UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

AMGEN INC.
Petitioner

v.

ALEXION PHARMACEUTICALS, INC.
Patent Owner

U.S. Patent No. 9,732,149

Case IPR2019-00741

DECLARATION OF DR. JOSEPH P. BALTHASAR, PH.D.

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I, Joseph P. Balthasar, Ph.D., hereby declare as follows

I. Introduction

1. I am over the age of eighteen (18) and competent to make this declaration.

2. I have been retained as an expert witness on behalf of Amgen Inc. ("Amgen") for the above-captioned *inter partes* review (IPR). I am being compensated for my time in connection with this IPR at my standard consulting rate, which is $400 per hour.

3. I understand that this Declaration accompanies a petition for IPR involving U.S. Patent No. 9,732,149 ("the '149 patent") (AMG1001). I understand that the '149 patent resulted from U.S. Patent Application No. 15/284,015 ("the '015 application"), which was filed on September 9, 2016. I also understand that the '149 patent is currently assigned to Alexion Pharmaceuticals, Inc. ("Alexion"). The '149 patents states that it is a continuation of a series of applications on its face page. The earliest of those applications has a filing date of March 15, 2007. I understand that that is the earliest date that Alexion can assert as a priority date. I refer to this date throughout this declaration and have performed my analysis based on this date.

4. In preparing this Declaration, I have reviewed the '149 patent and each of the documents cited in my declaration, in light of general knowledge in the art

before March 15, 2007. In formulating my opinions, I have relied upon my experience, education, and knowledge in the relevant art. In formulating my opinions, I have also considered the viewpoint of a person of ordinary skill in the art ("POSA") (*i.e.*, a person of ordinary skill in the field, as defined further below in §V) prior to March 15, 2007.

II. My background and qualifications

5. I am an expert in the development and evaluation of therapeutic antibodies, and have been since well before March 15, 2007. I have been actively working in the field of pharmaceutical sciences since the 1990s, and have over 20 years of experience in studying, developing, and evaluating therapeutic monoclonal antibodies (mAbs), including the clinical application of humanized mAbs.

6. My education includes obtaining a B.S. in Pharmacy and a Ph.D. in Pharmaceutics from the State University of New York at Buffalo in 1991 and 1996, respectively. I served as a Clinical Assistant Professor in the Department of Pharmaceutics at the State University of New York at Buffalo from January 1996 through February 1997. From February 1997 through December 1999, I was an Assistant Professor in the Department of Pharmaceutics and Pharmaceutical Chemistry at the University of Utah. I then returned to the State University of New York at Buffalo in December of 1999, where I have subsequently served as an

Assistant Professor (1999-2003), an Associate Professor (2003-2008), and Full Professor in the Department of Pharmaceutical Sciences.

7. I have served other roles in my professional career, including being a member of the Targeted Therapeutics Program at the Roswell Park Memorial Cancer Institute in Buffalo, New York from September 2006 to the present. From June 2007 to the present, I have been the Director of the Center for Protein Therapeutics at the University of Buffalo. And from January 2011 to the present, I have been the Associate Dean for Research for the School of Pharmacy and Pharmaceutical Sciences at the University at Buffalo. I am also on the editorial board for five different scientific journals, including the journal "mAbs," which is dedicated to antibody research and development.

8. In my various professional positions, I have made significant contributions to the fields of immunology and therapeutic antibodies. For example, in my role as the Director for the Center for Protein Therapeutics, I have been involved in the evaluation of at least 20 different monoclonal antibodies. And my own laboratory has performed research relating to:

- Engineering monoclonal antibodies for improved pharmacokinetic properties;
- Investigation of strategies to improve antibody distribution in solid tumors;

- Development of improved mathematical models for predicting the disposition and effects of monoclonal antibody drugs;
- Development of antibody conjugates for targeted, intra-cellular delivery of macromolecular toxins;
- Investigation of sources of inter-individual variability in monoclonal antibody pharmacokinetics;
- Investigation of the role of FcRn in the absorption, distribution, and elimination of IgG antibodies;
- Development of antibody-based therapies to treat and prevent infection;
- Optimization of bispecific T-cell engagers, with primary focus on the application of PKPD modeling to guide discovery and development; and
- Development of strategies to decrease off-target toxicity of antibody-drug conjugates and antibody-targeted nanoparticles.

9. My work has resulted in the publication of more than 80 peer-reviewed scientific articles, as well as three book chapters. In addition to my educational training and professional and research experiences described above, I have kept abreast of new developments relating to therapeutic monoclonal antibodies by reading scientific literature, conferring with colleagues in the field,

attending and presenting at scientific conferences, and presenting at invited lectures. Further information regarding my qualifications and credentials are set forth in my curriculum vitae, provided as AMG1003.

10. Accordingly, I am an expert in the development and evaluation of therapeutic monoclonal antibodies, and have been since prior to March 15, 2007. For that reason, I am qualified to provide an opinion as to what a POSA would have understood, known, or concluded before March 15, 2007.

III. Summary of opinions

11. Claim 1, the only claim in the '149 patent, is directed to an anti-C5 antibody 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 name of the anti-C5 antibody comprising SEQ ID NOs: 2 and 4 is eculizumab. AMG1015, 738 (Boone declaration ¶6); AMG1001, 4:60-67, 30:16-47.

12. Based on my review of the prior art, eculizumab was a well-known anti-C5 antibody that multiple clinical trials had demonstrated to be effective in treating PNH long before March 15, 2007. *See* §§VI.B-C. One reference that discloses using eculizumab to treat PNH is Alexion's patent application published as U.S. 2005/0191298 (AMG1005; "Bell"). Similarly, a publication by Hillmen et al. in the New England Journal of Medicine (AMG1004; "Hillmen"), "tested the clinical efficacy of eculizumab, a humanized antibody that inhibits the activation

of terminal complement components, in patients with PNH." AMG1004, Abstract. After the completion of the Hillmen trial, the same eleven patients were enrolled in an extension study described by Hill *et at.* (AMG1047; "Hill '05") in which the patients continued to receive 900 mg of eculizumab every other week for a period of 52 weeks. These publications disclosed that eculizumab was "well tolerated" and "particularly useful" therapeutic for treating PNH. AMG1004, Abstract, 558; AMG1005, ¶[0012]; AMG1047, 2565.

13. A declaration submitted by an Alexion employee (Dr. Laural Boone) confirmed that the eculizumab used in multiple clinical trial studies before the '149 patent was filed "contained the heavy and light chain sequences of SEQ ID NOs: 2 and 4." AMG1015, 736-738 (Boone declaration ¶6 and Table of Clinical Trials). Further, as a result of Alexion's multiple publications, including those describing the use of eculizumab to treat PNH, eculizumab's entire amino acid sequence was a matter of public knowledge. *See* §§VI.D-E below. U.S. Patent Application Publication No. 2003/0232972 (AMG1006; "Bowdish"), for example, lists the entire amino acid sequence of a humanized 5G1.1 antibody with a hybrid IgG2/IgG4 constant region (*i.e.*, eculizumab or an antibody like eculizumab) with the exception of the heavy chain CDR3 sequence. *See* §VI.E below. Bowdish further states that the CDR3 region of 5G1.1 had been replaced by a thrombopoietin ("TPO") peptide as a means for "presentation of" the TPO peptide,

and that further information regarding 5G1.1 can be found in U.S. Pat. No. 6,355,245 (AMG1007; "Evans"). *See* §VI.E below; AMG1006, ¶[0006].

14. Evans lists the entire heavy and light chain variable regions of humanized 5G1.1 antibodies, and provides the sequence for all six CDR regions of the 5G1.1 antibody, including the heavy chain CDR3 sequence missing from Bowdish. *See* §VI.E below. Evans further states that these 5G1.1 variable regions can be combined with hybrid IgG constant domains to form a full length antibody. AMG1007, 45:24-33.

15. Additionally, International Application Publication No. WO19997011971 ("Mueller") discloses the sequence of the claimed "non-naturally occurring, protein-engineered heavy chain" that Alexion alleged to be novel during prosecution. *See* §VI.E below. Based on these and other publications, I disagree with Alexion's assertion during prosecution of the '149 patent that the claims were patentable because "[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." AMG1015, 709.

16. Because the '149 patent claims nothing beyond the same antibody as was already disclosed in the prior art, it is my opinion that claim 1 of the '149 patent is anticipated or would have been obvious:

- Section X (Ground 1) details why claim 1 is anticipated by Hillmen.
- Section XI (Ground 2) details why claim 1 is anticipated by Hill '05.
- Section XII (Ground 3) details why claim 1 is anticipated by Bowdish.
- Section XIII (Ground 4) details why claim 1would have been obvious in view of Bell's disclosure of the anti-C5 antibody eculizumab and the antibody sequences disclosed in Bowdish and Evans.
- Section XIV (Ground 5) details why claim 6 would have been obvious in view of the antibody sequences disclosed in Evans and Mueller.

17. In preparing these grounds, I concluded that none of the evidence I have reviewed provides any objective evidence of non-obviousness of claim 1 of the '149 patent. *See* §XV below.

IV. Documents considered in formulating my opinions

18. In formulating my opinions, I have considered all the references and documents cited herein, including those listed below.

Exhibit No.	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)
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

Exhibit No.	Description
	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)
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
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)
1020	Pierangeli, S., *et al.*, "Requirement of activation of complement C3 and C5 for antiphospholipid antibody-mediated thrombophilia," *Arthritis & Rheumatism* 52: 2120-2124 (2005)
1021	Kaplan, M., "Eculizumab (Alexion)," *Curr. Opin. Investig. Drugs* 3: 1017-1023 (2002)
1022	Taylor, P. *et al.*, "The complement system," *Current Biology* 8:R259-R261 (1998)
1023	Thomas, T., *et al.*, "Inhibition of Complement Activity By Humanized Anti-C5 Antibody and Single-Chain Fv.," *Molecular Immunology,* 33: 1389- 1401 (1996)

Exhibit No.	Description
1024	FDA Medical Review for Application Number 125166 (eculizumab), available at https://www.accessdata.fda.gov/drugsatfda_docs/nda/2007/125166s0000TOC.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
1027	Opposition File History for European Patent No. 2359834
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)
1029	Wang, W., *et al*., "Antibody Structure, Instability, and Formulation," *Journal of Pharmaceutical Sciences* 96: 1-26 (2007)
1030	Daugherty, A. and Mrsny, R., "Formulation and delivery issues for monoclonal antibody therapeutics," *Advanced Drug Delivery Reviews* 58: 686-706 (2006)
1031	Mueller, P., *et al*., "Humanized Porcine VCAM-Specific Monoclonal Antibodies With Chimeric IgG2/G4 Constant Regions Block Human Leukocyte Binding To Porcine Endothelial Cells," *Molecular Immunology* 34: 441 -452 (1997)
1032	Rother, R., *et al*., "Antibodies and Fusion Proteins That Include Engineered Constant Regions," International Patent Application Publication No. WO 2005/007809 A2 (filed May 28 2004; published January 27, 2005)
1033	Rother, R., *et al*., "Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria," *Nature Biotechnology* 25: 1256-1264 (2007)
1034	Tacken, P., *et al*., "Effective induction of naive and recall T-cell responses by targeting antigen to human dendritic cells via a humanized anti-DC-SIGN antibody," *Blood* 106: 1278-1285 (2005)
1035	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)

Exhibit No.	Description
1036	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)
1037	Britton, W. and Demangel, C., "Compositions And Methods for Targeting Antigen-Presenting Cells with Antibody Single-Chain Variable Region Fragments," U.S. Patent Application Publication No. 2004/0146948 A1 (filed Oct. 17, 2003; published July 29, 2004)
1038	Bessler, M., *et al*., "Paroxysmal nocturnal haemoglobinuria (PNH) is caused by somatic mutations in the PIG-A gene," *The EMBO Journal* 13: 110-117 (1994)
1039	Matis, L. and Rollins, S., "Complement-specific antibodies: Designing novel anti-inflammatories," *Nature Medicine* 1: 839-842 (1995)
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/2007/125166s0000TOC.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)

Exhibit No.	Description
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)
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)
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

V. Person of Ordinary Skill in the Art

19. I understand that a POSA is a hypothetical person who is presumed to be aware of all pertinent art, thinks along conventional wisdom in the art, and is a person of ordinary creativity. A POSA in the field of the '149 patent had knowledge of the scientific literature and skills relating to that field, including the design and generation of antibodies, the complement system, and the application of

antibodies as therapeutics before March 15, 2007. A POSA also had knowledge of laboratory techniques and strategies used in immunology research such as molecular cloning and protein expression, and would have known of practical applications of the same. 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. 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.

VI. State of the art before March 15, 2007

20. I have been asked to provide a summary of the state of the art as it pertains to the '149 patent. In particular, the below presents a summary of the knowledge of the art before March 15, 2007 with regard to therapeutic antibodies, the complement system, eculizumab, and eculizumab's use to treat paroxysmal nocturnal hemoglobinuria ("PNH").

A. The art taught therapeutic use of humanized monoclonal antibodies

21. Before March 15, 2007, the basic structure of humanized monoclonal antibodies was well understood in the art. A standard antibody is a Y-shaped proteins made up of four proteins: two identical "heavy chain" polypeptide

sequences and two identical "light chain" polypeptide sequences. AMG1018, 7. Each of these heavy and light chain polypeptides can be divided into a constant region and a variable region. AMG1018, 11-12. The variable region was so-named because it contains the regions that differ between antibodies, while the constant region is consistent between antibodies. AMG1018, 11-12. The variable region for the light chain is denoted as V_L, the variable region for the heavy chain is denoted as V_H, the constant region for the light chain is denoted as C_L, and the constant region for the heavy chain is denoted as C_H. AMG1018, pp. 11-12.

22. The constant regions of an antibody heavy chain can be subdivided into different parts, referred to as C_{H1}, C_{H2}, and C_{H3}. AMG1040, 4. The light chain only contains one constant region (C_L). AMG1040, p. 4; AMG1018, 11-12. The number of subparts of the heavy chain constant region can differ depending on the particular isotype of antibody. The constant region, particularly C_{H2} and C_{H3} (*i.e.*, the Fc portion of an antibody), is responsible for interacting with the effector systems that facilitate antibody-mediated immune responses. AMG1040, 4-5. Proteins that interact with the constant region include complement proteins and Fc receptors. AMG1040, 4-5. Different antibody subclasses (e.g., IgG2 and IgG4) interact differently with the effector proteins and therefore induce different effector responses. For example, IgG4 was known for its low propensity to activate the complement system. AMG1023, 1399. The prior art also disclosed artificial

constant regions that had been generated to have specific effector responses, such as a hybrid IgG2/IgG4 constant region that has both a reduced ability to elicit unwanted inflammatory events and a lessened propensity to activate complement. AMG1032, 11, 19, 28; AMG1031 ("Mueller II"), 451.

23. The variable regions of an antibody contain the portions of the antibody that interact with its target, which is referred to as an antigen. AMG1018, 7, 9-10. More specifically, each variable region of an antibody (V_H and V_L) contains three complementarity determining regions, or "CDRs," which provide the antibody with its antigen-binding specificity. AMG1018, 11-12. The portions of the variable regions surrounding the CDRs are known as the "framework" regions.

24. A diagram depicting the basic antibody structure is shown in Figure 1 below:

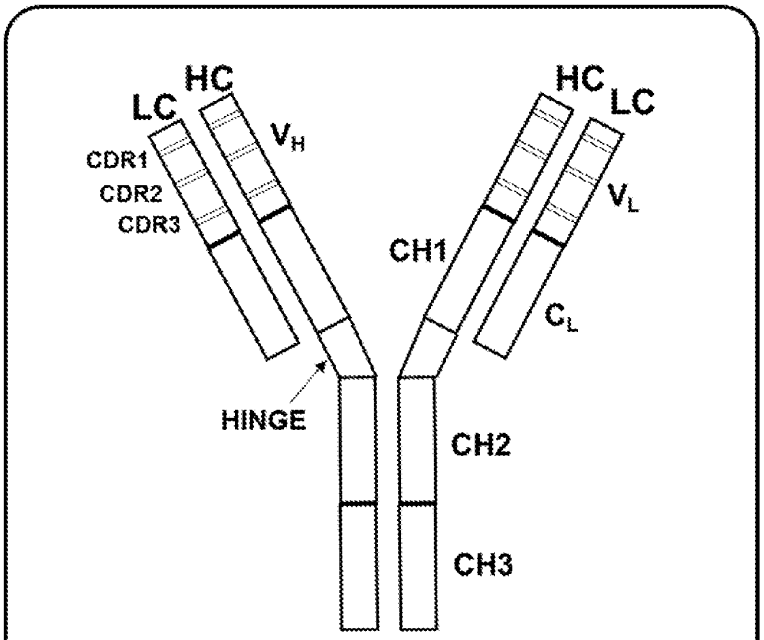


Figure 1: The basic domain structure of an antibody.

25. A humanized antibody is a type of antibody in which the CDR regions of a parent non-human antibody are integrated into the framework regions of a human antibody. AMG1040, 33; AMG1041, 140-141. Thus, a humanized antibody consists of human antibody sequence everywhere except in its CDR regions. The purpose of humanizing an antibody is to reduce the risk that a patient will recognize the antibody as "non-human" and mount a response against the antibody when the antibody is used as a therapeutic. AMG1040, 33-37; AMG1041, 139-141.

26. The therapeutic use of antibodies, including humanized antibodies, was well-established before March 15, 2007. For example, Wang et al.'s 2007

review in the *Journal of Pharmaceutical Sciences* lists 23 different monoclonal antibodies that had received FDA approval, and estimated that "200 or more are in development." AMG1029, Abstract, Table 1; *see also*, AMG1030, Abstract, Table 1. Knowledge of therapeutic antibodies included the understanding of how to develop formulations and doses for such antibodies. *See, e.g.*, AMG1026, 208-209; AMG1046, 2989; AMG1051, 1; AMG1050, 61; AMG1052; AMG1053, ¶[0043].

B. The normal and pathological roles of the complement system

27. The complement system is a part of the immune system that plays a "central role in host defense against many micro-organisms and in the modulation of inflammatory reactions." AMG1022, R259. The complement system was given its name because it serves a complementary role alongside antibodies in promoting the lysis of bacteria. AMG1022, R259. Activation of the complement system occurs via a cascade of "more than 20 serum proteins that interact in a precise series of enzymatic cleavage and membrane binding events leading to the generation of products with immunoprotective, immunoregulatory, proinflammatory and cytolytic properties." AMG1033, 1256 AMG1022, R259-R260. One endpoint of the complement system is the activation of the membrane attack complex on targeted cells and the subsequent lysis of those targeted cells. AMG1022, R259.

28. "Complement is activated by three pathways: the classical, alternative and mannan-binding lectin (MBL) pathways." AMG1022, R259. The different pathways initially involve different proteins, but "[a]ll pathways of complement activation converge at the cleavage of C5 into C5a and C5b by the C5 convertase enzyme complexes. This cleavage event initiates the terminal complement cascade." AMG1033, 1256; AMG1022, R259-R260; AMG1007, 10:59-64. A depiction of the complement cascade, adopted from a 1998 review by Taylor *et al.* (AMG1022) is provided below in Figure 2. AMG1022, R259.

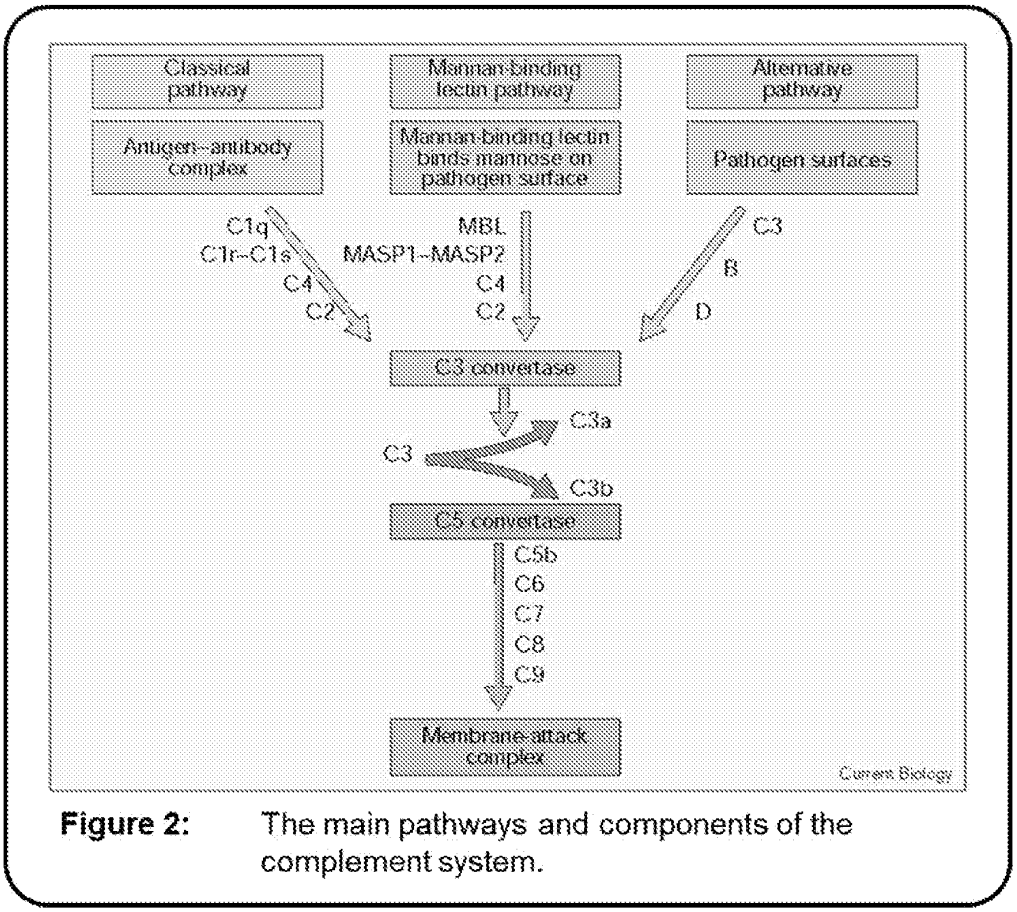


Figure 2: The main pathways and components of the complement system.

29. Before March 15, 2007, a POSA would have been aware that "complement can be both friend and foe." AMG1022, R259. The "foe" aspect of complement function is seen in the complement system's "contribut[ion] to many of the pathophysiological mechanisms underlying numerous acute and chronic inflammatory processes." AMG1023, 1389. Thus, "[t]he complement system provides critical immunoprotective and immunoregulatory functions but uncontrolled complement activation can lead to severe pathology." AMG1033, 1256.

30. Paroxysmal nocturnal hemoglobinuria ("PNH") is one complement-linked disorder known before March 15, 2007. AMG1004, 552-553; AMG1012, 1233-1234; AMG1005, ¶¶[0005]-[0008]. PNH arises from the mutation of one or more cyto-protective proteins (*e.g.*, the PIG-A protein) that prevent destruction of red blood cells ("RBCs"), platelets, and other blood cells from complement-mediated attack. AMG1004, 552-553; AMG1038, 110. The PIG-A protein "is essential for the synthesis of glycosylphosphatidylinositol (GPI), a lipid moiety that is embedded in the plasma membrane, where it serves to anchor a wide variety of proteins to the cell surface." AMG1004, 553. Loss of PIG-A function leads to loss of GPI-anchored proteins on the cell surface. AMG1004, 553. Two of these GPI-linked proteins are the complement control proteins (CCP) CD55 and CD59. AMG1047, 2559; AMG1004, 553. Due to the deficiency of these CCP on cells in

PNH, several cells, especially red blood cells, are particularly sensitive to attack and destruction by complement. AMG1047, 2559; AMG1004, 553. The loss of such red blood cells is the cause of the anemia that characterizes PNH. AMG1047, 2559; AMG1012, 1234. In other words, "[t]he clinical features of PNH result from the lack of one or more GPI-linked proteins that protect cells from complement-mediated attack." AMG1004, 553; AMG1033, 1256.

31. As a result of the destruction of RBCs and the resultant release of free hemoglobin into the blood, "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)." AMG1005, ¶[0007]. Based on this understanding of PNH's mechanism of action, researchers were actively developing therapeutics that targeted the complement system as a treatment for PNH long before March 15, 2007. *See, e.g.*, AMG1004, 552; AMG1012, 1233.

32. One known strategy for targeting the complement system was to inhibit a key step in the complement cascade—specifically the step of cleaving the complement protein C5. AMG1033, 1256; AMG1022, R259-R260; AMG1007, 10:59-64. Blockade at C5 was attractive because "Therapeutic inhibition of the complement cascade at C5 would block the formation of the potent inflammatory mediators C5a and C5b-9 via both the classical and alternative pathways."

AMG1039, 840-841; *see also*, AMG1033, 1257. Blockade at C5 was also attractive because it "preserves the early components of complement that are essential for the opsonization of microorganisms and clearance of immune complexes." AMG1004, 553; AMG1039, 840-841.

33. Researchers had been pursuing anti-C5 antibodies as a therapeutic strategy for blocking complement activity since at least 1995. *See, e.g.*, AMG1033, 1257; AMG1039, 840; AMG1023, 1389. Before March 15, 2007, these efforts included developing the anti-C5 antibody eculizumab, and they included testing eculizumab's efficacy in treating PNH and other complement-related disorders. *See, e.g.*, AMG1004, AMG1005; AMG1011; AMG1012; AMG1013; AMG1028; AMG1034; AMG1042, AMG1043; AMG1044; AMG1047.

