine at least two weeks prior to initiation of Soliris therapy and revaccinated according to current medical guidelines for vaccine use. [*see Warnings and Precautions (5.1)*].

2.1 Recommended Dosage Regimen

Soliris therapy consists of

- 600 mg every 7 days for the first 4 weeks, followed by
- 900 mg for the fifth dose 7 days later, then
- 900 mg every 14 days thereafter.

Soliris should be administered at the recommended dosage regimen time points, or within two days of these time points. [*see Warnings and Precautions (5.5)*]

2.2 Preparation for Administration

Soliris must be diluted to a final admixture concentration of 5 mg/mL using the following steps:

- Withdraw the required amount of Soliris from the vial into a sterile syringe.
- Transfer the recommended dose to an infusion bag.
- Dilute Soliris to a final concentration of 5 mg/mL by adding the appropriate amount (equal volume of diluent to drug volume) of 0.9% Sodium Chloride Injection, USP; 0.45% Sodium Chloride Injection, USP; 5% Dextrose in Water Injection, USP; or Ringer's Injection, USP to the infusion bag.

The final admixed Soliris 5 mg/mL infusion volume is 120 mL for 600 mg doses or 180 mL for 900 mg doses. Gently invert the infusion bag containing the diluted Soliris solution to ensure thorough mixing of the product and diluent. Discard any unused portion left in a vial, as the product contains no preservatives.

Prior to administration, the admixture should be allowed to adjust to room temperature [18°-25° C, 64-77° F]. The admixture must not be heated in a microwave or with any heat source other than ambient air temperature. The Soliris admixture should be inspected visually for particulate matter and discoloration prior to administration.

2.3 Administration

Do Not Administer As An Intravenous Push Or Bolus Injection

The Soliris admixture should be administered by intravenous infusion over 35 minutes via gravity feed, a syringe-type pump, or an infusion pump. Admixed solutions of Soliris are stable for 24 hours at 2-8° C (36-46° F) and at room temperature.

If an adverse reaction occurs during the administration of Soliris, the infusion may be slowed or stopped at the discretion of the physician. If the infusion is slowed, the total infusion time should not exceed two hours. Monitor the patient for at least one hour following completion of the infusion for signs or symptoms of an infusion reaction.

3 DOSAGE FORMS AND STRENGTHS

Soliris is supplied as 300 mg single-use vials each containing 30 mL of 10 mg/mL sterile, preservative-free eculizumab solution.

4 CONTRAINDICATIONS

Do not initiate Soliris therapy in patients:

- with unresolved serious *Neisseria meningitidis* infection.
- who are not currently vaccinated against *Neisseria meningitidis*.

5 WARNINGS AND PRECAUTIONS

5.1 Serious Meningococcal Infections

The use of Soliris increases a patient's susceptibility to serious meningococcal infections (septicemia and/or meningitis). All patients without a history of prior meningococcal vaccination must receive the meningococcal vaccine at least 2 weeks prior to receiving the first dose of Soliris and revaccinated according to current medical guidelines for vaccine use. Quadravalent, conjugated meningococcal vaccines are strongly recommended. Vaccination may not prevent meningococcal infections.

All patients must be monitored for early signs and symptoms of meningococcal infections and evaluated immediately if an infection is suspected. Physicians should strongly consider discontinuation of Soliris during the treatment of serious meningococcal infections.

In clinical studies, 2 out of 196 PNH patients developed serious meningococcal infections while receiving treatment with Soliris; both had been vaccinated. [*see Adverse Reactions (6.1)*].

5.2 Other Infections

Soliris blocks terminal complement; therefore patients may have increased susceptibility to infections, especially with encapsulated bacteria. Use caution when administering Soliris to patients with any systemic infection.