C. Eculizumab's ability to treat PNH had been extensively evaluated in the clinic before March 15, 2007

34. Before March 15, 2007, a large number of clinical trials had been performed to test eculizumab, including multiple trials testing its use as a therapy for PNH. During prosecution of the '149 patent, the Examiner and Alexion discussed "17 different clinical studies using eculizumab that occurred prior to March 16, 2007." AMG1015, 735-737 (Boone declaration, ¶3); *see, also*, AMG1014, 764-766 (Boone declaration, ¶3), 590-591 (Bell declaration, ¶2). Nine of the trials discussed during prosecution related to treatment of PNH. AMG1015, 734-737 (Boone declaration, Table of Clinical Trials).

35. The results of one clinical trial testing eculizumab's effectiveness in treating PNH was published in a 2004 paper from Hillmen et al. (AMG1004; "Hillmen"). Hillmen states that its goal was to test "the clinical efficacy of eculizumab, a humanized antibody that inhibits the activation of terminal complement components, in patients with PNH." AMG1004, 552. Hillmen's trial enrolled 11 patients, and each patient "received infusions of eculizumab (600 mg) every week for four weeks, followed one week later by a 900-mg dose and then by 900 mg every other week through week 12." AMG1004, 552. To test for efficacy, Hillmen measured "whether eculizumab could reduce the incidence of intravascular hemolysis, hemoglobinuria, and transfusion requirements in patients with PNH." AMG1004, 553. To chemically and biologically measure hemolysis during the treatment regimen, Hillmen observed the patients' lactate dehydrogenase levels and the patients' need for blood transfusions. AMG1004, 555-556. Treated patients showed that a "decrease in lactate dehydrogenase began after a single dose of eculizumab in all patients," and that the mean transfusion rate dropped from 2.1 units per month to 0.6 units per month during eculizumab treatment. AMG1004, 555-556, Fig. 2, Table 1. Hillmen concluded that "[e]culizumab is safe and well tolerated in patients with PNH." AMG1004, 552. Hillmen further concluded that the antibody "reduces intravascular hemolysis, hemoglobinuria, and the need for

transfusion, with an associated improvement in the quality of life in patients with PNH." AMG1004, 552, 559.

36. Before Hillmen was published in 2004, Alexion also disclosed results from what appears to be the same trial as part of an abstract in the *British Journal of Haematology* in 2003. AMG1042 ("Hillmen '03"), Abstract. Hillmen '03, like Hillmen, discloses performing a pilot study of the effects of eculizumab on PNH in which 11 patients were given 600 mg of intravenous eculizumab weekly for four weeks, followed by 900 mg intravenous eculizumab one week later, and then 900 mg every other week. AMG1042. The results of the treatment were characterized as "remarkable both in reduction in haemolysis and transfusions and in the improvement in quality of life," and was labeled as "the first effective therapy for haemolysis in PNH." AMG1042, 87.

37. After the completion of the Hillmen trial, the same eleven patients were enrolled in Hill '05's extension study in which the patients continued to receive 900 mg of eculizumab every other week for a period of 52 weeks. AMG1047. This extension study found that "long term complement inhibition with eculizumab in this patient population has resulted in sustained reductions in hemolysis and blood transfusions and continued improvement in quality of life." AMG1047, 2565. Ten of the eleven patients continued in a second extension of the Hillmen study, in which they continued to receive 900 mg of eculizumab every

other week for another period of 52 weeks. AMG1011 ("Hill '04"), Abstract# 2823. Hill '04 concluded that "eculizumab is well tolerated and has persisting efficacy as demonstrated by decreased hemolysis and transfusions, as well as improvement in the symptoms of PNH, for over 2 years of therapy." AMG1011, Abstract# 2823.

38. Another publication that had disclosed methods of treating PNH patients with a pharmaceutical composition comprising eculizumab is Bell. AMG1005, ¶¶[0012], [0052], [0081]-[0096]. Bell discloses data that overlaps with that in Hillmen, Hill '04, and Hill '05. Like Hillmen, Bell discloses a Phase 2 Pilot Study involving 11 PNH patients treated with eculizumab over a period of 12 weeks. *Compare* AMG1005, ¶¶[0081]-[0082] *with* AMG1004, Abstract, 553-554. Both Bell and Hillmen used eculizumab, treated 6 male and 5 female patients, administered eculizumab intravenously, and used identical dosing and administration regimens (600 mg eculizumab weekly for four weeks, followed by a 900 mg dose, then 900 mg eculizumab every other week through week 12). AMG1005, ¶¶[0081]-[0096]; AMG1004, Abstract, 553-554. Based on these studies, Bell and Hillmen reported identical resulting data for the 12 week study. *Compare, e.g.*, AMG1005, ¶[0089] and AMG1004, 556:Table 1 (showing a decrease in transfusion rates from 2.1 units to 0.6 units per patient per month). Bell differs from Hillmen in that it also describes data from the two extension studies in which the 11 patients continued treatment for a total of two additional years in

which 900 mg eculizumab were administered every two weeks. AMG1005, ¶[0082]. The extension data and protocol described in Bell matches with the data and protocol in Hill '04 and Hill '05, which indicates that Bell's extension studies are the same as those presented in Hill '04 and Hill '05. *Compare, e.g.*, AMG1005, Fig. 1a-1b, ¶[0091]; AMG1047, Table 1 and Figure 2; AMG1011. In analyzing the results of these studies, Bell concluded that eculizumab is a "particularly useful" therapeutic for treating PNH. AMG1005, ¶[0012].

39. Hillmen also published the results of a larger Phase III clinical study, referred to as the "TRIUMPH" study, in 2006 (AMG1012; "Hillmen '06"). Hillmen '06 discloses treating 87 patients with an extended version of the dosing schedule used in Hillmen, in which "eculizumab was given at a dose of 600 mg weekly for 4 weeks, followed 1 week later by a 900-mg dose and then 900 mg every other week through week 26." AMG1012, 1233. Hillmen '06 measured the "primary end points" of hemoglobin levels and number of blood transfusions, and well as a general quality of life index. AMG1012, 1233. Based on these measurements, Hillmen '06 concluded simply and definitively that "[e]culizumab is an effective therapy for PNH." AMG1012, 1233.

40. Young et al. published the results from a second phase III trial of eculizumab's ability to treat PNH in 2006. AMG1013 ("Young"), Abstract. This second phase III trial was referred to as the "SHEPHERD" study. AMG1013,

Abstract. Young demonstrated that "eculizumab treatment markedly reduces intravascular hemolysis, thereby providing clinical benefit to treated [PNH] patients." AMG1013, Abstract.

41. I have created Table 1, shown below, to list a range of clinical trials performed before March 15, 2007 to test eculizumab's efficacy in treating PNH, and where those clinical trials were published. Table 1 presents my current understanding of the pre-March 15, 2007 clinical trials with eculizumab, although I have not performed an exhaustive search to identify all publications discussing clinical trials that used eculizumab. Therefore, there may be further clinical trials and further publications discussing clinical trials that are not included below.

Table 1 □Publications Disclosing the Claimed Antibody (Eculizumab)		
Study	**Alexion Study Number [1]**	**Exemplary disclosure**
Hillmen (AMG1004)	C02-001	"In this trial, we investigated whether *eculizumab* could reduce the incidence of

[1] Alexion's study numbers are provided based on the information in the Boone declaration submitted to the US Patent and Trademark Office during prosecution of the '148 patent, and based on Alexion's reference to clinical trials in regulatory filings. AMG1015, 736; AMG1024, 16, 50, 109; AMG1025, 2-3. The Boone declaration was also submitted during the prosecution of related U.S. Patent Nos. 9,725,504 and 9,718,880. AMG1016, 733-740; AMG1014, 763-766.

Table 1 Publications Disclosing the Claimed Antibody (Eculizumab)		
Study	**Alexion Study Number [1]**	**Exemplary disclosure**
Phase 2 Pilot Study 11 patients		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.
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.

Table 1 – Publications Disclosing the Claimed Antibody (Eculizumab)		
Study	**Alexion Study Number [1]**	**Exemplary disclosure**
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.

D. Eculizumab's structure – humanized anti-C5 monoclonal antibody (h5G1.1) containing a hybrid IgG2/IgG4 constant region – was publicly known prior to March 15, 2007

42. A POSA would have been aware that eculizumab was a humanized anti-C5 monoclonal antibody (h5G1.1) containing a hybrid IgG2/IgG4 constant region before March 15, 2007, based on Alexion's repeated publications about eculizumab and Alexion's patent applications relating to eculizumab.

43. Eculizumab is a humanized antibody. AMG1004, Abstract; AMG1012, 1233. Alexion developed eculizumab by humanizing the murine antibody 5G1.1 (i.e., "m5G1.1 mAb"). AMG1033, 1257; AMG1005, ¶[0052]. Therefore, eculizumab was also referred to as humanized 5G1.1 mAb (i.e., "h5G1.1-mAb"). *See, e.g.*, AMG1005, ¶[0052]; AMG1021, 1017; AMG1034, 1279. For example, Alexion's patent application published as U.S. 2005/0191298

(AMG1005; "Bell") references humanizing the 5G1.1 antibody to generate eculizumab:

> 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., 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.

AMG1005, ¶[0052].

44. In some publications before March 15, 2007, the term "5G1.1" was used to refer to the parental murine 5G1.1 antibody (*see, e.g.*, AMG1007, 19:47-49), but "5G1.1" was also regularly used to refer to eculizumab. For example, a 2005 publication by Tacken et al (AMG1034; "Tacken")[2] used the term "*h5G1.1-mAb*" synonymously with "5G1.1"—characterizing eculizumab as "*5G1.1, eculizamab [sic]*, Alexion Pharmaceuticals." AMG1034, 1279; *see also*, AMG1019, 56 ("*Eculizumab (5G1.1*; Alexion Pharmaceuticals) is a humanized monoclonal antibody."); AMG1020, 2123 ("*Eculizumab (5G1.1)*, the humanized

[2] Tacken's research study was supported by funding from Alexion.

AMG1034, 1278.

anti-C5 mAb."); AMG1021, 1017 ("*Synonyms 5G1.1, h5G1.1*, C5 complement inhibitor (Alexion), h5G 1.1 scFv").

45. Before March 15, 2007, a POSA also would have known that eculizumab contains a hybrid IgG2/IgG4 heavy chain constant region. AMG1049, 838-839; AMG1034, 1279. Alexion directly stated as much during prosecution of another application related to eculizumab (U.S. Patent Application No. 11/127,438), noting "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. Tacken confirms that eculizumab contains a hybrid IgG2/IgG4 constant region. AMG1034, 1278-1279. Tacken describes using "h5G1.1-mAb (5G1.1, eculizamab [sic]; Alexion Pharmaceuticals)" containing an "IgG2/IgG4 constant region." AMG1034, 1279.

46. A POSA also would have known that there were good reasons to use the IgG2/IgG4 hybrid structure of eculizumab. A POSA would have been aware that "the HuG2/G4 antibody design should prove useful in humanization of other antibodies intended for human use where elimination of FcR binding and C [*i.e.*, complement] activation may be desirable." AMG1031, 451; *see also*, AMG1034, 1280. Because the goal of using eculizumab in treating PNH is to reduce the level of complement activation, using a heavy chain constant region that avoids a

counter-productive activation of complement by the therapeutic antibody aligns well with the desired outcome. AMG1005, ¶¶[0003], [0012].

47. While the art taught other humanized antibodies that were generated from the murine 5G1.1 antibody, a POSA would have been readily able to distinguish those antibodies from eculizumab. For example, Thomas *et al.*'s publication in the journal *Molecular Immunology* in 1996 (AMG1023; "Thomas") disclosed generating "an intact humanized h5G 1.1 antibody" by transferring the CDR regions of murine 5G1.1 into a human antibody sequence. AMG1023, 1389, 1396. Thomas states that the humanized antibody was an "IgG4 isotype," and refers to it as "h5G1.1 HuG4." AMG1023, 1396. But a POSA would have known that this antibody was not eculizumab because, as discussed above, a POSA would have known that eculizumab has a hybrid IgG2/IgG4 constant region.

48. Accordingly, before March 15, 2007, a POSA would have been familiar with both how eculizumab was created and what the resulting antibody structure was.

E. Eculizumab's sequence was publicly known prior to March 15, 2007

49. A POSA would have been aware of the full amino acid sequence of eculizumab before March 15, 2007, based on Alexion's repeated publications about eculizumab and Alexion's patent applications relating to eculizumab. A declaration submitted by an Alexion employee (Dr. Laural Boone) stated that "the antibody

(eculizumab) used in each of [the eculizumab clinical trial] studies … contained the heavy and light chain sequences of SEQ ID NOs: 2 and 4." AMG1015, 736-738 (Boone declaration ¶6 and Table of Clinical Trials). Further, Alexion explicitly lists eculizumab's amino acid sequence in a series of publications and patent applications well before March 15, 2007.

50. One example of an Alexion publication that discloses eculizumab's sequence is U.S. Patent Application Publication No. 2003/0232972 (AMG1006; "Bowdish"). Bowdish is titled "Rationally Designed Antibodies," and published on December 18, 2003. The focus of Bowdish is the generation of "[i]mmunoglobulins or fragments thereof [that] have a peptide of interest inserted into a complementarity determining region (CDR) of an antibody molecule." AMG1006, ¶[0006]. The process requires a base antibody that "serves as a scaffold for presentation of the peptide." AMG1006, ¶[0006]. As one example of that process of peptide presentation, Bowdish describes generating an immunoglobulin to present a TPO peptide in which the TPO peptide was "transplanted into the heavy chain CDR3 of another antibody framework, 5G1.1." AMG1006, ¶¶[0191]-[0194], Figures 13A-13B. Evaluation of the heavy chain sequence shown in Figures 13A and 13B of Bowdish (*e.g.*, through the use of BLAST or a similar search tool) indicates very high identity with known human sequences outside of the CDR regions, and would have indicated to a POSA that the sequences shown in

Figures 13A-13B is humanized 5G1.1 with a hybrid IgG2/IgG4 heavy chain (*i.e.*, eculizumab). The humanized nature of Bowdish's 5G1.1 antibody is also seen in the use of an "anti-human IgG" in a binding assay to detect 5G1.1—and a POSA would have known that such a method would work only with an antibody that contained human sequences. AMG1006, ¶[0192] (disclosing detection of 5G1.1 in FACs analysis using an "anti-human IgG"). I have generated a schematic of Bowdish's immunoglobulin structure, as shown below as Figure 3, with green representing the claimed sequences and the blue shading indicating the location in which the TPO peptide was inserted in place of the 5G1.1 CDR3 region:

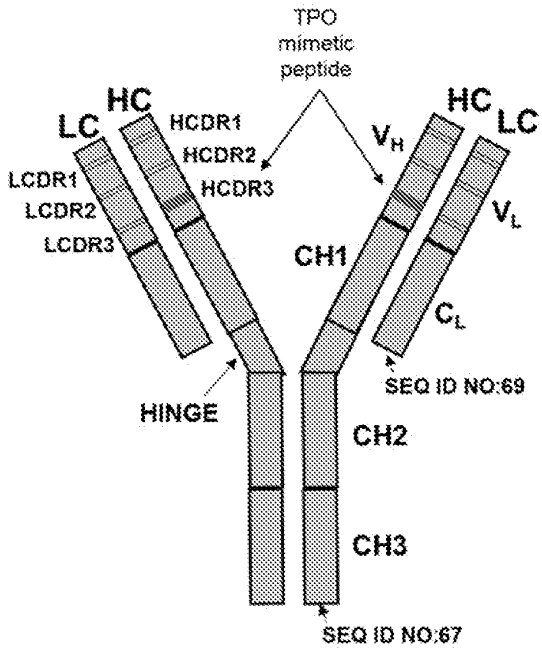


Figure 3: Diagram of the insertion of a TPO-mimetic peptide into the sequence of 5G1.1, as described in Bowdish.

51. Bowdish provides the sequence of the eculizumab-based immunoglobulin in Figure 13A (heavy chain; SEQ ID NO: 67) and Figure 13B (light chain; SEQ ID NO: 69). I note that the sequences in Figure 13A and Figure 13B contain leader sequences, identified as the portions of the sequence that are italicized. AMG1006, Figures 13A-13B. A POSA would have understood that leader sequences are cleaved off as a standard part of the maturation of an antibody. AMG1015, 546; AMG1014, 513; AMG1048, Figure 6; AMG1045, 582. I have aligned the mature sequences from Bowdish's construct with SEQ ID NOs: 2 and 4 from the '149 patent to generate Figures 4 and 5. As shown below in Figure 4, the mature portion of SEQ ID NO: 69 from Bowdish (*i.e.*, amino acids 23-236) aligns perfectly with the entirety of SEQ ID NO: 4 from the '149 patent.[3] Similarly, Figure 5 shows that the mature portion of SEQ ID NO: 67 from Bowdish (*i.e.*, amino acids 20 to 472) aligns perfectly with SEQ ID NO: 2 from the '149 patent outside of the heavy chain CDR3 region.

[3] The alignments presented in this declaration were prepared by entering the sequences in question into the EMBOSS Needle alignment tool, which is available at: https://www.ebi.ac.uk/Tools/psa/emboss_needle/.

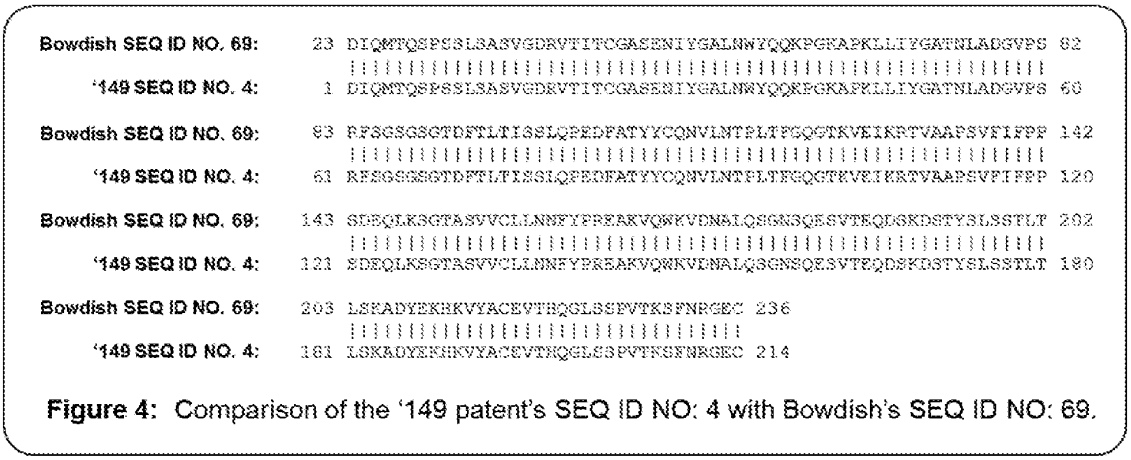


Figure 4: Comparison of the '149 patent's SEQ ID NO: 4 with Bowdish's SEQ ID NO: 69.

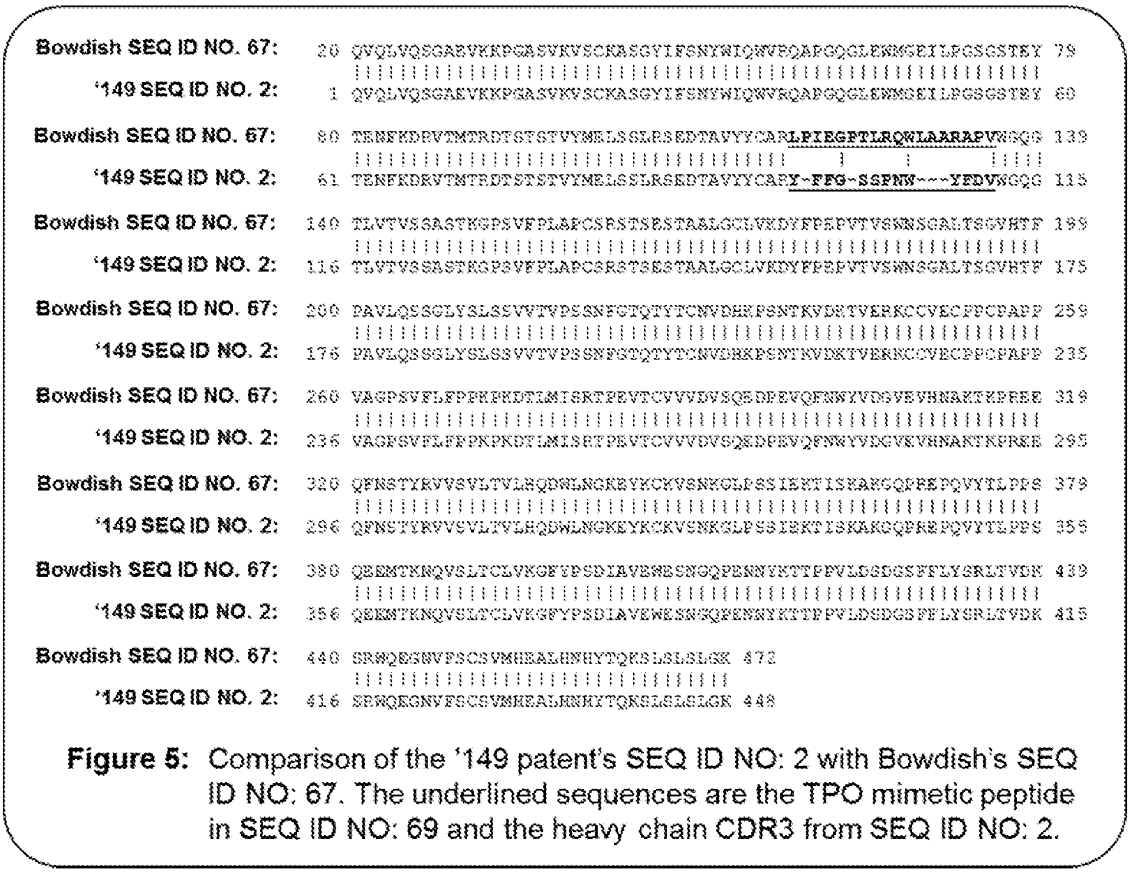


Figure 5: Comparison of the '149 patent's SEQ ID NO: 2 with Bowdish's SEQ ID NO: 67. The underlined sequences are the TPO mimetic peptide in SEQ ID NO: 69 and the heavy chain CDR3 from SEQ ID NO: 2.

52. While Bowdish does not provide the sequence of the heavy chain CDR3 sequence from h5G1.1, a POSA would have readily been able to find that

sequence from other publications. For example, Bowdish references U.S. Pat. No. 6,355,245 (AMG1007; "Evans") as describing the generation of the h5G1.1 antibody. AMG1006, ¶[0191] (referencing U.S. Patent Application No. 08/487,283, which issued as Evans); *see also*, AMG1005, ¶[0052]. Evans is titled "C5-Specific Antibodies for the Treatment of Inflammatory Diseases," and issued on March 12, 2002. Evans describes generating the murine 5G1.1 antibody, describes making a series of different humanized 5G1.1 scFv and full-length antibodies containing the CDR regions from the murine 5G1.1 antibody, and provides exemplary sequences of humanized scFv's and humanized light chain sequences.[4] AMG1007, 37:35-39:30, 42:59-45:33. Evans discloses the CDR regions used in the humanized 5G1.1 antibodies, which are underlined in the sequences provided in Figures 18 and 19 of Evans and identified by sequence number elsewhere. AMG1007, Figures 18-19, Example 11.

53. Evans discloses the sequences of humanized 5G1.1 scFv's, including an scFv whose variable regions align with eculizumab's variable regions.

[4] 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 ("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.

AMG1007, 42:59-45:33. The scFv designated "5G1.1 scFv CO12" contains all six 5G1.1 CDR regions and its sequence aligns perfectly with the eculizumab variable regions claimed by the '149 patent, as shown below in Figures 6 and 7. AMG1007, 44:4-23.

```
'149 SEQ ID NO. 2:     1 QVQLVQSGAEVKKPGASVKVSCKASGYIFSNYWIQWVRQAPGQGLEWMGE   50
                         ||||||||||||||||||||||||||||||||||||||||||||||||||
Evans SEQ ID NO. 20: 127 QVQLVQSGAEVKKPGASVKVSCKASGYIFSNYWIQWVRQAPGQGLEWMGE  176

'149 SEQ ID NO. 2:    51 ILPGSGSTEYTENFKDRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARYF  100
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||
Evans SEQ ID NO. 20: 177 ILPGSGSTEYTENFKDRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARYF  226

'149 SEQ ID NO. 2:   101 FGSSPNWYFDVWGQGTLVTVSS   122
                         |||||||||||||||||||||||
Evans SEQ ID NO. 20: 227 FGSSPNWYFDVWGQGTLVTVSS   248
```

Figure 6: Comparison of the heavy chain variable region disclosed in Evans as part of SEQ ID NO: 20 (*i.e.*, 5G1.1 scFv CO12) and the heavy chain variable region disclosed in the '149 patent as SEQ ID NO: 2. The underlined sequences are the CDRs identified in Evans.

```
'149 SEQ ID NO. 4:     1 DIQMTQSPSSLSASVGDRVTITCGASENIYGALNWYQQKPGKAPKLLIYG   50
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||
Evans SEQ ID NO. 20:   3 DIQMTQSPSSLSASVGDRVTITCGASENIYGALNWYQQKPGKAPKLLIYG   52

'149 SEQ ID NO. 4:    51 ATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQNVLNTPLTFGQ  100
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||
Evans SEQ ID NO. 20:   53 ATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQNVLNTPLTFGQ  102

'149 SEQ ID NO. 4:   101 GTKVEIK   107
                         |||||||
Evans SEQ ID NO. 20: 103 GTKVEIK   109
```

Figure 7: Comparison of the light chain variable region disclosed in Evans as part of SEQ ID NO: 20 (*i.e.*, 5G1.1 scFv CO12) and the light chain variable region disclosed in the '149 patent as SEQ ID NO: 4. The underlined portions are the CDRs identified in Evans.

54. A POSA also would have been aware of the sequence of the constant regions of eculizumab. The heavy and light chain constant regions are disclosed,

for example, as part of Bowdish. AMG1006, Figures 13A-13B. And, as discussed above, Tacken and other publications disclose that eculizumab's heavy chain has a hybrid IgG2/IgG4 constant region. AMG1033, 1257; AMG1049, 838-839; AMG1034, 1279; AMG1008, 12:35-37, Figure 15. While Tacken does not directly list the sequence of eculizumab, it cites a publication by Mueller *et al.* (AMG1031; "Mueller II") as providing information about the hybrid IgG2/IgG4 constant region portion of eculizumab. AMG1034, 1279, 1280, 1285; *see also*, AMG1033, 1257, 1263. Mueller II describes the hybrid domain as "containing the CH1 domain and hinge linker region of human IgG2 followed by the CH2 and CH3 domains of the human IgG4 constant region." AMG1031, 451; *see also*, AMG1032, 1, 4. Mueller II provides the amino acid sequence of the hybrid constant region, and provides guidance that this hybrid region "should prove useful in humaniz[ed] . . . antibodies intended for human use where elimination of FcR binding and [complement] activation may be desirable." AMG1031, 451, 449:Figure 9.

55. Alexion's International Application Publication No. WO1997/011971 (AMG1008; "Mueller") also discloses the sequence of eculizumab's hybrid IgG2/IgG4 heavy chain constant region, along with information on how to make and use an antibody with such a hybrid constant region. Mueller taught methods of developing recombinant antibodies to reduce immune-mediated organ transplant rejection, including antibodies comprising a hybrid IgG2/IgG4 constant region.

AMG1008, 1:4-19. Mueller provides the sequences of two antibodies targeting the P-selectin protein VCAM as examples, and notes that they have a heavy chain "containing the C1 and hinge region of human IgG2 and the C2 and C3 regions of human IgG4" that does not interact with the FcRI receptor and does not activate the endogenous complement system. AMG1008, 12:24-30; 34:11-13. And Mueller uses "h5G1.1 CO12 HuG2/G4 mAb" as a control antibody that shares that same hybrid IgG2/IgG4 constant region. AMG1008, 12:35-37, Figure 15. As part of these disclosures, Mueller provides the amino acid sequence of the hybrid IgG2/IgG4 constant region used in its antibodies. For example, the hybrid IgG2/IgG4 constant region sequence is included as part of the sequence of 3F4, one of its anti-VCAM antibodies.

56. As shown in Figure 8 below, alignment of the amino acid sequence of Mueller's hybrid heavy chain constant region from the 3F4 HuG2/G4 antibody and the '149 patent's SEQ ID NO: 2 shows that they disclose the same heavy chain constant region sequence.

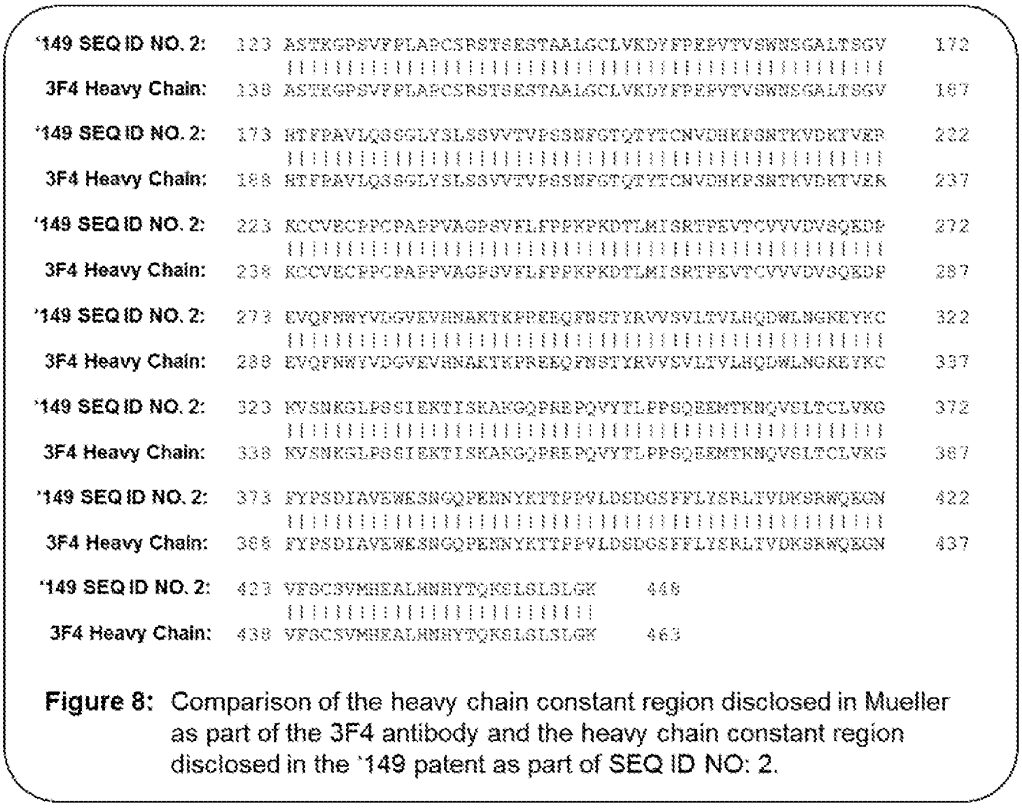


Figure 8: Comparison of the heavy chain constant region disclosed in Mueller as part of the 3F4 antibody and the heavy chain constant region disclosed in the '149 patent as part of SEQ ID NO: 2.

57. Mueller also discloses the light chain of the 3F4 HuG2/G4 antibody. As shown in Figure 9 below, the light chain constant region in 3F4 HuG2/G4 is the same as the light chain constant region disclosed in SEQ ID NO: 4 of the '149 patent.

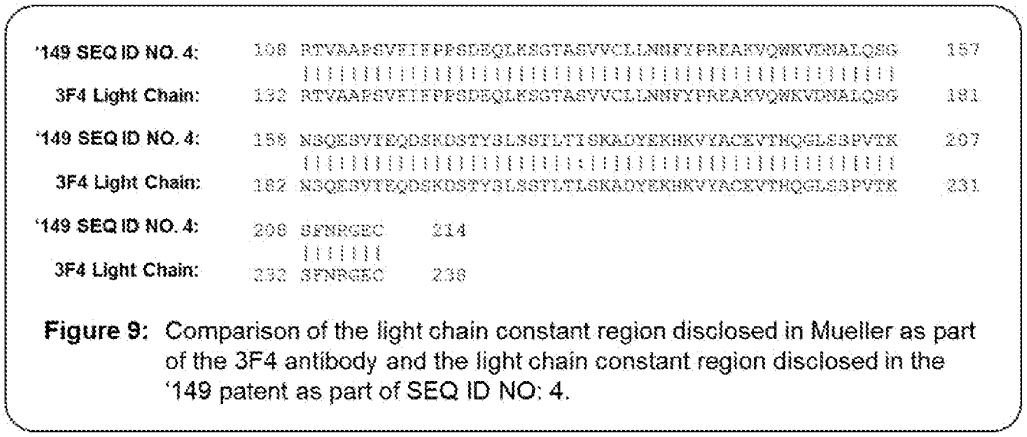


Figure 9: Comparison of the light chain constant region disclosed in Mueller as part of the 3F4 antibody and the light chain constant region disclosed in the '149 patent as part of SEQ ID NO: 4.

58. To better illustrate which sequences from a humanized 5G1.1 antibody are disclosed in Bowdish, Evans, and Mueller, I generated Figure 10 below. Figure 10 shows how large, overlapping portions of the claimed antibody sequences (shown in green) are disclosed by these references.

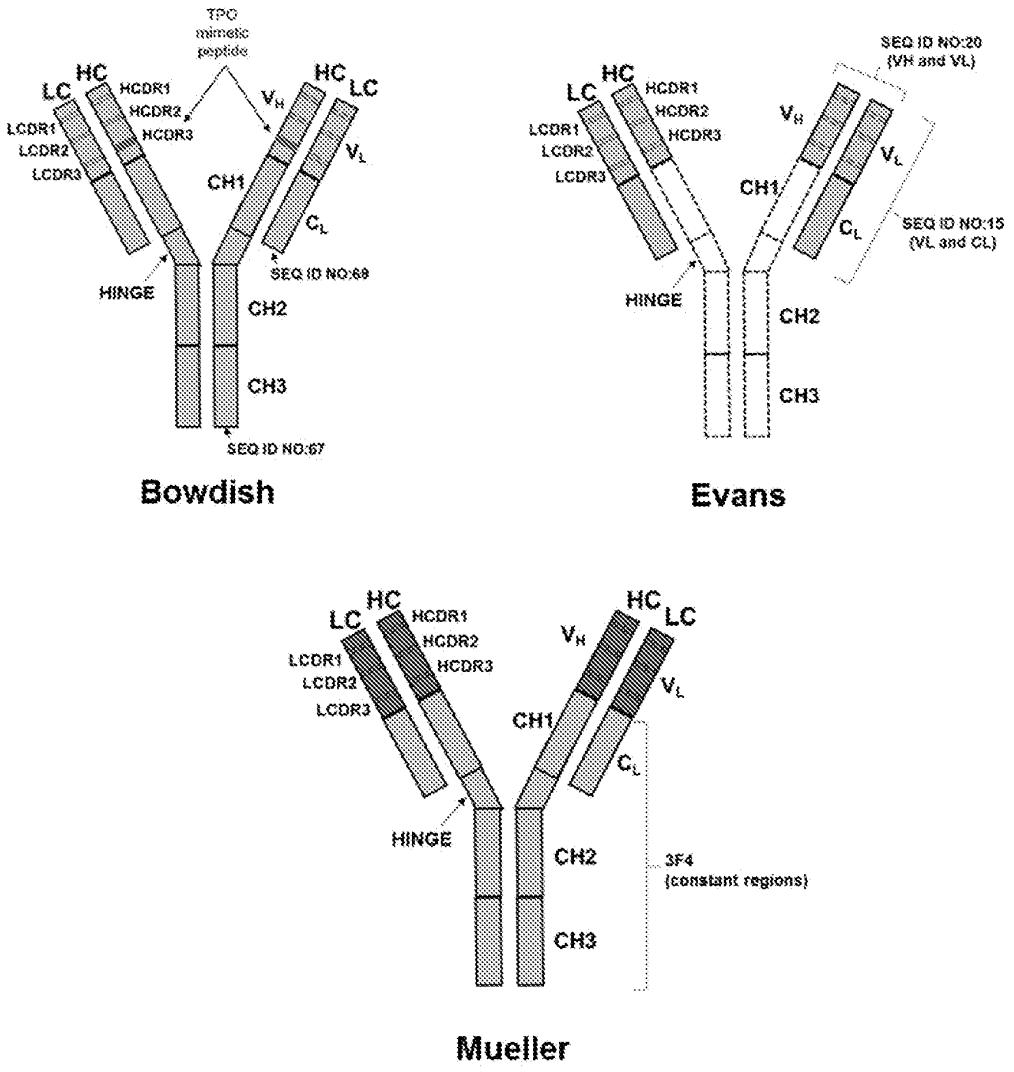


Figure 10: Diagram of the portions of the claimed antibody sequence disclosed in Bowdish (SEQ ID NOs: 67 and 69), Evans (SEQ ID NOs: 15 and 20), and Mueller (as part of the 3F4 antibody). *Green*: sequences matching SEQ ID NOs: 2 and 4 from the '149 patent; *blue*: other sequences

VII. The '149 patent specification and claims

59. The '149 patent is generally directed to an anti-C5 antibody comprising SEQ ID NOs: 2 and 4 (*i.e.*, the heavy and light chains of eculizumab).

AMG1001, 4:60-67, 30:16-47, 39:2-4; AMG1015, 596. The background section of the '149 patent describes a previous clinical trial with eculizumab, and notes that "[i]n a preliminary, 12-week, open-label clinical study in 11 PNH patients, eculizumab was shown to reduce intravascular hemolysis and transfusion requirements." AMG1001, 1:64-67 (citing AMG1004). The '149 patent specification reports the results of further clinical trials, and alleges that "[i]t has been surprisingly discovered that certain aspects of quality of life were unexpectedly improved by the treatment of PNH patients with eculizumab." AMG1001, 2:18-20.

60. Claim 1 of the '149 patent, the sole claim, is reproduced below:

> 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.

VIII. The meaning of claim terms

61. I understand that terms of the claims are to be given their ordinary and customary meaning in light of the specification and prosecution history—that is, the meaning that the term would have to a person of ordinary skill in the art in question at the time of the invention. Having reviewed the '149 patent specification, claims, and prosecution history, I do not view any term as requiring specific construction. Accordingly, where not specifically discussed I have given

all claim terms their plain and ordinary meaning to a POSA consistent with the specification and prosecution history of the '149 patent.

IX. The basis of my analysis

A. The basis of my analysis with respect to anticipation

62. I understand that a reference anticipates a claim if it discloses each and every element recited in the claim, arranged as in the claim. I understand that the disclosure of the elements of the claim can be either express or inherent. I also understand that for an element to be inherently present means that it is necessarily present, and that inherency requires more than probabilities or possibilities.

63. I understand that the anticipating reference must allow a POSA to make and use the claimed invention without the need for undue experimentation in light of the general knowledge available in the art. The factors that I have considered in determining whether a reference sets forth the elements of a claim in a sufficient manner such that a POSA could have made and used the claimed invention without undue experimentation include: the breadth of the claim, the nature of the invention, the state of the prior art, the level of one of ordinary skill, the level of predictability in the art, the amount of direction provided by the reference, the existence of working examples, and the quantity of experimentation needed to make or use the invention claimed.

B. The basis of my analysis with respect to obviousness

64. I understand that an obviousness analysis involves determining the scope and content of the prior art; comparing the differences between the claimed invention and the prior art; and resolving the level of ordinary skill in the pertinent art.

65. I also understand that obviousness can be established by combining or modifying the teachings of the prior art to achieve the claimed invention. I understand that the reason to combine prior art references must be shown. And I understand that where there is a reason to modify or combine the prior art to achieve the claimed invention, there must also be a reasonable expectation of success in so doing. I understand that the reason to combine prior art references can come from a variety of sources, not just the prior art itself or the specific problem the patentee was trying to solve. And I understand that the references themselves need not provide a specific hint or suggestion of the alteration needed to arrive at the claimed invention; the analysis may include recourse to logic, judgment, and common sense available to a person of ordinary skill that does not need to be explicit in any reference.

66. I also understand that a combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results. I also understand that when there is a design need or market pressure to

solve a problem and there are a finite number of identified, predictable solutions, a skilled person has good reason to pursue the known options within his or her technical grasp. If this leads to the anticipated outcome, then that outcome is likely a product not of innovation, but of ordinary skill and common sense, and therefore is considered obvious according to patent laws.

67. I understand that where the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable amount, range, or ratio. Further, I understand that where a range of conditions are disclosed in the prior art, a claimed amount, range, or ratio is considered obvious if it overlaps with or is encompassed by what is disclosed in the prior art. I also understand that a range can be disclosed in multiple prior art references instead of in a single prior art reference.

68. I understand that a finding of obviousness for a specific amount, range, or ratio in a patent can be avoided by showing criticality of the claimed amount, range, or ratio. Criticality can generally be demonstrated by showing that the claimed range achieves unexpected results relative to the prior art amount, range, or ratio.

69. I understand that when considering the obviousness of an invention, one should also consider whether there is any objective evidence that supports the non-obviousness of the invention. I understand that objective evidence of non-

obviousness (also known as secondary considerations of non-obviousness) includes factors such as failure of others, unexpectedly superior results, praise in the industry, long-felt but unmet need, copying, and commercial success.

X. Ground 1: Claim 1 is anticipated by Hillmen.

70. Claim 1 is unpatentable as being anticipated by Hillmen, which discloses every element of claim 1 expressly or inherently, and would have enabled a POSA to make the antibody of claim 1. Hillmen was published on February 4, 2004. Accordingly, Hillmen would have been publicly available before March 15, 2007, and I understand that it therefore qualifies as prior art to the '149 patent. My analysis below shows that Hillmen teaches every element of claim 1, and would have enabled a POSA to make the antibody of claim 1.

A. Hillmen discloses the claimed anti-C5 antibody

71. Hillmen discloses claim 1's limitation of an "antibody that binds C5." AMG1001, 39:2. The clinical study detailed in Hillmen "investigated whether eculizumab could reduce the incidence of intravascular hemolysis, hemoglobinuria, and transfusion requirements in patients with PNH." AMG1004, 553. Hillmen describes eculizumab as an "antibody against terminal complement protein C5." AMG1004, Abstract. Similarly, Hillmen states that eculizumab "binds specifically to the terminal complement protein C5." AMG1004, 553. Accordingly,

Hillmen discloses using eculizumab, an antibody that binds C5. AMG1004, Abstract, 554.

72. Hillmen's eculizumab is an antibody that meets claim 1's requirement of comprising "a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4." AMG1001, 39:2-7. Alexion confirmed that this during prosecution of the '149 patent. A declaration submitted by an Alexion employee (Dr. Laural Boone) listed a range of clinical trials, 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." AMG1015, 736-738 (Boone declaration ¶6 and Table of Clinical Trials). While Dr. Boone did not specifically mention Hillmen, she did list the study "C02-001" as one of the studies that used an antibody comprising SEQ ID NOs: 2 and 4. AMG1015, 736 (Boone declaration, Table of Clinical Trials). And regulatory documents for Alexion's Soliris® product from the U.S. Food and Drug Administration and the Australian Department of Health confirm that "Study C02-001" was published in "Hillmen, P et al.: Effect of Eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria. NEJM. 2004; 350:552-558." AMG1024, 109; AMG1025, 2. Therefore, a POSA would understand Dr. Boone's reference to the C02-001 clinical trial to also be a reference to Hillmen.

73. Dr. Boone's characterization of eculizumab is consistent with the eculizumab sequences disclosed in the prior art before March 15, 2007, which, as discussed in §§VI.D-E above, matched perfectly with the sequences listed in SEQ ID NOs: 2 and 4. Thus, by "test[ing] the clinical efficacy of eculizumab, a humanized antibody," Hillmen necessarily disclosed using an antibody with the property of having the heavy and light chain amino acid sequences of SEQ ID NOs: 2 and 4, respectively. AMG1004, Abstract. My understanding is that since Hillmen necessarily discloses an antibody "comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4," it is considered an inherent disclosure of that antibody for the purposes of an anticipation analysis.

B. Hillmen would have enabled a POSA to make eculizumab

74. I understand that, to anticipate a claim, the anticipatory reference must enable one skilled in the art to make the anticipating subject matter without undue experimentation. Hillmen's disclosures would have enabled a POSA to make eculizumab, and therefore to make the antibody of claim 1.

75. Hillmen enabled a POSA to make eculizumab because eculizumab's amino acid sequence was readily available and understood in the art. *See,* §VI.D-E above. A POSA—aware of all the pertinent art—would have known that the eculizumab sequences were publicly available in Alexion publications such as Bowdish, Evans, and Mueller. AMG1006, ¶¶[0191]-[0194], Figures 13A-13B,

SEQ ID NOs: 67 and 69; AMG1007, Figures 18-19, Example 11; SEQ ID NOs: 15 and 20; AMG1008, 52-53, 56-57; *see also*, §VI.E above. A POSA would have been able to construct eculizumab's sequence in at least two ways:

- First, 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 discloses the portion of eculizumab's sequence—the heavy chain CDR3 region—that is not disclosed in Bowdish. AMG1006, ¶¶[0191]-[0194], Figure 13A-13B (SEQ ID NOs: 67 and 69); AMG1007, 44:4-13 (SEQ ID NO:20). These sequences are discussed in more detail in §VI.E above.
- Second, a POSA would have known that Evans discloses eculizumab's heavy and light chain variable regions, while Mueller discloses the hybrid IgG2/IgG4 heavy chain and light chain constant regions of eculizumab. AMG1007, 44:4-13 (SEQ ID NO:20); AMG1008, 58-61. These sequences are discussed in more detail in §VI.E.

76. A POSA's level of skill in constructing antibody sequences would have been high based on the extensive knowledge in the art about antibody sequences in general, and about eculizumab's heavy and light chain sequences in particular. *See* §§VI.A, D-E. Constructing the sequence of eculizumab would have

required only standard molecular and cellular biology methods that were well known in the art to provide predictable results. For example, Mueller states that "[s]tandard molecular biology techniques" can be used to clone and express recombinant antibodies. AMG1008, 37:37-39. And Evans provides guidance on both the "Cloning" and "Construction and Expression of Recombinant mAbs." AMG1007, 21:4-24:61, Examples 10-11. And the results of constructing eculizumab's sequence from the disclosures of Evans and Mueller would have been predictable because molecular cloning and expression of antibodies were standard techniques. No experimentation with different sequences or different methods would have been needed because the art provided eculizumab's specific sequences. Therefore, a POSA would have been able to make eculizumab from the disclosures of its sequence in, *e.g.*, Evans, Bowdish, and Mueller using routine and ordinary skill, without any undue experimentation.

* * *

77. Given the above, Hillmen teaches every element of claim 1, and would have enabled a POSA to make the claimed antibody. Therefore, Hillmen anticipates claim 1.

XI. Ground 2: Claim 1 is anticipated by Hill '05.

78. Claim 1 is unpatentable as being anticipated by Hill '05, which discloses every element of claim 1 expressly or inherently, and would have enabled a POSA to make the antibody of claim 1. Hill '05 was published on October 1, 2005. Accordingly, Hill '05 would have been publicly available before March 15, 2007, and I understand that it therefore qualifies as prior art to the '149 patent. My analysis below shows that Hill '05 teaches every element of claim 1, and would have enabled a POSA to make the antibody of claim 1.

A. Hill '05 discloses the claimed anti-C5 antibody

79. Hill '05 discloses claim 1's limitation of an "antibody that binds C5." AMG1001, 39:2-4. Hill '05 discloses that eculizumab is an anti-C5 antibody, characterizing it as "a humanized monoclonal antibody that specifically targets the complement protein C5." AMG1047, 2559. The goal of the clinical study described in Hill '05 was to test the clinical efficacy of eculizumab, specifically in "a 1-year follow-up study" to Hillmen. AMG1047, 2559. The Hill '05 study was "designed to assess the long-term efficacy and safety of eculizumab in patients with PNH." AMG1047, 2559. To do this, patients received "a maintenance dose of eculizumab." AMG1047, 2560. The results of Hill '05's "extension study showed that eculizumab therapy continues to be safe and well tolerated in PNH patients."

AMG1047, 2565. Accordingly, Hill '05 discloses using eculizumab, an antibody that binds C5. AMG1047, 2559.

80. Hill '05's eculizumab is an antibody that meets claim 1's requirement of comprising "a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4." AMG1001, 39:2-7. As discussed in §X.B above, Alexion confirmed that Hill '05's eculizumab consists of heavy and light chains with the sequences in SEQ ID NOs: 2 and 4 during prosecution of the '149 patent. A declaration submitted by an Alexion employee (Dr. Laural Boone) listed a range of clinical trials, 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." AMG1015, 736-738) (Boone declaration ¶6 and Table of Clinical Trials). While Dr. Boone's declaration did not specifically mention Hill '05, she did list the extension study "E02-001" as one of the studies that used an antibody comprising SEQ ID NOs: 2 and 4. AMG1015, 736 (Boone declaration, Table of Clinical Trials). Dr. Boone characterized study E02-001 as "Extension Study of Eculizumab in Patients with Transfusion Dependent, Haemolytic, Paroxysmal E02-001 Nocturnal Haemoglobinuria Who Previously Participated in Study C02-001." AMG1015, 736. The results of study C02-001 are presented in Hillmen. *See* §X.B.

81. Hill '05 presents the results of the E02-001 extension study that continued the protocol disclosed in Hillmen / C02-001. This is seen, for example, in Hill '05's characterization of its clinical trial as an extension of Hillmen's Phase 2 Pilot Study, stating that:

> We previously reported the outcome of an open-label study of eculizumab in patients with PNH. [*citing Hillmen*] Results of this 12-week study demonstrated a dramatic reduction in hemolysis and a concomitant increase in the proportion of PNH type III RBCs. In addition, this initial study showed a marked decrease in the rates of paroxysms and blood transfusions and an improvement in quality of life. 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.

82. Similarly, Hill '05 also stated that its study was an "open-label extension" of the initial 12-week trial disclosed in Hillmen:

> The 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*. All 11 patients from the acute-phase study enrolled in the extension study.

AMG1047, 2560. Based on the above, a POSA would recognize that the study reported in Hill '05 was Alexion's E002-001 clinical trial. Therefore, Dr. Boone's reference to the E02-001 clinical trial was also a reference to Hill '05. By disclosing a clinical trial of eculizumab, Hill '05 necessarily disclosed using an antibody with the property of having the heavy and light chain amino acid sequences of SEQ ID NOs: 2 and 4, respectively. AMG1047, Abstract.