5.3 Monitoring After Soliris Discontinuation

Since Soliris therapy increases the number of PNH cells [in study 1, the proportion of PNH RBCs increased among Soliris-treated patients by a median of 28% from baseline (range from -25% to 69%)], patients who discontinue treatment with Soliris may be at increased risk for serious hemolysis. Serious hemolysis is identified by serum LDH levels greater than the pre-treatment level, along with any of the following: greater than 25% absolute decrease in PNH clone size (in the absence of dilution due to transfusion) in one week or less; a hemoglobin level of <5 gm/dL or a decrease of >4 gm/dL in one week or less; angina; change in mental status; a 50% increase in serum creatinine level; or thrombosis. Monitor any patient who discontinues Soliris for at least 8 weeks to detect serious hemolysis and other reactions.

If serious hemolysis occurs after Soliris discontinuation, consider the following procedures/treatments: blood transfusion (packed RBCs), or exchange transfusion if the PNH RBCs are >50% of the total RBCs by flow cytometry; anticoagulation; corticosteroids; or reinstitution of Soliris.

In clinical studies, 16 of 196 PNH patients discontinued treatment with Soliris. Patients were followed for evidence of worsening hemolysis and no serious hemolysis was observed.

5.4 Thrombosis Prevention and Management

The effect of withdrawal of anticoagulant therapy during Soliris treatment has not been established. Therefore, treatment with Soliris should not alter anticoagulant management.

5.5 Laboratory Monitoring

Serum LDH levels increase during hemolysis and may assist in monitoring Soliris effects, including the response to discontinuation of therapy. In clinical studies, six patients achieved a reduction in serum LDH levels only after a decrease in the Soliris dosing interval from 14 to 12 days. All other patients achieved a reduction in serum LDH levels with the 14 day dosing interval [*see Clinical Pharmacology (12.2) and Clinical Studies (14)*].

5.6 Infusion Reactions

As with all protein products, administration of Soliris may result in infusion reactions, including anaphylaxis or other hypersensitivity reactions. In clinical trials, no PNH patients experienced an infusion reaction which required discontinuation of Soliris. Soliris administration should be interrupted in all patients experiencing severe infusion reactions and appropriate medical therapy administered.

6 ADVERSE REACTIONS

6.1 Clinical Trial Experience

Meningococcal infections are the most important adverse reactions experienced by patients receiving Soliris therapy. In PNH clinical studies, two patients experienced meningococcal sepsis. Both patients had previously received a meningococcal vaccine. In clinical studies among patients without PNH, meningococcal meningitis occurred in an unvaccinated patient [*see Warnings and Precautions (5.1)*].

The data described below reflect exposure to Soliris in 196 adult patients with PNH, age 18-85, of whom 55% were female. All had signs or symptoms of intravascular hemolysis. Soliris was studied in a placebo-controlled clinical study (in which 43 patients received Soliris and 44, placebo); a single arm clinical study and a long term extension study. 182 patients were exposed for greater than one year. All patients received the recommended Soliris dose regimen.

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice. Table 1 summarizes the adverse reactions that occurred at a numerically

higher rate in the Soliris group than the placebo group and at a rate of 5% or more among patients treated with Soliris.

TABLE 1

ADVERSE REACTIONS REPORTED IN 5% OR MORE OF SOLIRIS TREATED PATIENTS AND GREATER THAN PLACEBO IN THE CONTROLLED CLINICAL STUDY

Reaction	Soliris N = 43 N (%)	Placebo N = 44 N (%)
Headache	19 (44)	12 (27)
Nasopharyngitis	10 (23)	8 (18)
Back pain	8 (19)	4 (9)
Nausea	7 (16)	5 (11)
Fatigue	5 (12)	1 (2)
Cough	5 (12)	4 (9)
Herpes simplex infections	3 (7)	0
Sinusitis	3 (7)	0
Respiratory tract infection	3 (7)	1 (2)
Constipation	3 (7)	2 (5)
Myalgia	3 (7)	1 (2)
Pain in extremity	3 (7)	1 (2)
Influenza-like illness	2 (5)	1 (2)

In the placebo-controlled clinical study, serious adverse reactions occurred among 4 (9%) patients receiving Soliris and 9 (21%) patients receiving placebo. The serious reactions included infections and progression of PNH. No deaths occurred in the study and no patients receiving Soliris experienced a thrombotic event; one thrombotic event occurred in a patient receiving placebo.

Among 193 patients with PNH treated with Soliris in the single arm, clinical study or the follow-up study, the adverse reactions were similar to those reported in the placebo-controlled clinical study. Serious adverse reactions occurred among 16% of the patients in these studies. The most common serious adverse reactions were: viral infection (2%), headache (2%), anemia (2%), and pyrexia (2%).

6.2 Immunogenicity

As with all proteins there is a potential for immunogenicity. Low titers of antibodies to Soliris were detected in 3/196 (2%) of all PNH patients treated with Soliris. No apparent correlation of antibody development to clinical response was observed. The immunogenicity data reflect the percentage of patients whose test results were considered positive for antibodies to Soliris in an enzyme linked immunosorbent assay (ELISA) and are highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of antibody positivity in the assay may be influenced by several factors including sample handling, timing of sample collection, concomitant medications and underlying disease. For these reasons, comparison of the incidence of antibodies to Soliris with the incidence of antibodies to other products may be misleading.

7 DRUG INTERACTIONS

Drug interaction studies have not been performed with Soliris.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Pregnancy Category C:

PNH is a serious illness. Pregnant women with PNH and their fetuses have high rates of morbidity and mortality during pregnancy and the postpartum period. There are no adequate and well-controlled studies of Soliris in pregnant women. Soliris, a recombinant IgG molecule (humanized anti-C5 antibody), is expected to cross the placenta. Animal studies using a mouse analogue of the Soliris molecule (murine anti-C5 antibody) showed increased rates of developmental abnormalities and an increased rate of dead and moribund offspring at doses 2-8 times the human dose. Soliris should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Animal reproduction studies were conducted in mice using doses of a murine anti-C5 antibody that approximated 2-4 times (low dose) and 4-8 times (high dose) the recommended human Soliris dose, based on a body weight comparison. When animal exposure to the antibody occurred in the time period from before mating until early gestation, no decrease in fertility or reproductive performance was observed. When maternal exposure to the antibody occurred during organogenesis, two cases of retinal dysplasia and one case of umbilical hernia were observed among 230 offspring born to mothers exposed to the higher antibody dose; however, the exposure did not increase fetal loss or neonatal death. When maternal exposure to the antibody occurred in the time period from implantation through weaning, a higher number of male offspring became moribund or died (1/25 controls, 2/25 low dose group, 5/25 high dose group). Surviving offspring had normal development and reproductive performance.

8.2 Labor and Delivery

No information is available on the effects of Soliris during labor and delivery.

8.3 Nursing Mothers

It is not known whether Soliris is secreted into human milk. IgG is excreted in human milk, so it is expected that Soliris will be present in human milk. However, published data suggest that breast milk antibodies do not enter the neonatal and infant circulation in substantial amounts. Caution should be exercised when Soliris is administered to a nursing woman. The unknown risks to the infant from gastrointestinal or limited systemic exposure to Soliris should be weighed against the known benefits of breastfeeding.

8.4 Pediatric Use

The safety and effectiveness of Soliris therapy in pediatric patients below the age of 18 have not been established.

8.5 Geriatric Use

In PNH studies, 15 patients 65 years of age or older were treated with Soliris. Although there were no apparent age-related differences observed in these studies, the number of patients aged 65 and over is not sufficient to determine whether they respond differently from younger patients.

10 OVERDOSAGE

No cases of Soliris overdose have been reported during clinical studies.

11 DESCRIPTION

Soliris is a formulation of eculizumab which is a recombinant humanized monoclonal $IgG_{2/4}\kappa$ antibody produced by murine myeloma cell culture and purified by standard bioprocess technology. Eculizumab contains human constant regions from human IgG2 sequences and human IgG4 sequences and murine complementarity-determining regions grafted onto the human framework light- and heavy-chain variable regions. Eculizumab is composed of two 448 amino acid heavy chains and two 214 amino acid light chains and has a molecular weight of approximately 148 kDa.