83. Dr. Boone's characterization of eculizumab is also consistent with the eculizumab sequences disclosed in the prior art before March 15, 2007, which, as discussed in §§VI.D-E above, matched perfectly with the sequences listed in SEQ ID NOs: 2 and 4. Thus, by disclosing a clinical trial of eculizumab, Hill '05 necessarily disclosed using an antibody with the property of having the heavy and light chain amino acid sequences of SEQ ID NOs: 2 and 4, respectively. AMG1047, Abstract. My understanding is that since Hill '05 necessarily discloses an antibody "comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4," it is considered an inherent disclosure of that antibody for the purposes of an anticipation analysis.

B. Hill '05 would have enabled a POSA to make eculizumab

84. Hill '05's disclosures would have enabled a POSA to make eculizumab, and therefore to make the antibody of claim 1, for the same reasons as discussed in §X.C above.

85. The prior art contained extensive disclosures on antibody structure and sequence in general, and on eculizumab's structure and sequence in particular. *See* §§VI.A, D-E. A POSA would have known that eculizumab's sequences were publicly available in Alexion publications such as Bowdish, Evans, and Mueller. AMG1006, ¶¶[0191]-[0194], Figures 13A-13B, SEQ ID NOs: 67 and 69; AMG1007, Figures 18-19, Example 11; SEQ ID NOs: 15 and 20; AMG1008, 52-53, 56-57; *see also*, §VI.E above. The high level of skill and knowledge in the art would have enabled a POSA to construct eculizumab's sequence, for example by using the sequences in Bowdish (all of eculizumab except the heavy chain CDR3) and Evans (eculizumab's heavy chain CDR3). AMG1006, ¶¶[0191]-[0194], Figure 13A-13B (SEQ ID NOs: 67 and 69); AMG1007, 44:4-13 (SEQ ID NO:20); *see* §VI.E above. Alternatively, a POSA would have been able to construct eculizumab's sequence using the sequences in Evans (variable regions) and Mueller (variable regions). AMG1007, 44:4-13 (SEQ ID NO:20); AMG1008, 58-61; *see* §VI.E above.

86. Constructing the sequence of eculizumab would have required only standard molecular and cellular biology methods that were well known in the art to provide predictable results. *See* §§VI.A, D-E; AMG1008, 37:37-39; AMG1007, 21:4-24:61, Examples 10-11. No experimentation with different sequences or different methods would have been needed because the art provided eculizumab's

specific sequences. Therefore, a POSA would have been able to make eculizumab from the disclosures of its sequence in, *e.g.*, Evans, Bowdish, and Mueller using routine and ordinary skill, without any undue experimentation.

* * *

87. Given the above, Hill '05 teaches every element of claim 1, and would have enabled a POSA to make the claimed antibody. Therefore, Hill '05 anticipates claim 1.

XII. Ground 3: Claim 1 is anticipated by Bowdish.

88. Claim 1 is unpatentable as being anticipated by Bowdish, which discloses every element of claim 1 expressly or inherently, and would have enabled a POSA to make the antibody of claim 1. Bowdish was published on December 18, 2003. Accordingly, Bowdish would have been publicly available before March 15, 2007, and I understand that it therefore qualifies as prior art to the '149 patent. My analysis below shows that Bowdish teaches every element of claim 1, and would have enabled a POSA to make the antibody of claim 1.

A. Bowdish discloses the claimed anti-C5 antibody

89. Bowdish discloses generating "[i]mmunoglobulins or fragments thereof [that] have a peptide of interest inserted into a complementarity determining region (CDR) of an antibody molecule." AMG1006, ¶[0006].

Bowdish's process requires a base antibody that "serves as a scaffold for presentation of the peptide." AMG1006, ¶[0006]. As one example of that process of peptide presentation, Bowdish describes generating an immunoglobulin to present a TPO peptide in which the TPO peptide was "transplanted into the heavy chain CDR3 of another antibody framework, 5G1.1." AMG1006, ¶¶[0191]-[0194], Figures 13A-13B. A POSA would have understood that Bowdish's "5G1.1" antibody is an anti-C5 antibody based on Bowdish's citation to Evans—which describes the anti-C5 antibody 5G1.1—for information about "[c]onstruction of 5G1.1." AMG1007, ¶[0191]. As discussed in detail below, Bowdish's "5G1.1" antibody necessarily comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4.

90. A POSA would have understood that the 5G1.1 antibody used in Bowdish is a humanized 5G1.1 antibody because Bowdish used an "anti-human" antibody to detect the presence of 5G1.1, and because such a detection method would not work on an antibody without human sequences. AMG1006, ¶[0192].

91. Bowdish provides almost all of the sequence of a 5G1.1 antibody, as part of its "5G1.1-TPO" antibody. AMG1006, ¶¶[0191]-[0194], Figures 13A-13B. The 5G1.1-TPO antibody has a TPO peptide inserted in place of the heavy chain CDR3 sequence of the 5G1.1 antibody. AMG1006, ¶¶[0191]-[0194], Figures 13A-13B. The sequences of the heavy and light chains of the 5G1.1+TPO antibody are

shown as SEQ ID NO:67 ("5G1.1-TPO Heavy Chain") and SEQ ID NO:69 ("5G1.1 Light Chain") in Bowdish's Figures 13A and 13B, respectively. AMG1006, Figures 13A-13B. As noted by Bowdish, the 5G1.1+TPO antibody is "the **5G1.1 full IgG construct** containing the TPO mimetic peptide in the heavy chain CDR3 positions." AMG1006, ¶[0193] (emphasis added). In Bowdish's Figures 13A and 13B, leader sequences are identified in italics, and a POSA would have understood that those leader sequences would be removed during normal protein maturation. *See* Figures 4 and 5 in §VI.E above; AMG1015, 546; AMG1014, 513; AMG1048, Figure 6; AMG1045, 582.

92. Bowdish identifies the TPO peptide as having the sequence of "Leu Pro Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Pro Val" (*i.e.*, "**LPIEGPTLRQWLAARAPV**"), and states that this peptide was used in place of the CDR3 sequence. AMG1006, ¶¶[0191], [0193]. Thus, a POSA would have known that the only portion of the sequence disclosed in Bowdish's Figures 13A-13B that is different from the original h5G1.1 "scaffold" antibody disclosed in Bowdish is the portion of SEQ ID NO: 67 that corresponds to the TPO peptide. AMG1006, ¶[0193]. I have generated Figure 11, shown below, to illustrate a comparison between Bowdish's 5G1.1-TPO antibody and its source h5G1.1 antibody.

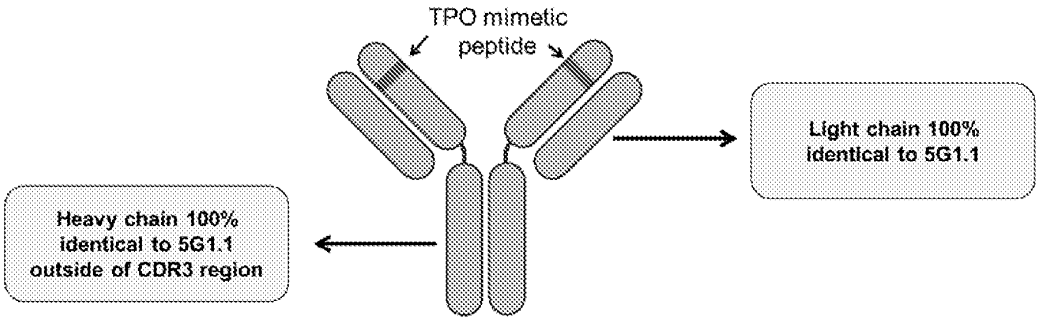


Figure 11: Summary of the 5G1.1-TPO antibody sequence relative to the 5G1.1 antibody. 5G1.1-TPO's areas of 100% identity to 5G1.1 are shown in green, and the TPO mimetic peptide is shown in blue.

93. Bowdish also discloses that its TPO-mimetic peptide was inserted in the place of the heavy chain CDR3 region that was removed from the source 5G1.1 antibody used to make the 5G1.1-TPO. AMG1006, ¶[0191]. 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] (emphasis added).

94. Bowdish references Evans for more information about the 5G1.1 antibody that was used as the scaffold, stating that "[c]onstruction of 5G1.1 is described in U.S. Appln. Ser. No. 08/487,283, incorporated herein by reference." AMG1006,¶[0191]. U.S. Application No. 08/487,283 issued as Evans. AMG1007, Face Page. I understand that when a patent incorporates by reference another

document (patent, patent application, or journal article, for example) it is akin to having the contents of that document also being recited within the patent that incorporated it. In this case, because Bowdish incorporates Evans by reference for the "[c]onstruction of 5G1.1", it is as if the teachings in Evans for construction of 5G1.1 were also recited within Bowdish.

95. Evans describes preparing antibodies and fragments thereof based on the murine 5G1.1 antibody, including humanized 5G1.1 sequences that are present in eculizumab. AMG1007, 19:47-49, 37:35-39:30, 40:31-45:4. In comparing Evans' sequences, a POSA would have seen that *all* of the 5G1.1 heavy chains in Evans contain the same CDR3 sequence: **YFFGSSPNWYFDV**. AMG1007, *e.g.*, 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. A POSA therefore would have understood that Bowdish (via its incorporation of Evans) teaches that **YFFGSSPNWYFDV** is the sequence of the 5G1.1 heavy chain CDR3 (i.e., the heavy chain CDR3 of eculizumab).

96. In view of the above, a POSA would have known that Bowdish's 5G1.1 antibody had a sequence comprising all of the sequence from the 5G1.1-TPO antibody except for the TPO peptide, with **YFFGSSPNWYFDV** instead placed in the heavy chain CDR3 region that is taught in Evans. I have generated Figure 12, shown below, to illustrate how a POSA would have understood the

structure of, and relationship between, Bowdish's 5G1.1 and 5G1.1-TPO constructs and the heavy chain CDR3 region of Evans.

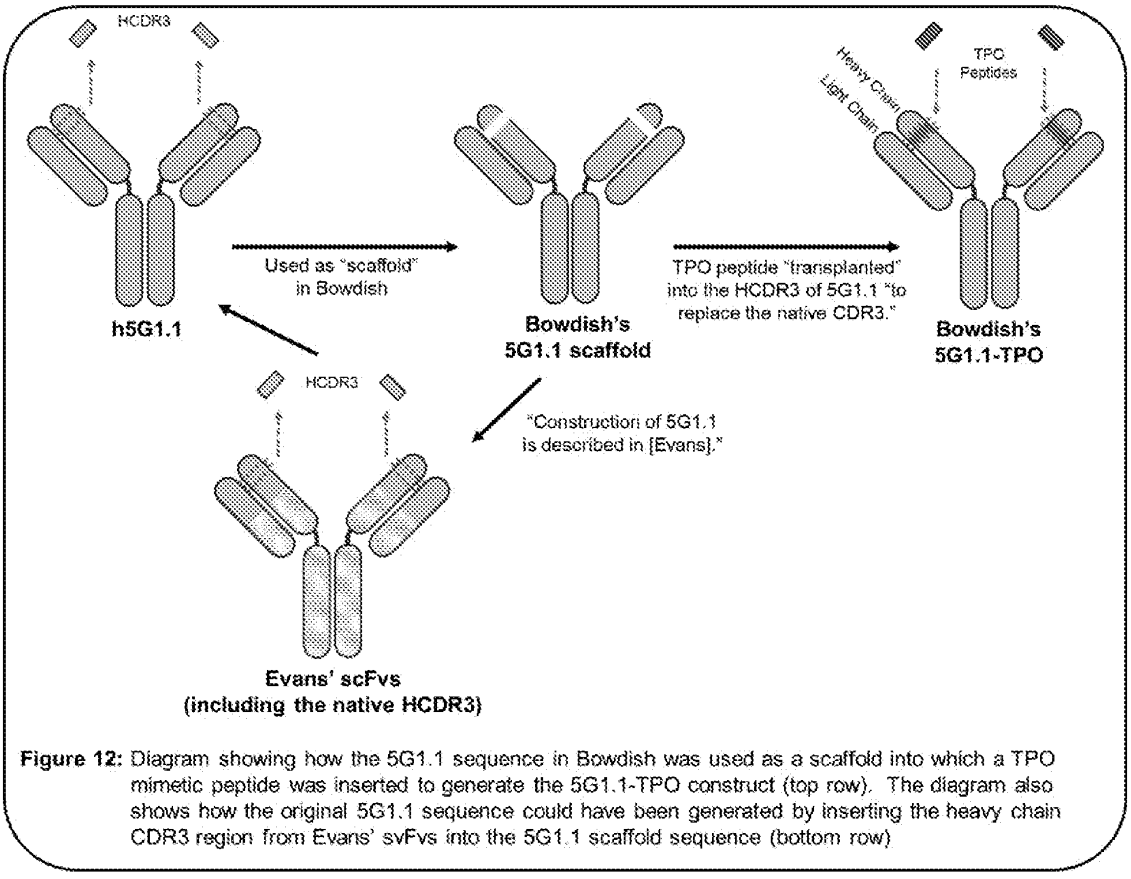


Figure 12: Diagram showing how the 5G1.1 sequence in Bowdish was used as a scaffold into which a TPO mimetic peptide was inserted to generate the 5G1.1-TPO construct (top row). The diagram also shows how the original 5G1.1 sequence could have been generated by inserting the heavy chain CDR3 region from Evans' svFvs into the 5G1.1 scaffold sequence (bottom row)

97. In Figure 12 and in other figures throughout this declaration, I have depicted combining antibody domains—these figures are intended to be a shorthand depiction of how a POSA could have spliced *DNA encoding* those regions together into different constructs for expressing the antibodies and antibody fragments shown in the figures.

98. The alignment in Figure 5, shown below and in §VI.E above, compares the mature heavy chain sequence of SEQ ID NO: 67 in Bowdish and SEQ ID NO: 2 from the '149 patent. The only difference in the two is the presence of the TPO-mimetic peptide (**LPIEGPTLRQWLAARAPV**) in SEQ ID NO: 67 instead of the CDR3 sequence (**YFFGSSPNWYFDV**). Thus, once a POSA replaced the TPO-mimetic peptide from Bowdish with the CDR3 region from Evans, she would have arrived at an antibody comprising SEQ ID NO: 2 from the '149 patent.

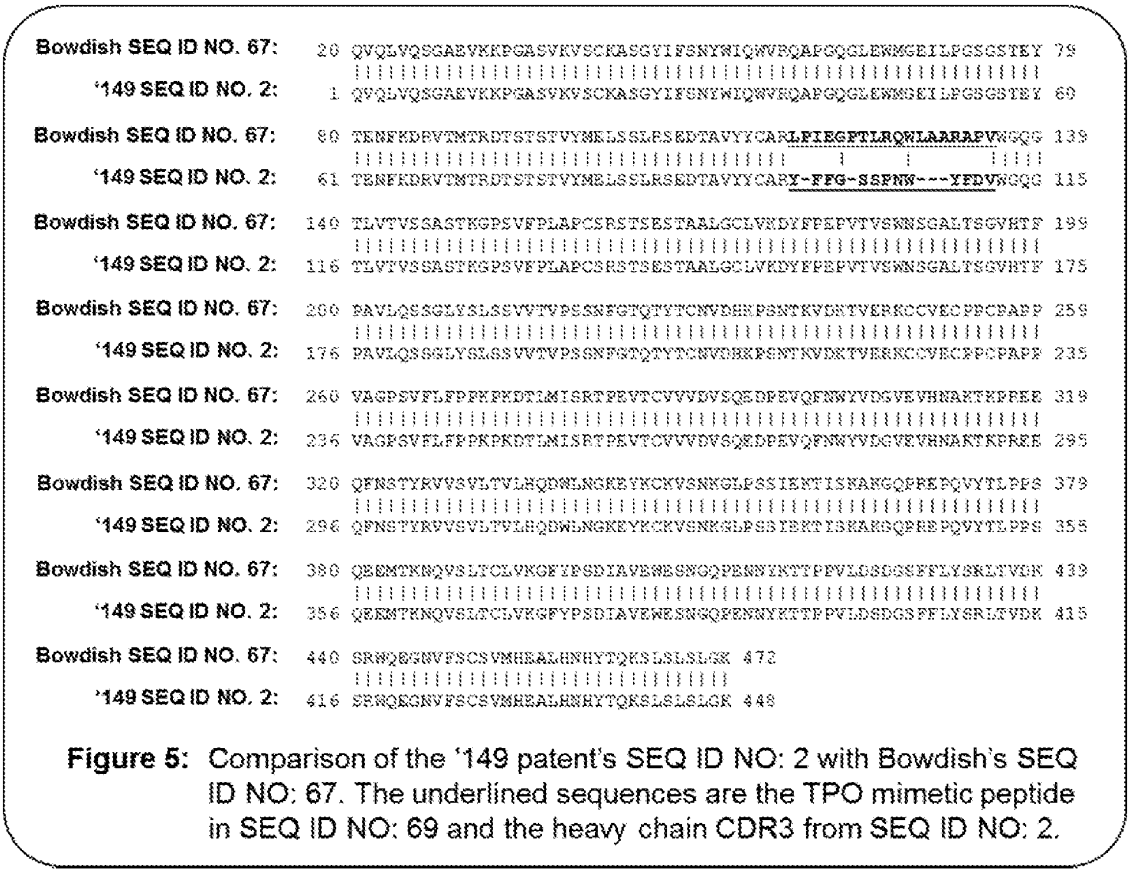


Figure 5: Comparison of the '149 patent's SEQ ID NO: 2 with Bowdish's SEQ ID NO: 67. The underlined sequences are the TPO mimetic peptide in SEQ ID NO: 69 and the heavy chain CDR3 from SEQ ID NO: 2.

99. I have generated the alignment shown in Figure 13 below to demonstrate that inserting Evans' heavy chain CDR3 sequence in place of the TPO peptide sequence in the mature form of Bowdish's SEQ ID NO:67 creates an heavy chain sequence that is 100% identical to SEQ ID NO: 2 from the '149 patent.

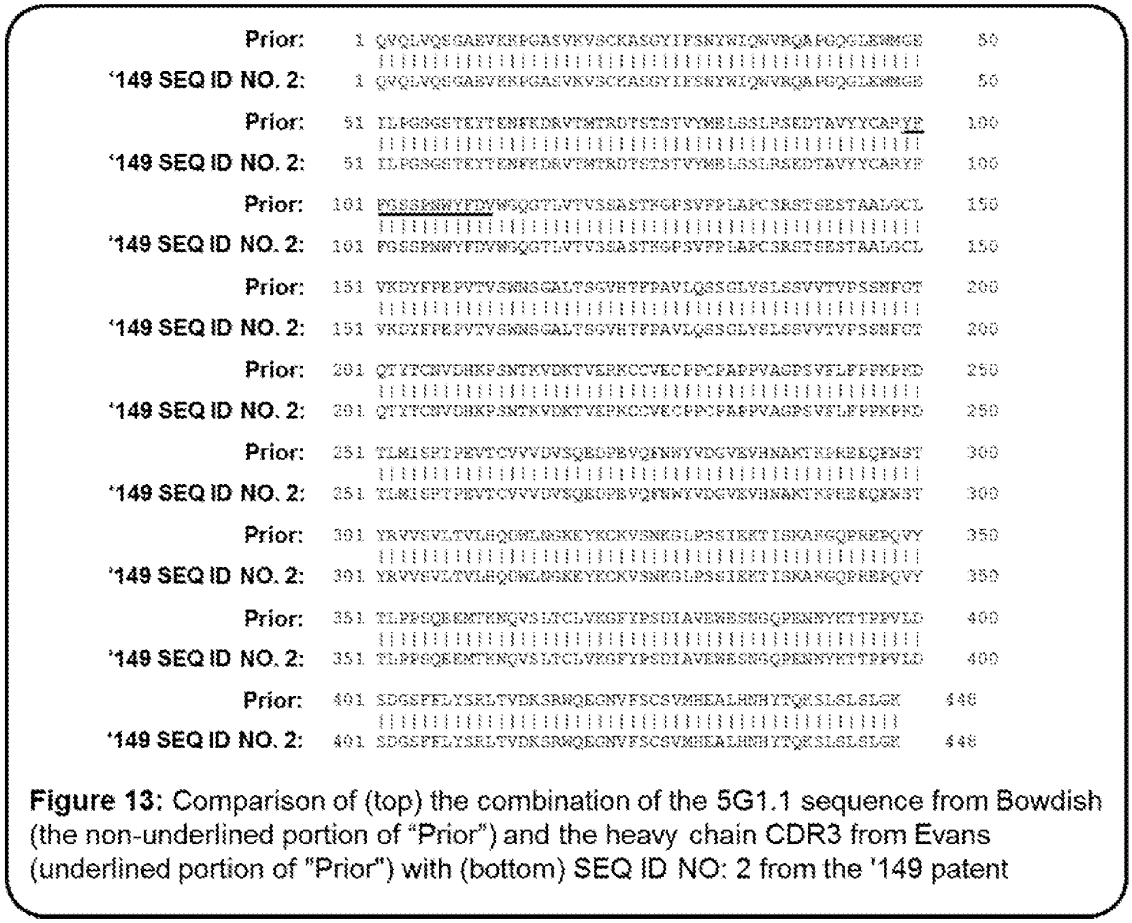


Figure 13: Comparison of (top) the combination of the 5G1.1 sequence from Bowdish (the non-underlined portion of "Prior") and the heavy chain CDR3 from Evans (underlined portion of "Prior") with (bottom) SEQ ID NO: 2 from the '149 patent

100. Further, the mature form of Bowdish's light chain SEQ ID NO:69 is a 100% match with SEQ ID NO:4 from the '149 patent—as shown in Figure 4 above. *See* §VI.E. These reconstituted sequences are what a POSA would have understood to be Bowdish's 5G1.1 source antibody. Accordingly, Bowdish's

disclosure of a "scaffold" 5G1.1 antibody is inherently a disclosure of an anti-C5 antibody comprising SEQ ID NO: 2 and SEQ ID NO: 4.

B. Bowdish would have enabled a POSA to make the claimed antibody

101. Bowdish's disclosures would have enabled a POSA to make eculizumab, and therefore to make the antibody of claim 1, for many of the same reasons as discussed in §§X.C and XI.C above.

102. A POSA would have known that Bowdish explicitly disclosed the entire amino acid sequence of 5G1.1 with the exception of the heavy chain CDR3 region, and that the incorporated reference Evans provided the sequence of that missing heavy chain CDR3 region. AMG1006, ¶¶[0191]-[0193], Figure 13A-13B (SEQ ID NOs:67 and 69); AMG1007, 44:4-13 (SEQ ID NO:20). Constructing the sequence of the source 5G1.1 antibody—which aligns perfectly with the the claimed antibody—would have required only standard molecular and cellular biology methods that were well known in the art to provide predictable results. *See* §§VI.A, D-E; AMG1008, 37:37-39; AMG1007, 21:4-24:61, Examples 10-11. No experimentation with different sequences or different methods would have been needed because Bowdish and its incorporated reference Evans provided specific sequences. Therefore, a POSA would have been able to make an anti-C5 antibody that binds C5 and has the claimed amino acid sequences from the disclosures in

Bowdish using routine and ordinary skill in this art, without any undue experimentation.

* * *

103. Given the above, Bowdish teaches every element of claim 1, and would have enabled a POSA to make the claimed antibody. Therefore, Bowdish anticipates claim 1.

XIII. Ground 4: Claim 1 would have been obvious over Bell, Bowdish, and Evans.

104. Claim 1 of the '149 patent would have been obvious to a POSA over the combination of Bell, Bowdish, and Evans.[5]

105. As I discuss below, Bell, Bowdish, and Evans teach all elements of '149 patent claim 1. Bell teaches "an antibody that binds C5" as claimed because it teaches treating PNH with a pharmaceutical composition comprising the anti-C5 antibody eculizumab. AMG1005, ¶¶[0052] and [0081]-[0096]. The only element

[5] Each of Bell, Bowdish, and Evans was published well before March 15, 2007. Bell was published on September 1, 2005. Bowdish was published on December 18, 2003. And Evans was published on March 12, 2002. Accordingly, each of these references would have been publicly available before March 15, 2007, and I understand that they therefore qualify as prior art to the '149 patent.

of claim 1 not expressly described in Bell is the requirement that the anti-C5 antibody "comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4." AMG1001, 39:2-4. This remaining element is taught by Bowdish and Evans. AMG1006, Figures 13A-13B, Example 4; AMG1007, 43:50-55, 44:4-13. In particular, Bowdish provides the full amino acid sequence of a humanized 5G1.1 antibody with a hybrid IgG2/IgG4 constant region (*i.e.*, eculizumab or an antibody like eculizumab) except for the heavy chain CDR3 sequence, and Evans provides the 5G1.1 antibody's heavy chain CDR3 sequence. AMG1006, ¶¶[0191]-[0194], Figs. 13A-13B, and SEQ ID NOs: 67 and 69; 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.

106. As discussed below, a POSA would have had reason to combine the disclosures of Bell, Bowdish and Evans to arrive at the antibody of claim 1, and would have had a reasonable expectation of doing so because these three references provide complementary information relating to an anti-C5 antibody that would have required only routine techniques to combine. Further, I am not aware of any objective evidence that weighs in support of patentability of the '149 patent claim. *See* §XV below. Accordingly, claim 1 of the '149 patent would have been obvious in view of Bell, Bowdish, and Evans.