Soliris is a sterile, clear, colorless, preservative-free 10 mg/mL solution for intravenous infusion and is supplied in 30-mL single-use vials. The product is formulated at pH 7 and each vial contains 300 mg of eculizumab, 13.8 mg sodium phosphate monobasic, 53.4 mg sodium phosphate dibasic, 263.1 mg sodium chloride, 6.6 mg polysorbate 80 (vegetable origin) and Water for Injection, USP.

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Eculizumab, the active ingredient in Soliris, is a monoclonal antibody that specifically binds to the complement protein C5 with high affinity, thereby inhibiting its cleavage to C5a and C5b and preventing the generation of the terminal complement complex C5b-9. Soliris inhibits terminal complement mediated intravascular hemolysis in PNH patients

A genetic mutation in PNH patients leads to the generation of populations of abnormal RBCs (known as PNH cells) that are deficient in terminal complement inhibitors, rendering PNH RBCs sensitive to persistent terminal complement-mediated destruction. The destruction and loss of these PNH cells (intravascular hemolysis) results in low RBC counts (anemia), and also fatigue, difficulty in functioning, pain, dark urine, shortness of breath, and blood clots.

12.2 Pharmacodynamics

In the placebo-controlled clinical study, Soliris when administered as recommended reduced hemolysis as shown by the reduction of serum LDH levels from 2200 ± 1034 U/L (mean ± SD) at baseline to 700 ± 388 U/L by week one and maintained the effect through the end of the study at week 26 (327 ± 433 U/L). In the single arm clinical study, Soliris maintained this effect through 52 weeks [*see Clinical Studies (14)*].

12.3 Pharmacokinetics

A population PK analysis with a standard 1-compartmental model was conducted on the multiple dose PK data from 40 PNH patients receiving the recommended Soliris regimen [*see Dosage and Administration (2.1)*]. In this model, the clearance of Soliris for a typical PNH patient weighing 70 kg was 22 mL/hr and the volume of distribution was 7.7 L. The half-life was 272 ± 82 hrs (mean ± SD). The mean observed peak and trough serum concentrations of Soliris by week 26 were 194 ± 76 mcg/mL and 97 ± 60 mcg/mL, respectively.

Studies have not been conducted to evaluate the PK of Soliris in special patient populations identified by gender, race, age (pediatric or geriatric), or the presence of renal or hepatic impairment.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Long-term animal studies have not been conducted to evaluate the carcinogenic and genotoxic potential of Soliris. Effects of Soliris upon fertility have not been studied in animals. Intravenous injections of male and female mice with a murine anti-C5 antibody at up to 4-8 times the equivalent of the clinical dose of Soliris had no adverse effects on mating or fertility.

14 CLINICAL STUDIES

The safety and efficacy of Soliris in PNH patients with hemolysis were assessed in a randomized, double-blind, placebo-controlled 26 week study (Study 1); PNH patients were also treated with Soliris in a single arm 52 week study (Study 2); and in a long term extension study. Patients received meningococcal vaccination prior to receipt of Soliris. In all studies, the dose of Soliris was 600 mg study drug every 7 ± 2 days for 4 weeks, followed by 900 mg 7 ± 2 days later, then 900 mg every 14 ± 2 days for the study duration. Soliris was administered as an intravenous infusion over 25 - 45 minutes.

Study 1:

PNH patients with at least four transfusions in the prior 12 months, flow cytometric confirmation of at least 10% PNH cells and platelet counts of at least 100,000/microliter were randomized to either Soliris (n = 43) or placebo (n = 44). Prior to randomization, all patients underwent an initial observation period to confirm the need for RBC transfusion and to identify the hemoglobin concentration (the "set-point") which would define each patient's hemoglobin stabilization and transfusion outcomes. The hemoglobin set-point was less than or equal to 9 g/dL in patients with symptoms and was less than or equal to 7 g/dL in patients without symptoms. Endpoints related to hemolysis included the numbers of patients achieving hemoglobin stabilization, the number of RBC units transfused, fatigue, and health-related quality of life. To achieve a designation of hemoglobin stabilization, a patient had to maintain a hemoglobin concentration above the hemoglobin set-point and avoid any RBC transfusion for the entire 26 week period. Hemolysis was monitored mainly by the measurement of serum LDH levels, and the proportion of PNH RBCs was monitored by flow cytometry. Patients receiving anticoagulants and systemic corticosteroids at baseline continued these medications.