A. Bell discloses all elements of claim 1 except the eculizumab amino acid sequence

107. Bell discloses that eculizumab is an anti-C5 antibody that blocks "the conversion of C5 into complement components C5a and C5b," and that eculizumab is also referred to as "h5G1.1-mAb" (*i.e.*, humanized 5G1.1-monoclonal antibody). AMG1005, ¶¶[0012], [0052]. Bell discloses methods of using eculizumab to treat hemolytic disease, and PNH in particular. AMG1005, ¶¶[0003], [0012], [0052], [0081]-[0096]. In particular, Bell describes treating 11 patients with eculizumab in a clinical trial, and evaluating the effects of eculizumab on PNH-related symptoms. AMG1005, ¶¶[0081]-[0096]. Bell's trial included 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. AMG1005, ¶[0082]. Bell concluded that "[p]atients in the two year study experienced a reduction in adverse symptoms associated with PNH." AMG1005, ¶[0096]. Bell's report of successfully treating PNH with eculizumab would have provided reason for a POSA to make eculizumab for the same purposes. Therefore, Bell discloses "an antibody that binds C5" as required by claim 1 in its disclosure of eculizumab, and provides a reason to make that antibody.

B. Bowdish and Evans disclose the entire amino acid sequence of eculizumab

108. The only element of claim 1 not expressly disclosed by Bell is the requirement that the antibody comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4.[6] However, Bowdish and Evans together disclose a heavy chain and a light chain of an anti-C5 antibody that consists of sequences that are identical to SEQ ID NOs: 2 and 4, respectively.

109. As discussed in Ground 3 above (*see* §XII), Bowdish provides the sequence its "5G1.1-TPO" antibody that was generated by replacing the heavy chain CDR3 region with a TPO-memetic peptide. AMG1006, ¶¶[0191]-[0194],

[6] As discussed in §VI.C above, the clinical studies taught in Bell include the clinical study (C02-001) disclosed in Hillmen. AMG1014, 763-770 (Boone declaration ¶6, Table of Clinical Trials); AMG1005, ¶[0082]. Therefore, the eculizumab used in Bell also comprises the amino acid sequences of SEQ ID NOs: 2 and 4, and Bell also anticipates claim 1 for the same reasons discussed in Ground 1 above for Hillmen. For the same reasons, the clinical trials disclosed in Hill '05 (study E02-001), Hill '04 (study X03-001), Hillmen '06 (C04-001), and Young 2006 (C04-002) also anticipate claim 1. AMG1014, 765, 767(Boone declaration ¶6); AMG1047, 2559-2560; AMG1012, 1235; AMG1011, Abstract; AMG1013, Abstract.

Figures 13A-13B. And Bowdish's Figures 13A and 13B show the sequences of the heavy and light chains of the 5G1.1+TPO antibody as SEQ ID NO:67 ("5G1.1-TPO Heavy Chain") and SEQ ID NO:69 ("5G1.1 Light Chain"), respectively. AMG1006, Figures 13A-13B. Bowdish identifies the TPO peptide as having the sequence of "Leu Pro Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala Pro Val" (*i.e.*, "**LPIEGPTLRQWLAARAPV**"), and states that this peptide was used in place of the CDR3 sequence. AMG1006, ¶¶[0191], [0193]. Based on the description of how the 5G1.1-TPO antibody was made, a POSA would have understood that the 5G1.1-TPO antibody is differs from the "scaffold" 5G1.1 antibody only in that it contains the TPO-mimetic peptide sequence in the CDR3 region.

110. A POSA would have understood, based on the description of the 5G1.1-TPO antibody, that removing the TPO-mimetic peptide present from the 5G1.1-TPO antibody would leave all of 5G1.1's sequence except for a gap in the CDR3 region. *See* Figure 11, above.

111. Also as discussed in Ground 3 above (*see* §XII), Evans provides the 5G1.1 heavy chain CDR3 region. Evans describes preparing antibodies and fragments thereof based on the murine 5G1.1 antibody, and *all* of the 5G1.1 heavy chains in Evans contain the same CDR3 sequence: **YFFGSSPNWYFDV**. AMG1007, *e.g.*, 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. A POSA therefore would have understood that **YFFGSSPNWYFDV** is the sequence of the heavy chain CDR3 of 5G1.1.

112. A POSA would have expected that replacing the TPO-mimetic peptide sequence in SEQ ID NO:67 of Bowdish with the 5G1.1 heavy chain CDR3 sequence from Evans would provide the complete heavy chain sequence of 5G1.1. The alignment in Figure 5, shown above in §VI.E, shows that the only difference between the heavy chain sequence of SEQ ID NO: 67 in Bowdish and SEQ ID NO: 2 from the '149 patent is the presence of the TPO-mimetic peptide (**LPIEGPTLRQWLAARAPV**) in SEQ ID NO: 67 instead of the CDR3 sequence (**YFFGSSPNWYFDV**). Thus, as shown in Figure 13's alignment above (*see* §XII.A), once a POSA replaced the TPO-mimetic peptide with the CDR3 region with the CDR3 region from Evans, she would have arrived at SEQ ID NO: 2 from the '149 patent. Further, the mature form of Bowdish's light chain SEQ ID NO:69 is a 100% match with SEQ ID NO:4 from the '149 patent—as shown in Figure 4's alignment above (*see* §VI.E). Accordingly, Bowdish and Evans together disclose an anti-C5 antibody having the claimed sequences.

113. I have generated Figure 14, shown below, to illustrate how a POSA would have arrived at the claimed sequence by placing the heavy chain CDR3 disclosed in Evans into the 5G1.1 antibody sequence disclosed in Bowdish.

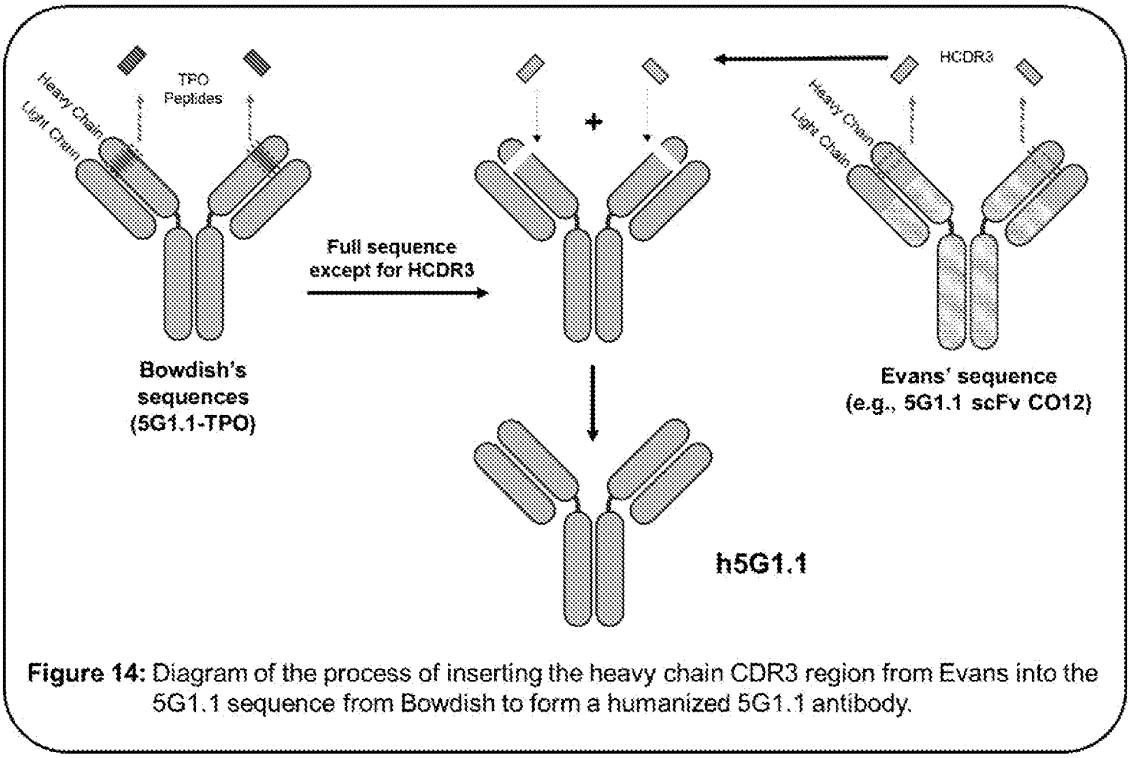


Figure 14: Diagram of the process of inserting the heavy chain CDR3 region from Evans into the 5G1.1 sequence from Bowdish to form a humanized 5G1.1 antibody.

C. A POSA would have had reason to combine Bell, Bowdish, and Evans to arrive at the antibody of claim 1

114. A POSA would have had a reason to make an anti-C5 antibody because Bell discloses that eculizumab—a known anti-C5 antibody—successfully treated PNH patients. AMG1005, ¶¶[0083]-[0096]. Because Bell does not provide the amino acid sequence of eculizumab, a POSA would have had reason to look to other references that did provide the sequence of an anti-C5 antibody in order to be able to express and produce an anti-C5 antibody for use in treating PNH. As discussed above, Bowdish and Evans are two publications that provide the

sequence of humanized 5G1.1 with a hybrid IgG2/IgG4 constant region (*i.e.*, the sequence of an antibody like eculizumab).

115. A POSA would have had reason to look to Bowdish because it provides most of the sequence of humanized 5G1.1 with a hybrid IgG2/IgG4 constant region as the framework of its 5G1.1-TPO antibody. AMG1006, ¶¶[0191]-[0194], Figures 13A-13B. And a POSA would have linked this disclosure to Bell because eculizumab was also known to a humanized 5G1.1 with a hybrid IgG2/IgG4 constant region. *See* §VI.D-E.

116. Knowing that the only change Bowdish made in creating the 5G1.1-TPO antibody was replacing the 5G1.1 heavy chain CDR3 region with a TPO-mimetic peptide, a POSA would have had reason to restore the original heavy chain CDR3 region into Bowdish's "scaffold" h5G1.1 in place of the TPO-mimetic peptide in order to make the source h5G1.1 antibody. A POSA would have had reason to look to Evans for the 5G1.1 heavy chain CDR3 sequence because both Bell and Bowdish incorporate Evans by reference while noting that Evans describes how the humanized 5G1.1 antibody was created. AMG1005, ¶[0052]; AMG1006, ¶[0191]. And identification of the h5G1.1 heavy chain CDR3 region in Evans was straightforward because the same heavy chain CDR3 region was used in all of Evans' humanized variants of the 5G1.1 antibody. 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.

117. This combination of references therefore would have led a POSA to generate a 5G1.1 anti-C5 antibody by replacing the TPO-mimetic peptide in the 5G1.1-TPO antibody with the heavy chain CDR3 sequence from Evans—thereby arriving at an antibody comprising SEQ ID NOs: 2 and 4 from the '149 patent.

D. A POSA would have had a reasonable expectation of success in practicing the antibody of claim 1

118. A POSA would have had a reasonable expectation of success in making the antibody of claim 1 from the disclosures of Bell, Bowdish, and Evans because doing so would have required only basic molecular and cellular biology techniques. Methods of cloning and producing antibodies were well-known in the art, and the required cloning would have required only basic molecular biology techniques such as PCR and restriction enzyme digests to replace the DNA encoding the TPO-mimetic peptide with DNA encoding the heavy chain CDR3 region from Evans. *See, e.g.*, AMG1006, ¶¶[0130]-[0131]; AMG1008, 37:37-39 (describing use of "[s]tandard molecular biology techniques" to prepare recombinant antibodies); AMG1007, 21:4-24:61, Examples 10-11 (describing use of "conventional recombinant DNA methods including restriction fragment subcloning and overlapping PCR procedures" to prepare recombinant antibodies).

Therefore, claim 1 would have been obvious over the combination of Bell, Bowdish, and Evans.

* * *

119. Given the above, Bell, Bowdish, and Evans teach every element of the antibody recited in claim 1, and a POSA would have had a reason to combine Bell, Bowdish, and Evans to arrive at the claimed antibody, with a reasonable expectation of success. Therefore, claim 1 would have been obvious to a POSA, notwithstanding any objective evidence (discussed in §XV below).

XIV. Ground 5: Claim 1 would have been obvious over Evans and Mueller.

120. Claim 1 of the '149 patent would have been obvious to a POSA over the combination of Evans and Mueller.[7]

121. As I discuss below, Evans and Mueller teach all elements of '149 patent claim 1. Evans and Mueller Bell teach "an antibody that binds C5." Evans is titled "C5-Specific Antibodies for the Treatment of Inflammatory Diseases," and

[7] Each of Evans and Mueller was published well before March 15, 2007. Evans published on March 12, 2002, and Mueller was published on April 3, 1997. Accordingly, both of these references would have been publicly available before March 15, 2007, and I understand that they therefore qualify as prior art to the '149 patent.

Mueller discloses "a humanized antibody directed against human C5." AMG1007, 1:1-3; AMG1008, 12:1-2. Further, Evans and Mueller together disclose claim 1's requirement that the anti-C5 antibody "comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4." AMG1001, 39:2-4; AMG1007, 43:50-55, 44:4-13; AMG1008, 7:28-8:26, 12:34-37, 58-61, Figure 9. In particular, Evans discloses variable regions from the 5G1.1 antibody, and Mueller discloses the constant regions from the 5G1.1 antibody.

122. Also as discussed below, a POSA would have had reason to combine the disclosures of Evans and Mueller to arrive at the antibody of claim 1 because Evan's teaches that its therapeutic 5G1.1 scFv fragments could be converted to full length antibodies, and because Mueller teaches that its hybrid constant regions are useful in humanized antibodies in which activation of complement is not desired. AMG1007, 45:24-33; AMG1008, 7:28-32, 8:23-26. And a POSA would have had a reasonable expectation of success in making the claimed antibody because doing so would have required only basic molecular and cellular biology techniques. Further, I am not aware of any objective evidence that weighs in support of patentability of the '149 patent claim. *See* §XV below. Accordingly, claim 1 of the '149 patent would have been obvious in view of Evans and Mueller.

A. A POSA would have had reason to look to Evans and Mueller for the sequence of an anti-C5 antibody

123. A POSA would have had reason to look to both Evans and Mueller for the sequence of an anti-C5 antibody, and would have had reason to combine the sequences from those references.

124. Evans discloses the creation of humanized antibodies and antibody fragments derived from the murine 5G1.1 antibody. AMG1007, 19:47-49, 37:35-39:30, 40:31-45:4. Evans states that these 5G1.1 antibodies are useful for "therapeutic treatments involving the pharmacologic inhibition of a patient's complement system." AMG1007, 1:16-17, 7:7-14; 19:47-67. Evans humanized antibody constructs include nine different humanized 5G1.1 scFv constructs, and Evans provides the amino acid sequences for each of those scFv's. AMG1007, 42:58-45:4 (construct numbers 2 and 11-18). An scFv is a well-known type of antibody fragment in which a single heavy chain variable region is connected to a single light chain variable region by a linker. *See, e.g.,* AMG1007, 6:39-41; AMG1040, 45-48. Therefore, in disclosing the sequence of humanized 5G1.1 scFv's, Evans provides humanized heavy and light chain variable region 5G1.1 sequences that could also be used in the context of a full length antibody. Evans itself teaches this, stating that the disclosed scFv's could be combined with constant domains, including hybrid IgG domains, to make a full length antibody. AMG1007, 45:24-33.

125. Mueller discloses developing new chimeric antibodies with a hybrid IgG2/IgG4 heavy chain constant region. AMG1008, 1:4-19, 8:20-26, 38:3-7. A chimeric antibody contains the variable regions from a non-human antibody and the constant regions from a human antibody. AMG1040, 29-30. Mueller's chimeric antibodies have hybrid IgG2/IgG4 heavy chain constant regions that "contain[] the C1 and hinge region of human IgG2 and the C2 and C3 regions of human IgG4." AMG1008, 12:24-30; 34:11-13, 8:23-26. Two of the antibodies Mueller describes as having the hybrid heavy chain constant region are the anti-C5 antibody "h5G1.1 CO12 HuG2/G4" and the anti-VCAM antibody 3F4. AMG1008, 8:23-26, 12:35-37, Figure 15, 52-53, 58-61. Mueller provides the amino acid sequence of the constant regions in such a hybrid G2/G4 antibody as part of its disclosure of the "3F4 HuG2/G4" antibody sequence. AMG1008, 52-53, 58-61.

126. A POSA would have had at least three reasons to combine the variable regions from Evans' scFv's with the constant regions from Mueller. First, a POSA would have viewed Mueller's hybrid constant region as useful because it does not activate the complement system. AMG1008, 7:28-32, 8:23-26. Preventing complement activation would have been important because the goal of administering an anti-C5 antibody such as those described in Evans was "the pharmacologic inhibition of a patient's complement system." AMG1007, 1:16-17. Activating the complement system while administering a complement inhibitor is

therefore inefficient and counterproductive. A POSA therefore would have recognized that using Mueller's hybrid constant region would allow more effective inhibition of the complement system than standard constant regions.

127. Second, Evans and Mueller are linked because both disclose humanized 5G1.1 antibodies. AMG1007, 19:47-49, 37:35-39:30, 40:31-45:4; AMG1008, 12:35-37, Figure 15. Further, Evans and Mueller use the same "CO12" nomenclature when referring to 5G1.1 antibodies. AMG1008, 12; AMG1007, 31:45-47, 44:4-13. The humanized sequences Evans provides include the variable regions in the scFv "5G1.1 scFv CO12" (SEQ ID NO: 20). AMG1007, 44:4-13; Certificate of Correction, at 42-44 (correction of SEQ ID NO: 20 at 121:32-123:48). And Mueller references the "h5G1.1 CO12 HuG2/G4" antibody. AMG1008, 12. Based on the overlap in inventors and the fact that Alexion is the assignee of both Mueller and Evans, a POSA would have understood that both publications were using the "CO12" nomenclature in the same way and that the "CO12" antibodies in each were linked. AMG1008, face page; AMG1007, face page. Therefore, while a POSA would have had reason to combine Mueller's constant regions with *any* of the humanized 5G1.1 variable sequences in Evans's scFv's, the nomenclature of the constructs would have provided a POSA with a further reason to use the variable regions from Evans' "5G1.1 scFv CO12."

128. Overlapping sequences between Mueller and Evans provide the third reason to combine the constant regions from Mueller with the variable regions from Evans' "5G1.1 scFv CO12." The light chain variable region from 5G1.1 scFv CO12 is also present in Evans' light chain sequence named "5G1.1 VL+O12" (SEQ ID NO: 15).[8] AMG1007, 43:50-55, Certificate of Correction at 31-33 (correction of SEQ ID NO: 15 at 103:55-107:9) and 42-44 (correction of SEQ ID NO: 20 at 121:32-123:48). The light chain constant region of 5G1.1 VL+O12 is, in turn, is the same as the light chain constant region in Mueller's 3F4 antibody. AMG1008, 52-53; AMG1007, 43:50-55, Certificate of Correction at 31-33 (correction of SEQ ID NO: 15 at 103:55-107:9). Therefore, a POSA would have known that the combination of Mueller's "h5G1.1 CO12 HuG2/G4" light chain constant region with the light chain variable region from Evans' "5G1.1 scFv CO12" *had already been made and tested* as the 5G1.1 VL+O12 light chain in Evans. Given this known pairing of light chain sequences, a POSA would have had reason to use that

[8] Amino acids 3-111 of 5G1.1 scFv CO12 (SEQ ID NO: 20) are the same as amino acids 1-109 of 5G1.1 VL+O12 (SEQ ID NO: 15). AMG1007, 43:50-55, Certificate of Correction at 31-33 (correction of SEQ ID NO: 15 at 103:55-107:9) and 42-44 (correction of SEQ ID NO: 20 at 121:32-123:48).

light chain and also make the analogous pairing between the h5G1.1 CO12 HuG2/G4 and 5G1.1 scFv CO12 heavy chain regions.

B. Evans and Mueller disclose the entire claimed anti-C5 antibody sequences

129. Evans and Mueller collectively disclose the complete heavy chain and light chain sequences of an anti-C5 5G1.1 full length antibody. Evans discloses the variable regions, while Mueller discloses the constant regions. AMG1007, 43:49-54, 44:4-13, SEQ ID NOs:15, 20; AMG1008, 58-61. Accordingly, as shown in Figure 15 below, Evans and Mueller taught complementary parts of an anti-C5 antibody sequence:

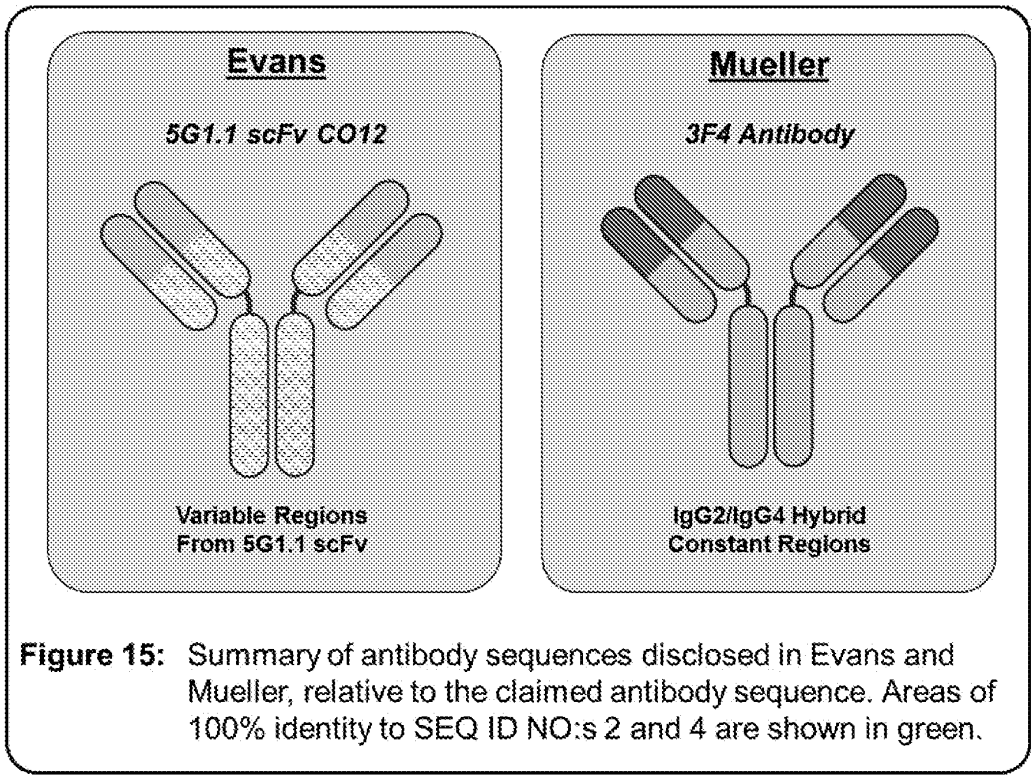


Figure 15: Summary of antibody sequences disclosed in Evans and Mueller, relative to the claimed antibody sequence. Areas of 100% identity to SEQ ID NO:s 2 and 4 are shown in green.

130. As discussed above, a POSA would have had reason to look to Evans' humanized 5G1.1 scFv construct named "5G1.1 scFv CO12" (SEQ ID NO: 20) for variable region sequences. AMG1007, 31:45-47, 44:4-13, Certificate of Correction at 42-44 (correcting SEQ ID NO: 20 sequence provided at 121:32-123:48 of the specification). As an scFv, 5G1.1 scFv CO12 contains (1) a humanized heavy chain variable region of 5G1.1, (2) a linker, and (3) a humanized light chain variable region of 5G1.1, but lacks any constant regions. AMG1007, 6:39-41; AMG1040, 45-48.

131. A POSA would have been able to readily identify the heavy and light chain variable region sequences in 5G1.1 scFv CO12 (*i.e.*, in SEQ ID NO: 20). SEQ ID NO: 20 contains 248 amino acids. A POSA would have known that the linker in 5G1.1 scFv CO12 is amino acids 112-126 of SEQ ID NO: 20, as these 15 amino acids (GGGGSGGGGSGGGGS) were generally known in the art as an scFv linker sequence. AMG1007, Certificate of Correction at 42-44 (correcting SEQ ID NO: 20 sequence provided at 121:32-123:48 of the specification); AMG1037, ¶¶[0021], [0097]. Alexion has also argued that a POSA would have understood that the first two amino acids of SEQ ID NO: 20 (*i.e.*, the "MA" sequence) are a leader sequence using the general knowledge that a characteristic kappa light chain starts with the sequence "DIQ" on its mature N terminus. AMG1015, 546; AMG1014, 513; AMG1048, 734:Figure 6. The remainder of the sequence in an

scFv is the two variable region sequences. Accordingly, a POSA would have understood that the mature light and heavy chain variable regions in 5G1.1 scFv CO12 correspond to amino acids 3-111 and 127-248 of SEQ ID NO: 20, respectively. AMG1007, 44:4-13; Certificate of Correction at 42-44 (correction of SEQ ID NO: 20 at 121:32-123:48).

132. As discussed above, a POSA would have had reason to pair the variable region sequences from Evans' 5G1.1 scFv CO12 with constant region sequences from Mueller to generate a full length 5G1.1 antibody. Mueller provides the amino acid sequence of the IgG2/IgG4 constant region as part of the sequence of the chimeric anti-VCAM "3F4" antibody—particularly the version of the 3F4 antibody with a hybrid IgG2/IgG4 heavy chain constant region ("3F4 HuG2/G4"). AMG1008, 58-61. Because 3F4 HuG2/G4 is a chimeric antibody, a POSA would have understood that the 3F4 HuG2/G4 antibody heavy chain includes the murine 3F4 variable regions attached to human constant regions. Mueller discloses the amino acid sequence of the 3F4 variable regions in Figure 9. AMG1008, Figure 9, 58-61. By aligning the 3F4 variable region sequences from Figure 9 with the sequences of the 3F4 HuG2/G4 antibody, a POSA would have identified the 3F4 variable regions 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. A POSA therefore would have immediately known that the remainder of the 3F4

HuG2/G4 heavy chain, specifically amino acids 138-463, is the IgG2/IgG4 constant region of that antibody. And a POSA also would have immediately known that the remainder of the 3F4 light chain, specifically amino acids 132-238, is the light chain constant region of that antibody.