Major baseline characteristics were balanced (see table 2).

TABLE 2
STUDY 1 PATIENT BASELINE CHARACTERISTICS

	Study 1	
Parameter	Placebo N = 44	Soliris N = 43
Mean age (SD)	38 (13)	42 (16)
Gender - female (%)	29 (66)	23 (54)
History of aplastic anemia or myelodysplastic syndrome (%)	12 (27)	8 (19)
Patients with history of thrombosis (events)	8 (11)	9 (16)
Concomitant anticoagulants (%)	20 (46)	24 (56)
Concomitant steroids/immunosuppressant	16 (36)	14 (33)

Parameter	Study 1 Placebo N = 44	 Soliris N = 43
treatments (%)		
Packed RBC units transfused per patient in previous 12 months (median (Q1,Q3))	17 (14, 25)	18 (12, 24)
Mean hgb level (g/dL) at setpoint (SD)	8 (1)	8 (1)
Pre-treatment LDH levels (median, U/L)	2,234	2,032
Free hemoglobin at baseline (median, mg/dL)	46	41

Patients treated with Soliris had significantly reduced ($p < 0.001$) hemolysis resulting in improvements in anemia as indicated by increased hemoglobin stabilization and reduced need for RBC transfusions compared to placebo treated patients (see table 3). These effects were seen among patients within each of the three pre-study RBC transfusion strata (4 - 14 units; 15 - 25 units; > 25 units). After 3 weeks of Soliris treatment, patients reported less fatigue and improved health-related quality of life. Because of the study sample size and duration, the effects of Soliris on thrombotic events could not be determined.

TABLE 3
STUDY 1 RESULTS

	Placebo N = 44	Soliris N = 43
Percentage of patients with stabilized hemoglobin levels	0	49
Packed RBC units transfused per patient (median)	10	0
(range)	(2 - 21)	(0 - 16)
Transfusion avoidance (%)	0	51
LDH levels at end of study (median, U/L)	2,167	239
Free hemoglobin at end of study (median, mg/dL)	62	5

Study 2 and Extension Study:
PNH patients with at least one transfusion in the prior 24 months and at least 30,000 platelets/microliter received Soliris over a 52-week period. Concomitant medications included anti-thrombotic agents in 63% of the patients and systemic corticosteroids in 40% of the patients. Overall, 96 of the 97 enrolled patients completed the study (one patient died following a thrombotic event). A reduction in intravascular hemolysis as measured by serum LDH levels was sustained for the treatment period and resulted in a reduced need for RBC transfusion and less fatigue. 187 Soliris-treated PNH patients were enrolled in a long term extension study. All patients sustained a reduction in intravascular hemolysis over a total Soliris exposure time ranging from 10 to 54 months. There were fewer thrombotic events with Soliris treatment than during the same period of time prior to treatment. However, the majority of patients received concomitant anticoagulants; the effects of anticoagulant withdrawal during Soliris therapy was not studied [*see Warnings and Precautions (5.4)*].

16 HOW SUPPLIED / STORAGE AND HANDLING

Soliris (eculizumab) is supplied as 300 mg single-use vials containing 30 mL of 10 mg/mL sterile, preservative-free Soliris solution per vial.

Soliris vials must be stored in the original carton until time of use under refrigerated conditions at 2-8° C (36-46° F) and protected from light. Do not use beyond the expiration date stamped on the carton. Refer to *Dosage and Administration* (2) for information on the stability and storage of diluted solutions of Soliris.

DO NOT FREEZE. DO NOT SHAKE.

NDC 25682-001-01 Single unit 300 mg carton: Contains one (1) 30 mL vial of Soliris (10 mg/mL).

17 PATIENT COUNSELING INFORMATION

See Medication Guide.

Prior to treatment, patients should fully understand the risks and benefits of Soliris, in particular the risk of meningococcal infection. Ensure that patients receive the Medication Guide.

Patients should be informed that they are required to receive a meningococcal vaccination at least 2 weeks prior to receiving the first dose of Soliris, if they have not previously been vaccinated. They are required to be revaccinated according to current medical guidelines for meningococcal vaccine use while on Soliris therapy. Patients should also be informed that vaccination may not prevent meningococcal infection. Patients should be educated about any of the signs and symptoms of meningococcal infection, and strongly advised to seek immediate medical attention if these signs or symptoms occur. These signs and symptoms are as follows:

- moderate to severe headache with nausea or vomiting
- moderate to severe headache and a fever
- moderate to severe headache with a stiff neck or stiff back
- fever of 103° F (39.4° C) or higher
- fever and a rash
- confusion
- severe muscle aches with flu-like symptoms, and eyes sensitive to light

Patients should be informed that they would be provided with the Patient Safety Card that they should carry with them at all times. This card describes symptoms which, if experienced, should prompt the patient to immediately seek medical evaluation.

Patients should be informed that there is a potential for serious hemolysis when Soliris is discontinued and that they will be monitored by their healthcare professional for at least 8 weeks following Soliris discontinuation.

Manufactured by:

Alexion Pharmaceuticals, Inc.

352 Knotter Drive

Cheshire, CT 06410 USA

US License Number 1743

Patients receiving Soliris treatment should carry this card at all times.

INFORMATION FOR THE PATIENT

You should call your doctor (#_____) right away if you have any of these symptoms:

- **moderate to severe headache with nausea or vomiting**
- **moderate to severe headache and a fever**
- **moderate to severe headache with a stiff neck or stiff back**
- **fever of 103° F (39.4° C) or higher**
- **fever and a rash**
- **confusion**
- **severe muscle aches with flu-like symptoms, and eyes sensitive to light**

If you cannot reach your doctor right away, go to an emergency room and show them this card.

INFORMATION FOR THE PHYSICIAN

This patient was prescribed Soliris (eculizumab) therapy. Soliris is an antibody that inhibits terminal complement activation and prevents the formation of C5b-9 complex. Before beginning treatment, this patient should have received a meningococcal vaccine, but may still be susceptible to meningococcal infections.

For more information about Soliris, see the full prescribing information available at www.soliris.net\soliris.pdf, or call 1-888-SOLIRIS (1-888-765-4747)



Food and Drug Administration
Center for Drug Evaluation and Research
Office of Biotechnology Products

FACSIMILE TRANSMITTAL SHEET

DATE: May 8, 2009

To: Mr. Arthur D. Edwards	**From:** LCDR Andrew Shiber Project Manager
Company: Alexion Pharmaceuticals Corporation	Office of Biotechnology Products
Fax number: 203-271-8191	**Fax number:** (301) 796-9743
Phone number: 203-272-2596	**Phone number:** (301) 796-4798

Subject: STN 125166/36

Total no. of pages including cover: 2

Comments: Please find attached the action letter for the above supplement for Soliris®(eculizumab).

Document to be mailed: X☐ YES ☐ NO

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DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration
Rockville, MD 20857

STN 125166-36

May 8, 2009

Alexion Pharmaceuticals
Attention: Mr. Arthur D. Edwards
Executive Director, Regulatory Affairs
352 Knotter Drive
Cheshire, CT 06410

Dear Mr. Edwards:

Your request to supplement your biologics license application for Soliris®(eculizumab), to extend the shelf-life of the drug product from 24 to 30 months when stored at 2 – 8 degrees Celsius, has been approved.

Please refer to http://www.fda.gov/cder/biologics/default.htm for information regarding therapeutic biological products, including the addresses for submissions.

This information will be included in your biologics license application file.

Sincerely,

Kathleen A. Clouse

Kathleen A. Clouse, Ph.D.
Director
Division of Monoclonal Antibodies
Office of Biotechnology Products
Office of Pharmaceutical Science
Center for Drug Evaluation and Research