133. I have generated Figure 16, shown below, to illustrate how a POSA would have combined Evan's heavy chain sequences with Mueller's variable region sequences.

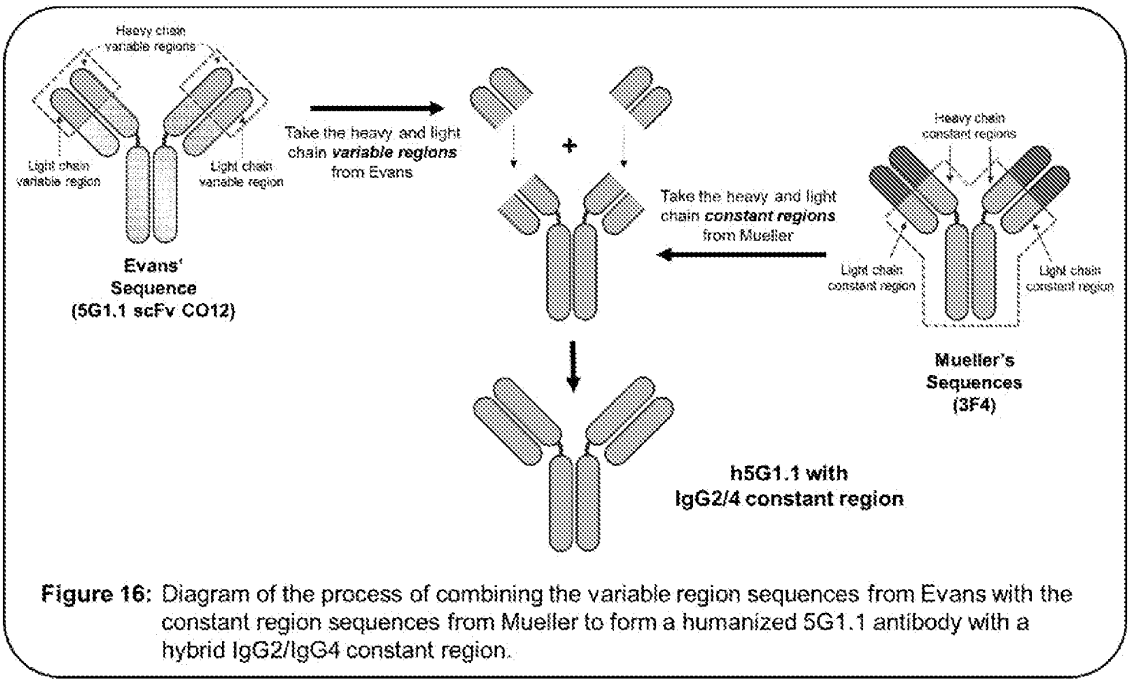


Figure 16: Diagram of the process of combining the variable region sequences from Evans with the constant region sequences from Mueller to form a humanized 5G1.1 antibody with a hybrid IgG2/IgG4 constant region.

134. Combining the heavy chain variable region of SEQ ID NO: 20 from Evans (*i.e.*, a.a. 127-248 of SEQ ID NO: 20, shown as underlined sequence of "Prior") with the IgG2/IgG4 constant region from Mueller (*i.e.*, portion of "Prior" not underlined) yields a sequence identical to SEQ ID NO: 2 in claim 1 of the '149

patent. I have generated the alignment depicted in Figure 17 below to illustrate how these heavy chain sequences come together to form a sequence 100% identical to SEQ ID NO: 2:

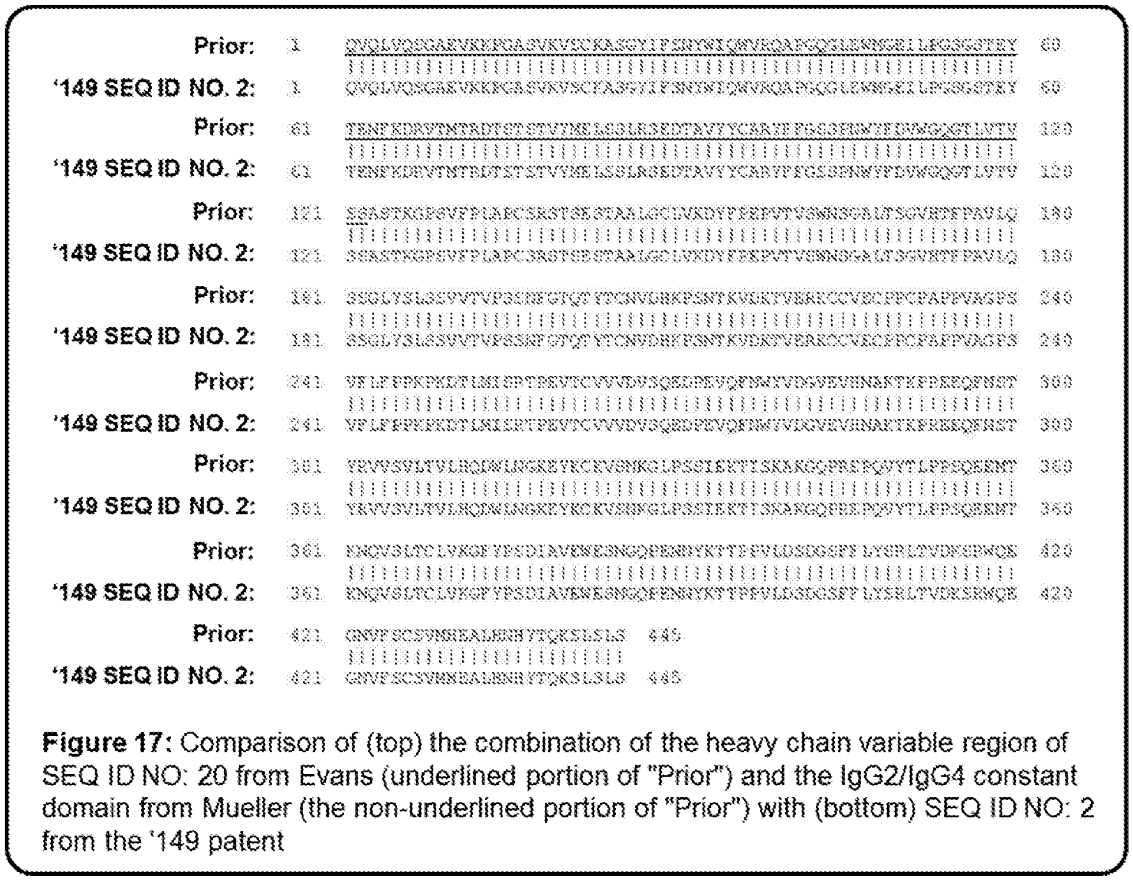


Figure 17: Comparison of (top) the combination of the heavy chain variable region of SEQ ID NO: 20 from Evans (underlined portion of "Prior") and the IgG2/IgG4 constant domain from Mueller (the non-underlined portion of "Prior") with (bottom) SEQ ID NO: 2 from the '149 patent

135. Similarly, combining the light chain variable region of SEQ ID NO: 20 from Evans (*i.e.*, a.a. 3-111 of SEQ ID NO: 20, shown as underlined sequence of "Prior") with the light chain region from Mueller (*i.e.*, portion of "Prior" not underlined) would yield a sequence identical to SEQ ID NO: 4 in claim 1 of the '149 patent. I have depicted this alignment in Figure 18 below.

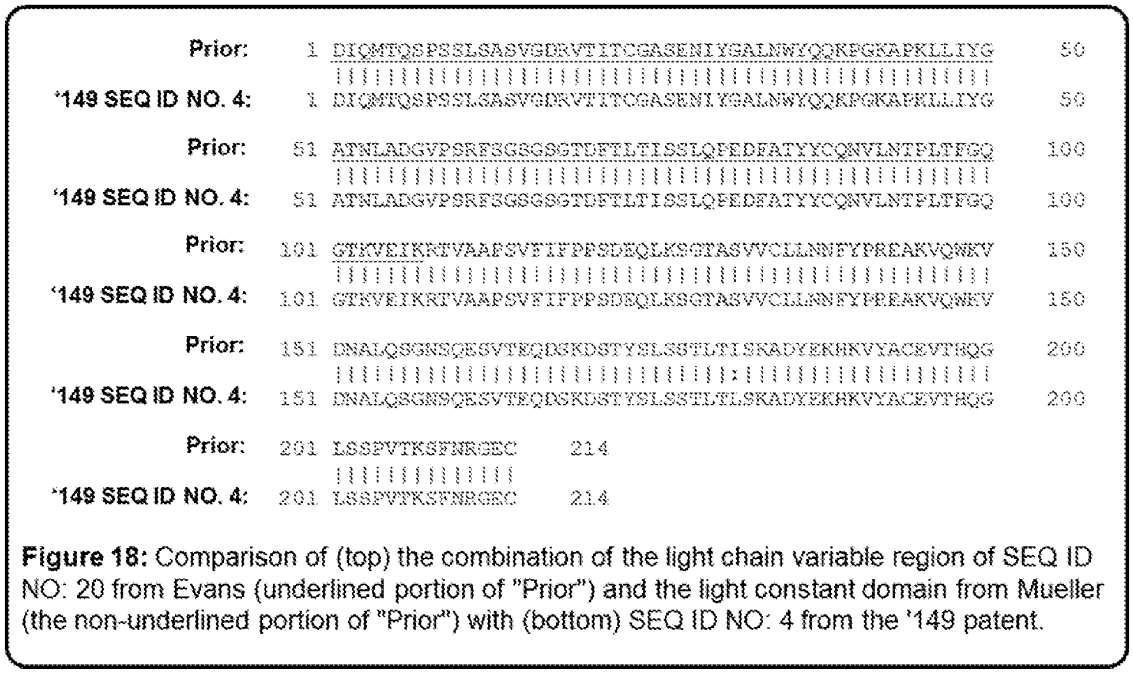


Figure 18: Comparison of (top) the combination of the light chain variable region of SEQ ID NO: 20 from Evans (underlined portion of "Prior") and the light constant domain from Mueller (the non-underlined portion of "Prior") with (bottom) SEQ ID NO: 4 from the '149 patent.

C. A POSA would have had a reasonable expectation of success in combining the antibody sequences from Evans and Mueller

136. As discussed above, a POSA desiring to perform Bell's method would have had reason to look to Evans' humanized 5G1.1 svFv's because Bell incorporated Evans by reference for methods of making humanized 5G1.1. And a POSA would have had reason to look to Mueller's constant regions because a POSA would have understood that Mueller's disclosures relating to "h5G1.1 CO12 HuG2/G4 mAb" to be about an anti-C5 antibody like eculizumab, and because Evans teaches using such hybrid domains in combination with its humanized 5G1.1 variable regions. This was a known strategy in the art, as seen in Alexion's patent application, WO 2005/007809, which contained 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. Accordingly, a POSA would have had reasonable expectation of success in using the complementary sequence elements of the anti-C5 antibodies from those references to generate a full length anti-C5 antibody. A POSA also would have had a reasonable expectation of success in obtaining a full-length antibody by combining the sequences in Evans and Mueller because doing so would have required only basic molecular and cellular biology techniques. Methods of cloning and producing antibodies were well-known in the art. *See, e.g.*, AMG1006, ¶¶[0130]-[0131]. The required cloning would have required only basic molecular biology techniques such as PCR and restriction enzyme digests to attach the variable regions from Evans to the constant regions from Mueller. *See, e.g.*, AMG1006, ¶¶[0130]-[0131]; AMG1008, 37:37-39 (describing use of "[s]tandard molecular biology techniques" to prepare recombinant antibodies); AMG1007, 21:4-24:61, Examples 10-11 (describing use of "conventional recombinant DNA methods including restriction fragment subcloning and overlapping PCR procedures" to prepare recombinant antibodies)

* * *

137. Given the above, Evans and Mueller teach every element of the antibody recited in claim 1, and a POSA would have had a reason to combine Evans and Mueller to arrive at the claimed antibody, with a reasonable expectation

of success. Therefore, claim 1 would have been obvious to a POSA, notwithstanding any objective evidence (discussed in §XV below).

XV. No Objective Evidence Supports Non-Obviousness of Claim 1.

138. In reaching my opinions regarding obviousness described in this declaration, I considered whether there were any objective indicia (i.e., objective evidence) of non-obviousness.

139. In reviewing the prosecution history of the related U.S. Patent No. 9,725,504, I noted that Alexion argued that none of the references cited by the examiner "suggests the unique, non-naturally occurring, protein-engineered heavy chain of eculizumab, which confers important, unexpected functional effects." AMG1014, 588. Specifically, Alexion asserted that the claimed antibody has "decreased effector function, reduced immunogenicity and increased half-life." AMG1014, 587. I understand that when unexpected results are used as evidence of non-obviousness, the results must be attributable to a novel feature of the claims. For the reasons discussed below, it is my opinion that the results discussed during prosecution (1) were not attributable to a novel feature of the claims; and (2) were not unexpected.

140. First, the allegedly unexpected decreased effector function, reduced immunogenicity and increased half-life were not unexpected because they are all

known properties of eculizumab, and attributable to the sequence of eculizumab—which was known in the art before the priority date of the '149 patent. Additionally, these properties are attributable to a non-novel heavy chain design. As discussed in §VI.C above, eculizumab has the same hybrid IgG2/IgG4 heavy chain that was used in other prior art antibodies, such as those described in Mueller II, Tacken, Bowdish, and Mueller. AMG1031; AMG1032; AMG1034; AMG1006; AMG1008. The properties that Alexion asserts to be unexpected were actually well-established properties of antibodies with this hybrid heavy chain. For example, Mueller II states that antibodies with this hybrid IgG2/IgG4 heavy chain design "[do] not contain the antibody sequences necessary for FcR binding," the interaction needed for antibody effector function. AMG1031, 448, 451. Mueller II also notes that a hybrid IgG2/IgG4 heavy chain would not contain "any new epitopes that would likely be immunogenic." AMG1031, 448, 451. And a POSA would have known that a hybrid IgG2/IgG4 heavy chain would "have increased half-life." AMG1032, 5, 19. These improved properties are likely why Mueller II commented that the hybrid IgG2/IgG4 structure be "should prove useful in humanization of other antibodies intended for human use." AMG1031, 451. Therefore, all of the allegedly unexpected results would have been entirely expected in view of the prior art.

141. I have also considered whether there is any other objective evidence of nonobviousness, such as a long-felt but unmet need, failure of others, skepticism of experts, copying, or praise in the field. I am not aware of any such evidence.

XVI. Conclusion

142. For the reasons discussed above, claim 1 of the '149 patent is anticipated or would have been obvious in view of the cited art.

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.

Respectfully submitted,

Joseph P. Balthasar, Ph.D.

Date: February 27, 2019

12700950

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452 Kapoor Hall
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State University of New York
716-645-4807
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Home Address
5630 William Street
Lancaster, New York
14086

EDUCATION

Ph.D. Pharmaceutics
State University of New York at Buffalo
- January, 1996
- Dissertation: Optimization of intraperitoneal cancer chemotherapy with anti-drug antibodies

Advisor: Dr. Ho-Leung Fung
Committee: Dr. Ho-Leung Fung, Dr. William Jusko, Dr. Robert Straubinger, Dr. Thomas Kalman

Bachelor of Science, Pharmacy
State University of New York at Buffalo
- May 1991

POSITIONS HELD

January 2011 – present: Associate Dean for Research, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo

July 2008 – present: Professor, Department of Pharmaceutical Sciences, University at Buffalo

June 2007 – present: Director, Center for Protein Therapeutics, University at Buffalo

September 2006 – present: Member, Targeted Therapeutics Program, Roswell Park Memorial Cancer Institute, Buffalo, New York

July 2005 – July 2008: Director of Graduate Studies, Department of Pharmaceutical Sciences, University at Buffalo, State University of New York

July 2003 – July 2008: Associate Professor, Department of Pharmaceutical Sciences, University at Buffalo

December 1999 – June 2003: Assistant Professor, Department of Pharmaceutical Sciences, University at Buffalo, State University of New York

February 1997 – December 1999: Assistant Professor, Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah

January 1996 – February 1997: Clinical Assistant Professor, Department of Pharmaceutics, State University of New York at Buffalo

March 1993 – September 1995: Part-time Staff Pharmacist, Buffalo General Hospital, Buffalo, New York

January 1994: Pharmacokinetics Consultant, Buffalo General Hospital, Buffalo, New York

Samsung Bioepis Exhibit No. 1002
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TEACHING

UNIVERSITY OF UTAH

Class Coordinator & Chief Lecturer

PHCEU 405: Clinical Pharmacokinetics, Department of Pharmaceutics and Pharmaceutical Chemistry (Spring 1998).
PHCEU 789: Research Seminar; Department of Pharmaceutics and Pharmaceutical Chemistry (Fall 1997-Fall 1999).
PHCEU 5513: Introduction to Pharmacokinetics and Biopharmaceutics, Department of Pharmaceutics and Pharmaceutical Chemistry (Fall 1999).

Lecturer

PHM 7325: Applied Pharmacokinetics, University of Utah, Department of Pharmacy Practice (Spring 1999)
PHCEU 7030: Drug Delivery, University of Utah, Department of Pharmaceutics and Pharmaceutical Chemistry (Fall 1998, Fall 1999)
PHTX 6610: Principles of Pharmacology and Toxicology, University of Utah, Department of Pharmacology and Toxicology (Fall 1998, Fall 1999)
PHRM 5113: Basics in Pharmaceutical Sciences, Department of Pharmacy Practice (Fall 1998, Fall 1999)
CL PH 705: Applied Pharmacokinetics; Department of Pharmacy Practice (Spring 1998)
PHCEU 614: Systems Approach to Drug Delivery; Department of Pharmaceutics and Pharmaceutical Chemistry (Spring 1997)
PHARM 411: Introduction to Pharmaceutical Sciences, Department of Pharmacy Practice (Spring 1998)

UNIVERSITY AT BUFFALO, THE STATE UNIVERSITY OF NEW YORK

Class Coordinator & Chief Lecturer

PHC 412: Introduction to Pharmacokinetics and Biopharmaceutics 2, Department of Pharmaceutical Sciences (yearly, Spring 2001-2016)
PHC 413: Pharmaceutics Seminar, Department of Pharmaceutical Sciences (Fall 2000, Fall 2016)
PHC 414: Pharmaceutics Seminar, Department of Pharmaceutical Sciences (Spring 2001)
PHC 532: Introduction to Pharmacokinetics and Biopharmaceutics 2, Department of Pharmaceutical Sciences (yearly, Spring 2001-2018)
PHC 607: Intermediate Pharmacokinetics, Department of Pharmaceutical Sciences (yearly, Fall 2000-2017)
PHC 613: Pharmaceutics Seminar, Department of Pharmaceutical Sciences (Fall 2000, Fall 2016)
PHC 614: Pharmaceutics Seminar, Department of Pharmaceutical Sciences (Spring 2001, Spring 2017)

Lecturer

PHC 608: Advanced Pharmacokinetics, Department of Pharmaceutical Sciences (even-numbered years, Spring 2002-2018)
PHC 609: Pharmacodynamics, Department of Pharmaceutical Sciences (odd-numbered years, Spring 2001-2017)
PHC407: Selected Topics in Pharmaceutics, Department of Pharmaceutics (Spring 1996)

Teaching Assistant

PHC 411: Introduction to Pharmacokinetics, Department of Pharmaceutics (Fall 1993, Fall 1994)

TEACHING, MENTORING STUDENTS

Note: the following lists service as primary advisor (only); students mentored as a committee member are not included

GRADUATE STUDENTS

1. Ryan J. Hansen, Ph.D., Pharmaceutical Sciences, University at Buffalo
   Joined lab June 1998, successfully defended Ph.D. dissertation 5/2002
   Intravenous immunoglobulin effects in experimental models of immune thrombocytopenia.

2. Evelyn Lobo, Ph.D., Pharmaceutical Sciences, University at Buffalo
   Joined lab August 1997, successfully defended Ph.D. dissertation 8/2002 (2)
   Anti-methotrexate Fab fragments for the optimization of intraperitoneal chemotherapy

3. Rong Deng, Ph.D., Pharmaceutical Sciences, University at Buffalo
   Joined lab June 2001, successfully defended Ph.D. dissertation 8/2005 (4)
   Antibody-coated liposomes as a new treatment for ITP

4. Jin Chen, Ph.D., Pharmaceutical Sciences, University at Buffalo
   Joined lab August 2001, successfully defended Ph.D. dissertation 6/2005 (3)
   Optimization of ip topotecan chemotherapy via inverse targeting

5. Feng Jin, Ph.D., Pharmaceutical Sciences, University at Buffalo
   Joined lab February 2002, successfully defended Ph.D. dissertation 1/2006 (5)
   Acceleration of anti-platelet antibody elimination in immune thrombocytopenia

6. Amit Garg, Ph.D., Pharmaceutical Sciences, University at Buffalo
   Joined lab March 2003, successfully defended Ph.D. dissertation 8/2007 (6)
   Dissertation title: *Application of PBPK to characterize the influence of FcRn on IgG tissue distribution and elimination*

7. Saed Khammash, graduate student, Pharmaceutical Sciences, University at Buffalo
   Joined lab August 2003; resigned May 2005

8. Kate Getman, BS/MS, Pharmaceutical Sciences, University at Buffalo
   Joined lab June 2002, Successfully completed MS July 2004
   Project title: *Evaluation of the effects of a novel FcRn inhibitor on the disposition of monoclonal IgG antibodies in rats*

9. Shweta Urva, Ph.D., Pharmaceutical Sciences, University at Buffalo
   Joined lab February 2005, Successfully defended Ph.D. dissertation 4/2009 (7)
   Dissertation title: *Antibody mediated targeting for the treatment and diagnosis of colorectal cancer*

10. Dhaval Shah, Ph.D., Pharmaceutical Sciences, University at Buffalo
    Joined lab February 2006; Successfully defended Ph.D. dissertation 8/2010 (8)
    Dissertation title: *Pharmacokinetic strategies to improve the safety and efficacy of intraperitoneal chemotherapy.*

11. Jenna Voellinger, BS/MS student, Pharmaceutical Sciences, University at Buffalo
    Entered BS/MS program January 2007; Successfully defended MS project 8/2008
    Project title: *Optimization of anti-FcRn antibodies*

12. Yang Chen, Ph.D., Pharmaceutical Sciences, University at Buffalo
    Joined lab February 2007; Successfully defended PhD dissertation 3/2012 (9)
    Dissertation title: *FcRn-IgG Interaction: Development of FcRn Inhibitors and PBPK Modeling*

13. Lubna Abuqayyas, Ph.D., Pharmaceutical Sciences, University at Buffalo
    Joined lab February 2007; Successfully defended PhD dissertation 8/2012 (10)
    Dissertation title: *Evaluation of the mechanistic determinants of antibody exposure in tissues*

14. Tommy Li, Ph.D., Pharmaceutical Sciences, University at Buffalo
    Joined lab February 2011; Successfully defended PhD thesis January 2018 (13)

Tentative dissertation title: *FcRn expression and turnover: Implications for the development of FcRn inhibitors*

15. Naveen Mangal, graduate student, Pharmaceutical Sciences, University at Buffalo
    Joined lab February 2011; Successfully completed MS 8/2012
    Project title: *Development of anti-topotecan monoclonal antibodies*

16. Matthew Franchini, graduate student, Pharmaceutical Sciences, University at Buffalo
    Joined lab February 2011; resigned from lab August 2012
    Tentative project title: *Investigation of complement disposition in the eye*

17. Frank Engler, Ph.D., Pharmaceutical Sciences, University at Buffalo
    Joined lab May 2011; Successfully defended PhD thesis October 2015 (11)
    Dissertation title: *Development and evaluation of catch-and-release anti-carcinoembryonic antigen monoclonal antibodies.*

18. Patrick Glassman, Ph.D. Candidate, Pharmaceutical Sciences, University at Buffalo
    Joined lab December 2011; successfully defended PhD thesis August 2016 (12)
    Dissertation title: *Physiologically-based pharmacokinetic modeling to guide the development of therapeutic antibodies*

19. Veena Thomas, Ph.D. Candidate, Pharmaceutical Sciences, University at Buffalo
    Joined lab December 2011, Successfully completed MS 8/2013 – transferred into Ph.D. program, successfully defended dissertation 4/2018 (15)
    Dissertation title: *Investigation of sources of inter-individual variability in mAb pharmacokinetics*

20. Shunxin Lin, Ph.D. Candidate, Pharmaceutical Sciences, University at Buffalo
    Joined lab December 2012, successfully defended PhD dissertation 1/2018 (14)
    Dissertation title: *Pharmacokinetics and pharmacodynamics of monoclonal antibodies for eradication of bacterial infection.*

21. Brittany Walker, BS/MS student, Pharmaceutical Sciences, University at Buffalo
    Joined lab December 2012, Successfully completed MS 5/2014
    BS/MS Project: *Delivery of anti-Rac1b SiRNA*

22. Anita Moein, MS student, Pharmaceutical Sciences, University at Buffalo
    Joined lab December 2012, Successfully completed MS 5/2014
    MS Project: *Evaluation of antibody distribution to the brain*

23. Femi Adesina, graduate student, Pharmaceutical Sciences, University at Buffalo
    Joined lab December 2014, resigned May 2015
    Ph.D. Project: *Optimization of antibody penetration into solid tumors*

24. J. Ryan Polli, Ph.D. Candidate, Pharmaceutical Sciences, University at Buffalo
    Joined lab December 2014
    Ph.D. Project: *Engineered antibody-drug conjugates for cancer therapy*

25. Yujie Yang, graduate student, Pharmaceutical Sciences, University at Buffalo
    Joined lab December 2014, successfully defended MS 12/2016 – transferred into Ph.D. program
    Tentative dissertation title: *PK/PD evaluation of bispecific T-cell engaging mAb in mouse models*

26. Toan Duc Nguyen, BS/MS student, Pharmaceutical Sciences, University at Buffalo

Joined lab December 2015
BS/MS Project: *Development and characterization of anti-CD123 monoclonal antibodies*

27. Brandon Bordeau, graduate student, Pharmaceutical Sciences, University at Buffalo
Joined lab December 2015
Ph.D. Project: *Evaluation of new strategies to enhance mAb penetration in tumors*

28. Jue Gong, graduate student, Pharmaceutical Sciences, University at Buffalo
Joined lab February 2017
Ph.D. Project: *Development and characterization of anti-PTK7 antibodies and antibody conjugates*

POST-DOCTORAL FELLOWS

1. Qiang Lu, Ph.D., Medicinal Chemistry, Ohio State University, 2005.
July 2005 – June 2007

2. Weiyan Zhang, Ph.D., Pharmaceutical Sciences, West Virginia University, 2005.
April 2006 – November 2009

3. Beom Soo Shin, Ph.D., Pharmacokinetics, Sungkyunkwan University, Korea, 2005.
May 2006 – August 2007

4. Xioyan Zhang, Ph.D., Pharmacology, University of Louisville, 2008.
April 2008 – November 2009

5. Deong Weong Song, Ph.D., Pharmacology, Roswell Park Cancer Institute, 2005.
April 2008 – May 2009

6. Shang-Chiung Chen, Ph.D., Molecular Pharmacology and Cancer Therapeutics, Roswell Park Cancer Institute, 2008.
June 2008 – July 2010

7. Bo Zheng, Ph.D., Pharmaceutical and Biomedical Sciences, University of Georgia, 2009.
January 2010 – January 2012

8. Alison Gaudy, Ph.D., Pharmacology, University of Rochester, 2010.
July 2010 – June 2012

9. Tao Ji, Ph.D., Medicinal Chemistry, University at Buffalo, The State University of New York, 2005.
October 2011 – present

10. Nathaniel Page, Ph.D., Pharmaceutical Sciences, University at Buffalo, State University of NY, 2011.
December 2011 – December 2012

VISITING SCIENTISTS

1. Yoshimasa Ishida, M.S., Chugai Pharmaceutical Co., Ltd., January 2004 – May 2004. Research project: Population pharmacokinetic/pharmacodynamic modeling for lenograstim (rhG-CSF) in healthy volunteers and lung cancer patients.

2. Weirong Wang, Ph.D., Merck & Company, Inc., March 2007 – May 2007. Research project: Pharmacokinetic / pharmacodynamic modeling of monoclonal antibody drugs.

3. Ellen Wang, Ph.D., Pfizer, Inc., August 2007 – April 2008. Research project: Pharmacokinetic / pharmacodynamic modeling of monoclonal antibody drugs. Dr. Wang will be investigating the use of target-mediated disposition models to assist in the prediction of antibody doses to achieve minimal anticipated biological effect levels (MABEL) for first-in-man clinical trials.

UNDERGRADUATE STUDENTS

1. Robert Horton, participant in the Department of Pharmaceutics and Pharmaceutical Chemistry Summer Research Program, University of Utah, Summer 1997

2. Fred Dawson, participant in the Department of Pharmaceutics and Pharmaceutical Chemistry Summer Research Program, University of Utah, Summer 1999

3. Zia R. Tayab, Department of Pharmaceutical Sciences, University at Buffalo, 6/00 – 6/03

4. Chris Wallen, Department of Pharmaceutical Sciences, University at Buffalo, 2/01 –6/01

5. Agnes Chan, Department of Pharmaceutical Sciences, University at Buffalo, 8/01 – 12/01

6. Kenneth Chen, Department of Pharmaceutical Sciences, University at Buffalo, 8/01 – 5/02

7. Eric Sawey, Department of Pharmaceutical Sciences, University at Buffalo, 8/01 – 5/02

8. Danny McNatty, Pharm.D. candidate, School of Pharmacy, University at Buffalo,1/03 – 6/05

9. Sadat Yarose, Pharm.D. candidate, School of Pharmacy, University at Buffalo, 6/05 – 6/06

10. Jenna Voellinger, Department of Pharmaceutical Sciences, University at Buffalo, 6/06 – 12/06

11. Stephanie McCutcheon, Pharm.D. candidate, University at Buffalo, 8/06 – 5/08

12. Deborah Lianna, Pharm.D. candidate, School of Pharmacy, University at Buffalo, 8/06 – 5/08

13. Rebekah Tate, Pharm.D. candidate, School of Pharmacy, University at Buffalo, 8/09 – 5/10

14. Patrick Glassman, Department of Pharmaceutical Sciences, University at Buffalo, 1/10 – 8/10

15. Catherine Slota, Department of Pharmaceutical Sciences, University at Buffalo, 1/10 – 8/11

16. Femi Adesina, Pharm.D. candidate, School of Pharmacy, University at Buffalo, 1/12 – 6/14

17. Brian Baur, Pharm.D. candidate, School of Pharmacy, University at Buffalo, 1/12 – 5/12

18. Yi Ting Lien, Department of Pharmaceutical Sciences, University at Buffalo, 6/12 – 6/14

19. Brittany Walker, Department of Pharmaceutical Sciences, University at Buffalo, 8/12 – 12/12

20. Melissa Rozek, Pharm.D. candidate, School of Pharmacy, University at Buffalo, 1/13 – 12/14

GRANTS AWARDED (*as Principal Investigator, starting with NIH R01 awards*)

1. NIH, R01 HL67347
   Role: Principal Investigator
   Pharmacology and bioengineering of new treatments of ITP
   April 11, 2001 – March 31, 2005, $1,046,706 (total)

2. NIH, R01 AI60687
   Role: Principal Investigator
   FcRn Inhibitors for Antibody-Mediated Immune Conditions,
   May 1, 2004 – April 30, 2008, $1,375,382 (total)

3. NIH, R01 CA118213
   Role: Principal Investigator
   Pharmacokinetic strategies to optimize IP chemotherapy
   September 13, 2006 – August 31, 2010, *$1,443,115* (total)

4. NIH, R01 2HL67347
   Role: Principal Investigator
   Pharmacology and Bioengineering of New Treatments of ITP
   September 24, 2007 – May 31, 2011, *$1,578,001* (total)

5. NIH, R01 CA204192
   Role: Principal Investigator
   Catch and Release Immunotoxins: CAR-Bombs for cancer
   December 1, 2016 – November 30, 2021, *$1,827,130* (total)

6. Industry Support from Genentech, Inc.
   Role: Principal Investigator
   Center for Protein Therapeutics
   December 1, 2007 – November 30, 2010, $450,000 (total)

7. Industry Support from Roche Pharmaceuticals
   Role: Principal Investigator
   Center for Protein Therapeutics
   July 1, 2008 – June 30, 2018, $1,950,000 (total)

8. Industry Support from Merck and Company
   Role: Principal Investigator
   Center for Protein Therapeutics
   July 1, 2008 – June 30, 2018, $1,350,000 (total)

9. Industry Support from Pfizer, Inc.
   Role: Principal Investigator
   Center for Protein Therapeutics
   July 1, 2008 – June 30, 2014, $900,000 (total)

10. Industry Support from Eli Lilly and Company
    Role: Principal Investigator
    Center for Protein Therapeutics
    July 1, 2008 – June 30, 2018, $1,800,000 (total)

11. Industry Support from Abbott/AbbVie, Inc.
    Role: Principal Investigator
    Center for Protein Therapeutics
    January 1, 2010 – June 30, 2018, $1,650,000 (total)

12. Industry Support from Boehringer Ingelheim

Role: Principal Investigator
Center for Protein Therapeutics
July 1, 2010 – June 30, 2011, $150,000 (total)

13. Industry Support from Amgen
Role: Principal Investigator
Center for Protein Therapeutics
January 1, 2012 – June 30, 2018, $1,050,000 (total)

14. Industry Support from Janssen, Inc.
Role: Principal Investigator
Center for Protein Therapeutics
January 1, 2013 – June 30, 2018, $825,000 (total)

15. Industry Support from Sanofi Aventis
Role: Principal Investigator
Center for Protein Therapeutics
January 1, 2013 – June 30, 2018, $750,000 (total)

16. Industry Support from Novartis Pharma AG
Role: Principal Investigator
Laboratory for Protein Therapeutics
July 1, 2007 – June 30, 2010, $900,000 (total)

17. Industry Support from Novartis Pharma AG
Role: Principal Investigator
FcRn as a determinant of IgG Bioavailability
June 1, 2007 – May 31, 2008, $100,000 (total)

18. Industry Support from Protherics PLC
Role: Principal Investigator
Voraxaze Effects on Methotrexate-Induced Weight Loss in Mice
October 1, 2006 – September 30, 2007, $8,345 (total)

19. American Association of Colleges of Pharmacy
Role: Principal Investigator
Fellowship for Danny McNatty: *Humanization of anti-methotrexate monoclonal antibodies*
Awarded May, 2003; $5,500 (total), $5,500 (direct)

20. Kapoor Foundation
Role: Principal Investigator
Pharmacology of adjuvants for inverse targeting
Awarded June, 2002; $20,000 (total), $20,000 (direct)

21. American Association of Colleges of Pharmacy, New Investigators Program for Pharmacy Faculty
Role: Principal Investigator
Optimization of intraperitoneal chemotherapy through the use of anti-drug antibody therapy.
Awarded October, 1997; $12,500 (total), $12,500 (direct)

22. Parenteral Drug Association, Faculty Development Grant
Role: Principal Investigator

Inverse targeting with anti-drug antibodies: A new approach of optimizing chemotherapy.
Awarded June, 1997; $20,000 (total), $20,000 (direct)

23. University of Utah, College of Pharmacy Research Support Grant
Role: Principal Investigator
Antineoplastic diffusivity: Implications for intraperitoneal chemotherapy of ovarian cancer.
Awarded June, 1997; $5,000 (total), $5,000 (direct)

24. University of Utah, Research Instrumentation Committee
Role: Principal Investigator
Equipment for pharmacokinetic and pharmacodynamic analyses.
Awarded June, 1997; $50,000 (total), $50,000 (direct)

25. University of Utah, Research Project Grant
Role: Principal Investigator
Optimized IP chemotherapy of experimental ovarian cancer.
Awarded May, 1997; $6,000 (total), $6,000 (direct)

26. SUNY at Buffalo, Invention Commercialization Enhancement Grant
Role: Principal Investigator
Anti-resistance antibiotic oligonucleotides
Awarded August, 1995; $9,500 (total), $9,500 (direct)

GRANTS AWARDED (*as Co- Investigator*)

27. NIH, R01 HL70227
Role: Co-Investigator (PI: S. V. Balasubramanian)
Development and pharmacology of novel lipidic rAHF,
April 1, 2002 – March 31, 2006, $762,736 (total), $500,000 (direct)

28. NIH, R01CA114612
Role: Co-PI (PI: Dr. Victor Yang, University of Michigan)
PTD-Mediated Protein or Drug Delivery for Cancer Therapy
April 1, 2005 – March 31, 2009 (UB contract: 4/1/05 – 3/31/09; $200,000 total costs for subcontract)

29. NIH, R01 HL55461
Role: Co-PI (PI: Dr. Victor Yang, University of Michigan)
Triggered Local Release of Active Thrombolytic Agents
February 1, 2005 – January 31, 2009 (UB contract: 2/1/05 – 1/31/08; $150,000 total costs for subcontract)

30. NIH, R01 2HL70227
Role: Co-Investigator (S. V. Balasubramanian, PI)
Development and pharmacology of novel lipidic rAHF
January 1, 2007 – December 31, 2010, $1,085,688 (total)

31. Industry Support from Pfizer, Inc.
Role: Protein Therapeutics Project Leader and Member of Steering Committee
UB/Pfizer Strategic Alliance: Center of Excellence in Pharmacokinetics and Pharmacodynamics
November 1, 2005 – June 30, 2010, $5,000,000 (total)

32. NIH, 2-R01CA114612

Role: Co-PI (PI: Dr. Victor Yang, University of Michigan)
PTD-Mediated Protein or Drug Delivery for Cancer Therapy
June 1, 2011 –May 31, 2016, $1,273,180.00 (total)

33. NIH, R01DE023080
Role: Co-Investigator (PI: Dr. Chunhao Li, University at Buffalo, School of Dental Medicine)
Exploring new virulence factors of the oral spirochete Treponema denticola
July 1, 2013 – June 30, 2018, $1,951,240.00 (total)

34. NIH, 2 R42CA176951 02A1
Role: Co-Investigator (PI: Dr. Kate Rittenhouse-Olson, University at Buffalo, School of Dental Medicine)
Clinical translation of an anti-metastatic antibody for breast cancer therapy
May 1, 2015 – April 30, 2016, $1,986,991 (total)

35. NIH, 1R01DE027073-01A1
Role: Co-Investigator (PI: Dr. Michelle B. Visser, University at Buffalo, School of Medicine)
The role of oral spirochete virulence factors in the impairment of neutrophil response
September 1, 2018 – August 31, 2023, $1,894,065 (total)

NEW TECHNOLOGY DISCLOSURES

R-5318: Anti-resistance antibiotic oligonucleotides
University at Buffalo
September, 1995
This disclosure was for the invention of anti-sense oligonucleotides designed to inhibit vancomycin resistance.

U-2872: Reduction of toxicity with lipid administration
University of Utah
July, 1999
This disclosure was for the invention of strategies for using lipid emulsions to alter the pharmacokinetics and toxicity of lipophilic toxins.

U-2873: Reduction of intraperitoneal toxicity with lipid administration
University of Utah
July, 1999
This disclosure was for the invention of strategies for using lipid emulsions to alter the pharmacokinetics and toxicity of chemotherapeutics following intraperitoneal dosing.

R-5720: Monoclonal anti-methotrexate antibody fragments
University at Buffalo
June, 2001
This disclosure was for the invention of a monoclonal antibody, W8, with high affinity for methotrexate. This antibody has been shown to alter the pharmacokinetics and toxicity of methotrexate (e.g., Lobo ED and Balthasar JP, Application of anti-methotrexate Fab fragments for the optimization of intraperitoneal methotrexate therapy in a murine model of peritoneal cancer. *J Pharm Sci* 94:1957-64,2005).

R-5721: Anti-topotecan antibodies
University at Buffalo
June, 2001
This disclosure was for the invention of a monoclonal antibody, 8C2, with high affinity for topotecan. This antibody has been shown to alter the pharmacokinetics topotecan, and the antibody may be useful for decreasing systemic toxicity associated with intraperitoneal administration of drug (e.g., for treatment of peritoneal tumors).

R-5780: Bioreactor for accelerated immunotoxicotherapy
University at Buffalo
August, 2002
This disclosure was for the invention of an extra-corporeal device for rapid removal of antibody-toxin conjugates. This bioreactor device would be useful for rapid removal of toxic substances from patients.

R-5832: Anti-FcRn therapy for treatment of auto/allo immune conditions
University at Buffalo
April, 2003
This disclosure was for the invention of antibodies that non-competitively inhibit the FcRn receptor. This receptor protects immune gamma globulin (IgG) antibodies from intracellular catabolism, and this protection contributes to the long biological half-life of IgG (~25 days in humans). Inhibition of FcRn accelerates the clearance of IgG antibodies (e.g., Getman K and Balthasar JP, Pharmacokinetic effects of 4C9, an anti-FcRn antibody, in rats: Implications for the use of FcRn inhibitors for the treatment of humoral autoimmune and alloimmune conditions. *J Pharm Sci*, 94(4): 718-729, 2005). As such, anti-FcRn antibodies may have utility in the treatment of patients with humoral allo-immune or autoimmune conditions, which are typically mediated by pathogenic IgG antibodies.
Note: This technology has been licensed to VLST, Inc. and, subsequently, to Momenta, Inc.

R-5962: Opsisomes for immune thrombocytopenia
University at Buffalo
October, 2004
This disclosure was for the invention of antibody-coated liposomes for the treatment of immune thrombocytopenia. In our preclinical investigations, we have found that antibody-coated liposomes ("opsisomes") are able to produce substantial increases in platelet counts in animal models of immune thrombocytopenia (e.g,. Deng R. and Balthasar JP, Comparison of the effects of antibody-coated liposomes, IVIG and anti-RBC immunotherapy in a murine model of passive chronic immune thrombocytopenia. *Blood*, 109(6):2470-2476, 2007).

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33. Deng R and Balthasar JP, Investigation of antibody-coated liposomes in vivo and in vitro as a therapy for immune thrombocytopenia. *AAPS PharmSci* 5(4), 2003.

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37. Garg A and Balthasar JP, Application of a physiologically-based pharmacokinetic (PBPK) model to study the effects of FcRn and IVIG on IgG tissue disposition. *AAPS Journal* **6**(4), 2004.

38. Garg A and Balthasar JP, Investigation of the Tissue Disposition of 7E3, a Murine Monoclonal IgG1 Antibody, in Wild-Type and FcRn-Knockout Mice. *AAPS Journal* 7(S2), 2005.

39. Deng R and Balthasar JP, Pharmacokinetic/pharmacodynamic modeling of IVIG effects in a murine model of immune thrombocytopenia. *AAPS Journal 7*(S2), 2005.

40. Jin F, Getman K and Balthasar JP, Pharmacokinetic modeling of an anti-FcRn antibody and its effect on IgG disposition in rats. *AAPS Journal 7*(S2), 2005.

41. Jin F and Balthasar JP, Development and in vitro evaluation of antibodies directed against the human neonatal Fc receptor (hFcRn). *AAPS Journal 7*(S2), 2005.

42. Urva S, Lu Q, and Balthasar JP, Preclinical investigation of a new immune complex therapy for treatment of immune thrombocytopenic purpura (ITP). *AAPS Journal 8*(S2), 2006.

43. Garg A and Balthasar JP, Application of a physiologically-based pharmacokinetic (PBPK) model to predict IgG tissue kinetics in wild-type and FcRn-knockout mice. *AAPS Journal87*(S2), 2006.

44. Garg A and Balthasar JP, PBPK Analysis of the effects of IVIG and FcRn on IgG tissue disposition. AAPS National Biotechnology Meeting, San Diego, California, 2007.

45. Shah D, Chen Y, Shin BS, Chen J, Balthasar JP, Simulations of the influence of antibody-ligand binding kinetics on the pharmacokinetics of "high-clearance" ligands. Pfizer/UB Alliance Annual Meeting, 2007.

46. Urva SR, Shin BS, Yang VC, and Balthasar JP, High Performance Liquid Chromatographic Assay for Doxorubicin. AAPS Annual Meeting, Atlanta, GA, 2008.

47. Urva SR, Yang VC, and Balthasar JP, Physiologically-Based Pharmacokinetic(PBPK) Model Incorporating Target Mediated Disposition Component. AAPS Annual Meeting, Atlanta, GA, 2008.

48. Shah DK, Shin BS, and Balthasar JP, A Pharmacokinetic Strategy to Increase the Efficacy of Intraperitoneal Chemotherapy. AAPS Annual Meeting, Atlanta, GA, 2008.

49. Shah DK, Shin BS, Bernacki RJ, Veith J, and Balthasar JP, Improving Therapeutic Benefits of Intraperitoneal Chemotherapy by Co-administration of Antiangiogenic Therapy. AAPS Annual Meeting, Atlanta, GA, 2008.

50. Urva SR, Yang VC, and Balthasar JP, Role of target mediated disposition of T84.66 (anti-carcinoembryonic antigen antibody) in diagnosis of colorectal tumors. AAPS Annual Meeting, Los Angeles, CA, 2009.

51. DK, Shin BS, Bernacki RJ, Veith J, and Balthasar JP, Treating ovarian cancer using combination of antiangiogenic therapy and intraperitoneal chemotherapy. AAPS Annual Meeting, New Orleans, LA, 2010.

52. Abuqayyas L, and Balthasar JP. Investigation of the Influence of FcR on the Distribution of IgG to the Brain. AAPS Annual Meeting, New Orleans, LA, 2010.

53. Abuqayyas L, and Balthasar JP. Investigation of the Influence of FcγR on the Elimination and Disposition of IgG. AAPS Annual Meeting, New Orleans, LA, 2010.

54. Chen Y, and Balthasar JP. A physiologically-based pharmacokinetic (PBPK) model for disposition of IgG with altered FcRn binding kinetics. AAPS Annual Meeting, New Orleans, LA, 2010.

55. Abuqayyas L and Balthasar JP. Use of Modeling and Simulation to Investigate the Influence of Co-administered Soluble Antigen Fragments on the Tumor Penetration of mAbs. AAPS National Biotechnology Conference, San Francisco, CA, 2011.

56. Oxley T, Lappin G, Chen S-C, Balthasar J, Target Mediated Disposition of 14C-anti-CEA using Accelerator Mass Spectrometry. AAPS Annual Meeting, Washington DC, 2011.

57. Chen Y and Balthasar JP, Comparison of two PBPK models with assumptions of equilibrium and non-equilibrium IgG/FcRn interaction: Implications for antibody engineering for improved pharmacokinetics. AAPS Annual Meeting, Washington DC, 2011.

58. Abuqayyas L and Balthasar JP, Pharmacokinetic mAb-mAb Interaction: Anti-VEGF mAb Decreases Tumor Uptake of an Anti-CEA mAb in Colorectal Tumor Xenografts. AAPS Annual Meeting, Washington DC, 2011.

59. Engler FA, Zheng B, and Balthasar JP, The influence of nephropathy on mAb disposition in a mouse model of diabetic nephropathy. AAPS National Biotechnology Conference, San Diego, California, 2013

60. Engler FA and Balthasar JP, Investigation of Gelonin Pharmacokinetics in Mice with Use of a New Enzyme Linked Immunosorbent Assay. AAPS National Biotechnology Conference, San Diego, California, 2013

61. Glassman PM and Balthasar JP, Application of a catenary PBPK model to describe the disposition of "catch and release" antibodies, AAPS National Biotechnology Conference, San Diego, California, May 2014

62. Engler FA and Balthasar JP, Development and characterization of a pH-dependent anti-CEA monoclonal antibody, AAPS National Biotechnology Conference, San Diego, California, May 2014

63. Li T and Balthasar JP, Comparison of FcRn expression in transgenic mice, Swiss Webster mice, and in human tissues, AAPS National Biotechnology Conference, San Francisco, California, May 2015

64. Thomas VA, Yang Y, and Balthasar JP, Investigation of the influence of systemic lupus erythematosus on monoclonal antibody elimination in a mouse model, AAPS National Biotechnology Conference, San Francisco, California, May 2015

65. Glassman PM and Balthasar JP, PBPK Modeling of Monoclonal Antibodies Conjugated to Residualizing and Non-Residualizing Radioisotopes, AAPS National Biotechnology Conference, Francisco, California, May 2015

66. Thomas VA and Balthasar JP, Sorafenib Decreases the Tumor Uptake and Distribution of an Anti-carcinoembryonic Antigen Monoclonal Antibody in a Mouse Model of Colorectal Cancer, AAPS National Biotechnology Conference, Boston, Massachusetts, May 2016

67. Nguyen TD and Balthasar JP, Development of anti-CD123 monoclonal antibodies. Celebration of Student Academic Excellence, Center for the Arts, University at Buffalo, November 2016

67. Polli JR, Engler FA, An B, Qu J, Balthasar JP, Development and characterization of a "Catch-and-Release" murine anti-hCEA monoclonal antibody. Gordon Research Conference: DMPK; Holderness School, New Hampshire, July 2017

INVITED SEMINARS AND SPECIAL LECTURES

Application of mechanistic modeling to support the discovery and development of therapeutic antibodies, University of Missouri at Kansas City, May 2018, Kansas City, Missouri

Application of mechanistic modeling to support the discovery and development of therapeutic antibodies, University of Pittsburgh, December 2017, Pittsburgh, Pennsylvania

Pharmacokinetics and Pharmacodynamics of Antibodies (lecture series), Roche, November 2017, Basel, SWITZERLAND

Monoclonal Antibody Pharmacokinetics (lecture series), Merck, October 2017, Upper Gwynedd, Pennsylvania

Nanoparticles: Disruptive Technologies, Pfizer, October 2017, Boston, Massachusetts

Mechanistic Modeling and Avidity Effects, Sanofi, September 2017, Bridgewater, New Jersey

Antibody Pharmacokinetics (lecture series), Boehringer Ingelheim, September 2017, Biberach, GERMANY

Protein Pharmacokinetics (lecture series), Genentech, July 2017, San Francisco, CA

Antibody Pharmacokinetics Lecture Series, University at Buffalo, May 2017, Niagara Falls, New York

Tissue distribution and PBPK modeling, AAPS National Biotechnology Conference, May 2017, San Diego, California

Pharmacokinetic and pharmacodynamics considerations for the use of monoclonal antibodies in the treatment of infectious disease, University at Buffalo, Jacobs School of Medicine and Biomedical Sciences, May 2017, Buffalo, New York

Use of pH-dependent, “Catch-and-Release” antibodies for enhanced biological persistence and increased tumor exposure, University of Pennsylvania, School of Medicine, February 2017, Philadelphia, PA

Monoclonal Antibody Pharmacokinetics (lecture series), Amgen, February 2017, San Francisco, CA

Determinants of antibody distribution to the brain, AbbVie, November 2016, Worcester, MA

Mechanistic Determinants of Monoclonal Antibody Disposition, AbbVie, November 2016, Worcester, MA

Physiologically-based pharmacokinetic modeling to predict the disposition of monoclonal antibodies, AbbVie, November 2016, Worcester, MA

Mechanistic Determinants of the Pharmacokinetics of Therapeutic Proteins, GPEN Conference, University of Kansas, November 2016, Lawrence, KS

Physiologically-based pharmacokinetic modeling to predict the disposition of monoclonal antibodies, GPEN Conference, University of Kansas, November 2016, Lawrence, KS

Going beyond mAbs – PK opportunities and challenges, Bayer, September 2016, Berlin, GERMANY

Monoclonal Antibody Pharmacokinetics: Understanding between-patient and between-mAb PK variability, Pfizer, August 2016, Andover, Massachusetts

Albumin-Binding Strategies to Enhance Biologics Half-Life, Sanofi Aventis, July 2016, Frankfurt, GERMANY

PBPK Models for mAb, Amgen, June 2016, Cambridge, Massachusetts

PBPK Models for mAb: Discussion of the Promise, the Problems and the Pitfalls, New England Drug Metabolism Discussion Group, June 2016, Burlington, Massachusetts

Antibody Pharmacokinetics Lecture Series, University at Buffalo, May 2016, Niagara Falls, New York

Monoclonal Antibody Pharmacokinetics Lecture Series, Merck & Co, Inc., May 2016, North Wales, Pennsylvania

Protein Pharmacokinetics Lecture Series, Roche R&D, April 2016, Basel, SWITZERLAND

Use of Mechanistic Mathematical Models to Predict Monoclonal Antibody Pharmacokinetics, Delaware Valley Drug Metabolism Discussion Group, March 2016, Langhorne, Pennsylvania

Strategies for Predicting the Pharmacokinetics of Monoclonal Antibody Drugs, Bayer, September 2015, Duesseldorf, GERMANY

Trends in Biotherapeutic Research and Development, Sanofi Aventis, June 2015, Paris, FRANCE

Pharmacological Implications of Bispecific Antibodies, BIO Meeting, May 2015, Philadelphia, Pennsylvania

Application of PKPD Modeling to Guide the Development of New Antibody-Based Therapies, Canadian Society of Pharmaceutical Scientists, May 2015, Toronto, Ontario, CANADA

Antibody Pharmacokinetics Lecture Series, University at Buffalo, May 2015, Niagara Falls, New York

Mechanistic Models of Antibody Pharmacokinetics: Applications in drug discovery and optimization, University of Kansas, October 2014, Lawrence, Kansas

Physiologically-Based Pharmacokinetic Modeling for Monoclonal Antibody Drugs, ADAPT Workshop, University at Buffalo, May 2014, Niagara Falls, New York

Antibody Pharmacokinetics Lecture Series, University at Buffalo, June 2014, Niagara Falls, New York

Physiologically-Based Modeling of Monoclonal Antibodies: Challenges for application to pediatric patients, ASCPT Annual Meeting, April 2014, Atlanta, GA

Application of PKPD modeling and simulation to facilitate discovery and development of biologics, Celegene Inc., March 2014, San Diego, CA

Use of Mechanistic Studies to Inform the Development of Physiologically-Based Models for mAb Pharmacokinetics, PopSim 2014, January 2014, Copenhagen, DENMARK

Pharmacokinetic Models for Protein Therapeutics, Danish Society of Pharmacology, January 2014, Odense, DENMARK

Physiologically-based models for predicting mAb pharmacokinetics, AAPS ADME-BioTx Workshop, August 2013, Buffalo, NY

Protein Elimination and Catabolism, AAPS ADME-BioTx Workshop, August 2013, Buffalo, NY

Pharmacokinetics and Pharmacodynamics Course, Genentech Inc., July 2013, San Francisco, CA

Antibody Pharmacokinetics and Pharmacodynamics Lecture Series, University at Buffalo, May 2013, Niagara Falls, New York

Application of PBPK Modeling to Predict Monoclonal Antibody Disposition in Plasma and Tissues in Mouse Models of Human Colorectal Cancer, 2013 National Biotechnology Conference, May 2013, San Diego, California

Mechanistic Considerations for Assessing DDI Risks for ADCs, 2013 National Biotechnology Conference, May 2013, San Diego, California

In pursuit of predictive models for mAb pharmacokinetics: Striving for progress through mechanistic studies, Plenary Lecture, DMdg Symposium, May 2013, Basel, SWITZERLAND

Use of mechanistic studies and mathematical modeling to characterize and predict antibody distribution in tissues, CIHR Biological Therapeutics Training Program Symposium, University of Toronto, May 2013, Toronto, CANADA

Pharmacokinetics of Proteins and Peptides, New Jersey Drug Metabolism Discussion Group Symposium on Models, Pharmacogenomics, and Pharmacokinetics, April 2013, Somerset, New Jersey

Use of PKPD modeling in the development of new monoclonal antibodies for treatment and detection of disease, University at Buffalo, School of Dental Medicine, February 2013, Buffalo, New York

Pharmacokinetics and Pharmacodynamics of Protein Drugs - Lecture Series, Novimmune, January 2013, Geneva, SWITZERLAND

Monoclonal Antibody Pharmacokinetics - Lecture Series, University of Paris V, January 2013, Paris, FRANCE

Physiologically- based pharmacokinetic modeling to expedite the development of targeted nano-therapies, 2012 Chinese Pharmaceutical Conference, September 2012, Chengdu, CHINA

Antibody Pharmacokinetics: Mechanistic determinants & strategies for predicting mAb disposition in humans, Bayer Pharmaceuticals, May 2012, Berlin, GERMANY

Antibody Pharmacokinetics and Pharmacodynamics Lecture Series, University at Buffalo, May 2012, Niagara Falls, New York

Overview of Antibody Pharmacokinetics, Vaccinex, March 2012, Rochester, NY

Application of PK/PD modeling to Guide the Development of New Immunotherapies, BIOMED Lecture, University of Leuven (KU Leuven), March 2012, Leuven, BELGIUM

Application of PKPD Modeling to Estimate Effective Clinical Doses of mAb, Roche, January 2012, Nutley, NJ

Overview of Ongoing Research in the Center for Protein Therapeutics, Roche, January 2012, Basel, SWITZERLAND

Monoclonal Antibody Lecture Series, University of Paris 5, January 2012, Paris, FRANCE

Application of PKPD modeling to expedite the discovery and early development of novel drug therapies, Korean Society of Pharmaceutical Sciences and Technology, 41st Annual Meeting, November 2011, Cheongju, SOUTH KOREA

An academic view of therapeutic protein drug interactions, 17th North America Regional ISSX Meeting, October 2011, Atlanta, GA.

Mechanistic considerations for drug-drug interactions with biological, Centocor Drug-Drug Interaction Symposium, October 2011, Raritan, NJ

Overview of the determinants of mAb clearance & methods for characterizing target mediated mAb disposition, Roche, September 2011, Schlieren, SWITZERLAND

Preclinical considerations for mAbs, Annual Meeting of the American Clinical College of Pharmacy, September 2011, Chicago, IL

Application of PKPD modeling to estimate effective clinical doses of mAb, MedImmune Inc., July 2011, Gaithersburg, MD

Monoclonal Antibody Pharmacokinetics – Lecture Series, Ferring Inc., June 2011, Copenhagen, DENMARK

Translation of TMD from animals to humans: Where are we and what is our vision? Hofmann La Roche, May 2011, Nutley, NJ

Unusual and unexpected PK behavior of mAbs: Examples and possible mechanisms, AAPS National Biotechnology Conference, May 2011, San Francisco, CA

Antibody Pharmacokinetics and Pharmacodynamics Lecture Series, University at Buffalo, May 2011, Buffalo, New York

Antibody Pharmacokinetics and Pharmacodynamics Lecture Series, Amgen Pharmaceuticals Inc., May 2011, Thousand Oaks, CA

Investigation of the influence of FcRn on the pharmacokinetics of monoclonal antibodies, Abbott, February 2011, Chicago, IL

Monoclonal Antibody Pharmacokinetics – Lecture Series, Pfizer, January 2011, Andover, MA

Antibody Pharmacokinetics Lecture Series, University of Paris 5, January 2011, Paris, FRANCE

Applications of Imaging for Investigation of mAb Pharmacokinetics and Pharmacodynamics, Roche, January 2011, Basel, SWITZERLAND

Mechanistic Modeling of Monoclonal Antibodies: Applications in discovery and early development, AAPS Annual Meeting, November 2010, New Orleans, LA

Antibody Pharmacokinetics Lecture Series, Amgen, November 2010, Seattle, WA

Strategies for Rational Optimization of mAb Bioavailability, Persistence, and Efficacy, Eisai Pharmaceuticals, October 2010, Research Triangle Park, NC

Application of PKPD Modeling to Guide the Discovery and Early Development of mAbs, UNC / Eisai Distinguished Lecture in Drug Delivery, University of North Carolina, October 2010, Chapel Hill, NC

Monoclonal Antibodies: PK Questions and Applications of PKPD Modeling, Bayer Pharmaceuticals, September 2010, Berkeley, CA

Investigation of the influence of FcRn on the pharmacokinetics of monoclonal antibodies, Pfizer, September 2010, Cambridge, MA

Antibody Pharmacokinetics, NovoNordisk, August 2010, Copenhagen, DENMARK

Pharmacokinetic strategies for improved cancer therapy and detection, University of Kentucky, August 2010, Lexington, Kentucky

Application of PKPD to assist in the development of immunotherapies, Twelfth Buffalo Pharmaceutics Symposium, July 2010, Buffalo, New York

Antibody Pharmacokinetics and Pharmacodynamics Lecture Series, University at Buffalo, May 2010, Buffalo, New York

SC Bioavailability of mAb: Investigations with Knockout Mice and Engineered mAb, May 2010, AAPS National Biotechnology Conference, San Francisco, California

Scaling the Pharmacokinetics of Biologics Across Species, May 2010, AAPS National Biotechnology Conference, San Francisco, California

An Academic View of Therapeutic Protein Drug-Drug Interactions, May 2010, AAPS National Biotechnology Conference, Workshop on Therapeutic Protein Drug-Drug Interactions, San Francisco, California

Antibody PKPD Lecture Series, Boerhinger-Ingelheim, April 2010, Ridgeway, Connecticut

Monclonal Antibody PKPD Lecture Series, Boerhinger-Ingelheim, March 2010, Biberach, GERMANY

Effects of FcR on the Disposition of Monoclonal Antibodies, Roche, March 2010, Basel, SWITZERLAND

Antibody Pharmacokinetics and Pharmacodynamics Lecture Series, Schering-Plough, December 2009, Kenilworth, New Jersey

Target Mediated Disposition of Antibodies, Abbott, November 2009, Worcester, MA

Pharmacology and Mechanisms of IVIG Action Emphasis on the Use of IVIG for ITP, American Association of Blood Banks Annual Meeting, October 2009, New Orleans, Louisiana

Predicting the Human Pharmacokinetics of Monoclonal Antibodies: Concepts and Examples, ISSX Annual Meeting, October 2009, Baltimore, Maryland

Organized Plenary Symposium titled: Disposition and Dynamics of Biologics: Where Are We Headed? ISSX Annual Meeting, October 2009, Baltimore, Maryland

Antibody Pharmacokinetics and Pharmacodynamics Lecture Series, Merck, September 2009, West Point, Pennsylvania

Antibody Pharmacokinetics and Pharmacodynamics Lecture Series, Roche, September 2009, Nutley, New Jersey

Pharmacokinetics and Pharmacodynamics of Monoclonal Antibodies, Land O' Lakes Meeting, September 2009, Merrimac, Wisconsin

Antibody Pharmacokinetics and Pharmacodynamics Lecture Series, Glaxo Smith Kline, August 2009, London, ENGLAND

Application of PKPD M&S to Discovery/Development of Large Molecules: Learning from Antibodies, Gordon Conference on Drug Metabolism, July 2009, Holderness, NH

Antibody Pharmacokinetics and Pharmacodynamics Lecture Series, OncoMed, July 2009, South San Francisco, California

Antibody Pharmacokinetics and Pharmacodynamics Lecture Series, Centocor, July 2009, Malvern, Pennsylvania

Antibody Pharmacokinetics and Pharmacodynamics Lecture Series, University at Buffalo, May 2009, Buffalo, New York

Antibody Pharmacokinetics and Pharmacodynamics, Pharmacokinetic – Pharmacodynamic Concepts and Applications Course, University at Buffalo, May 2009, Buffalo, New York

Antibody Pharmacokinetics and Pharmacodynamics Lecture Series, Pfizer, March 2009, New London, Connecticut

Use of PKPD modeling to guide the development of new strategies for cancer therapy, Biogen-IDEC, February 2009, Cambridge, Massachusetts

Antibody Pharmacokinetics and Pharmacodynamics Lecture Series, University of Paris 5, January 2009, Paris, FRANCE

Target Mediated Disposition of Monoclonal Antibodies, Biogen-IDEC, December 2008, Cambridge, Massachusetts

Antibody Pharmacokinetics and Pharmacodynamics Lecture Series, Pfizer, March 2008, Sandwich, ENGLAND

Protein Pharmacokinetics, Allergan Pharmaceuticals, May 2008, Irvine California

Antibody Pharmacokinetics and Pharmacodynamics Lecture Series, Pfizer, March 2008, Groton, Connecticut

Introduction to Antibody PK/PD: Determinants of Mab Absorption, Distribution, and Elimination, American College of Clinical Pharmacology, Annual Meeting, September 2007, San Francisco, California

PKPD of Antibodies: Examples, American College of Clinical Pharmacology, Annual Meeting, September 2007, San Francisco, California

Comparability Assessment of Therapeutic Proteins, Amgen, July 2007, Thousand Oaks, California

Intricacies in Pharmacokinetics and Pharmacodynamics of Peptides and Proteins, Amgen, July 2007, Thousand Oaks, California

Pharmacokinetics of Peptides and Proteins, Amgen, July 2007, Thousand Oaks, California

Bimolecular Interactions in Dynamics, Amgen, July 2007, Thousand Oaks, California

Target-Mediated Disposition of Monoclonal Antibodies, Amgen, July 2007, Thousand Oaks, California
Physiologically-based Pharmacokinetic Models for Monoclonal Antibodies, Amgen, July 2007, Thousand Oaks, California

Pharmacodynamic Models Relevant to Proteins, Amgen, July 2007, Thousand Oaks, California

Utility of PK/PD Modeling in the Comparability Assessment of Therapeutic Proteins – An Academic Perspective, AAPS National Biotechnology Meeting, June 2007, San Diego, California

Where Did my Mab Go?, AAPS National Biotechnology Meeting, June 2007, San Diego, California

Monoclonal Antibody Pharmacokinetics, Pfizer, June 2007, Groton, Connecticut

PK/PD Considerations for the Optimization of Immunotoxicotherapy, Pfizer, June 2007, Groton, Connecticut

PD Models Relevant to Monoclonal Antibodies, May 2007, Genentech, Pharmacokinetic – Pharmacodynamic Concepts and Applications Course, San Francisco, California

Target-Mediated Disposition of Antibodies, May 2007, Genentech, Pharmacokinetic – Pharmacodynamic Concepts and Applications Course, San Francisco, California

Dynamics of Bimolecular Interactions: Considerations for Immunotoxicotherapy, May 2007, Genentech, Pharmacokinetic – Pharmacodynamic Concepts and Applications Course, San Francisco, California

Pharmacokinetics of Peptides and Proteins, May 2007, Genentech, Pharmacokinetic – Pharmacodynamic Concepts and Applications Course, San Francisco, California

Pharmacokinetics and Pharmacodynamics of Monoclonal Antibodies, May 2007, University at Buffalo, Pharmacokinetic – Pharmacodynamic Concepts and Applications Course, Buffalo, New York

Monoclonal Antibody Pharmacokinetics, New York Academy of Sciences, Symposium on The Future of Monoclonal Antibody Biotherapeutics Production and Development, May 2007, New York, New York

Alphabet Soup: ITP, IVIG, FcRn, & Mab PK, May 2007, University of Kentucky, Lexington, Kentucky

Mechanistic Insights into the ADME Properties of Antibodies and Other Biologics, Merck, March 2007, West Point, Pennsylvania

Target-Mediated Disposition and Dynamics of Biologics: Principles and Applications, Merck, March 2007, West Point, Pennsylvania

Pharmacokinetics and Pharmacodynamics of Antibodies: Work Underway in the Balthasar Laboratory, Amgen, November 2006, Thousand Oaks, California

Monoclonal Antibody Pharmacodynamics, F.Hoffmann-La Roche Ltd, November 2006, Basel, SWITZERLAND

Monoclonal Antibody Pharmacokinetics, F.Hoffmann-La Roche Ltd, November 2006, Basel, SWITZERLAND

Physiologically-based Modeling: General Considerations and Examples, Novartis Pharma AG, October 2006, Basel, SWITZERLAND

Investigations of the influence of FcRn on IgG tissue and systemic disposition, Novartis Pharma AG, October 2006, Basel, SWITZERLAND

Use of Simulation to Guide the Selection of Optimal Monoclonal Antibodies for Immunotoxicotherapy, Novartis Pharma AG, October 2006, Basel, SWITZERLAND

Pharmacokinetic / Pharmacodynamic Modeling and Immunotoxicotherapy, Novartis Pharma AG, October 2006, Basel, SWITZERLAND

Target-Mediated Monoclonal Antibody Disposition, Novartis Pharma AG, October 2006, Basel, SWITZERLAND

Monoclonal Antibody Pharmacokinetics & Pharmacodynamics, Novartis Pharma AG, October 2006, Basel, SWITZERLAND

Monoclonal Antibodies: Pharmacokinetic & Pharmacodynamic Modeling, Cognigen, September 2006, Amherst, New York

Distribution, Clearance, and PK/PD Modeling: Special Considerations for Proteins and Peptides, Merck, June 2006, North Wales, Pennsylvania.

Mab PK Overview, AAPS Workshop on Current Trends in Monoclonal Antibody Development and Manufacturing, AAPS National Biotechnology Meeting, June 2006, Boston, Massachusetts.

Application of FcRn Inhibitors for the Treatment of Humoral Autoimmune Diseases, AAPS National Biotechnology Meeting, June 2006, Boston, Massachusetts.

Pharmacokinetics and Pharmacodynamics of Monoclonal Antibodies, May 2006, University at Buffalo, Pharmacokinetic – Pharmacodynamic Concepts and Applications Course, Buffalo, New York

Antibody Pharmacokinetics and Pharmacodynamics: Focus on Issues Relevant for Immunotoxicotherapies, May 2006, Wyeth, Collegeville, Pennsylvania.

PK/PD Correlations of Monoclonal Antibodies, April 2006, 5th International Symposium on Measurement and Kinetics of In Vivo Drug Effects, Noordwijkerhout, The NETHERLANDS.

Antibody Pharmacokinetics, April 2006, Pfizer, Ann Arbor, Michigan.

FcRn Inhibition: An Important Mechanism for IVIG Effects in ITP? March 2006, Department of Oral Biology, University at Buffalo, The State University of New York, Buffalo, New York.

Pharmacokinetic Strategies to Optimize IP Chemotherapy for Ovarian Cancer, January 2006, Rutgers University, New Brunswick, New Jersey.

Studies assessing the effects of an anti-FcRn antibody on the disposition of anti-platelet antibodies and on anti-platelet antibody-induced thrombocytopenia in a mouse model of sustained ITP, December 2005, ITP Study Group Meeting (in conjunction with the Annual Meeting of the American Society of Hematology), Atlanta, Georgia.

Comparison of anti-RBC immunotherapy, IVIG, and antibody-cated liposome therapies in a mouse model of sustained ITP, December 2005, ITP Study Group Meeting (in conjunction with the Annual Meeting of the American Society of Hematology), Atlanta, Georgia.

Antibody Pharmacokinetics and Pharmacodynamics: Are there important differences relative to small molecule PK/PD?, November 2005, Schering Plough Research Institute, Kenilworth, New Jersey.

FcRn-inhibition: An important mechanism for IVIG effects in immune thromobocytopenia? September 2005, University of Minnesota, Minneapolis, Minnesota

Development of FcRn inhibitors for autoimmunity, August 2005, Genentech, San Francisco, California.

Antibody Pharmacokinetics and Pharmacodynamics, June 2005, Wyeth, Boston, Massachusetts.

Application of pharmacokinetic / pharmacodynamic modeling to expedite the discovery and development of monoclonal antibodies for immunotoxicotherapy, June 2005, National Biotechnology Conference of the American Association of Pharmaceutical Scientists, San Francisco, California

Pharmacokinetics and Pharmacodynamics of Monoclonal Antibodies, May 2005, University at Buffalo, Pharmacokinetic – Pharmacodynamic Concepts and Applications Course, Buffalo, New York

Pharmacokinetic Strategies to Optimize IP Chemotherapy, February 2005, Pharmacology and Therapeutics Department, Grace Cancer Drug Center, Roswell Park Memorial Cancer Institute, Buffalo, New York.

Pharmacokinetics and Pharmacodynamics of Antibodies, January 2005, International Symposium for Life Sciences and Medicine, Keio University, Tokyo, JAPAN

Mechanisms and Kinetics of IgG Elimination, November 2004, Sunrise School, Annual Meeting of the American Association of Pharmaceutical Scientists, Baltimore, MD

Pharmacokinetics and Pharmacodynamics of Monoclonal Antibodies, May 2004, University at Buffalo – Leiden/Amsterdam Center for Drug Research Pharmacokinetic – Pharmacodynamic Concepts and Applications Course, Buffalo, New York

Inverse Targeting for Optimization of Intraperitoneal Chemotherapy, May 2004, Department of Biopharmaceutical Sciences, University of Illinois, Chicago, Illinois

Inverse Targeting with Anti-Drug Antibodies, April 2004, University of Kentucky, Division of Pharmaceutical Sciences, College of Pharmacy, Lexington, Kentucky

Pharmacodynamic Analyses for Assessment of Equivalence of Biotech Products, November 2003, Expert Panel on Complex Activities, United States Pharmacopoeia, Rockville, Maryland

Competitive Inhibition of FcRn: An important mechanism of IVIG action?, November 2003, Annual Meeting of the American Association of Blood Banks, San Diego, California

Pharmacokinetics and Pharmacodynamics of Monoclonal Antibodies, May 2003, University at Buffalo – Leiden/Amsterdam Center for Drug Research Pharmacokinetic – Pharmacodynamic Concepts and Applications Course, Buffalo, New York

Application of PK/PD to Investigate Mechanisms of IVIG Action in ITP, May 2003, Division of Pharmaceutics, College of Pharmacy, The Ohio State University, Columbus, Ohio.

Application of Kinetic/Dynamic Mathematical Models to Guide the Development of New Therapies of Immune Thrombocytopenia, March 2003, Department of Chemical Engineering, University of Maryland, College Park, Maryland.

Pharmacodynamic Analyses for Assessment of Equivalence of Biotech Products, January 2003, Expert Panel on Complex Activities, United States Pharmacopoeia, Rockville, Maryland

Role of FcRn in IgG Metabolism: Implications for Therapeutic Antibodies and Autoimmunity, January 2003, Molecular Biopharmaceutics Meeting, Waikiki, Hawaii

Antibody PK/PD: New Insights & Applications, November 2002, Buffalo Pharmaceutics Symposium, University at Buffalo, The State University of New York, Amherst, New York

Evidence for a New Mechanism of IVIG Action, October 2002, The University of Michigan, Department of Pharmaceutical Sciences, College of Pharmacy, Ann Arbor, Michigan

Bioreactor Accelerated Immunotoxicotherapy, August 2002, Industrial Science & Technology Network Inc., York, Pennsylvania

Investigation of IVIG Mechanism of Action in ITP: Application of new findings to identify targets for therapy, June 2002, The University of Michigan, Yang Laboratory, Department of Pharmaceutical Sciences, College of Pharmacy, Ann Arbor, Michigan

Application of Kinetic – Dynamic Modeling to Predict Antibody Effects in an Inverse Targeting Strategy, May 2002, University of North Carolina, Division of Drug Delivery and Disposition, College of Pharmacy, Chapel Hill, North Carolina

Case Study: Application of PK/PD Modeling to Delineate Mechanisms of IVIG Action in Immune Thrombocytopenia, May 2002, Georgetown University Center for Drug Development Science: Workshop on 'Physiologically Based Pharmacokinetics in Drug Development and Regulatory Science', Washington, DC

Monoclonal Antibodies, May 2002, University at Buffalo – Leiden/Amsterdam Center for Drug Research Pharmacokinetic – Pharmacodynamic Concepts and Applications Course, Buffalo, New York

New Insights Into the Mechanism of IVIG Action in Immune Thrombocytopenia, April 2002, University of Kentucky, Division of Pharmaceutical Sciences, College of Pharmacy, Lexington, Kentucky

Pharmacokinetics and Pharmacodynamics of Monoclonal Antibodies, May 2001, University at Buffalo – Leiden/Amsterdam Center for Drug Research Pharmacokinetic – Pharmacodynamic Concepts and Applications Course, Buffalo, New York

Pharmacokinetics and Pharmacodynamics of Antibodies, May 2000, University at Buffalo – Leiden/Amsterdam Center for Drug Research Pharmacokinetic – Pharmacodynamic Concepts and Applications Course, Buffalo, New York

Pharmacology and Bioengineering of New Treatments for Immune Thrombocytopenia, January 2000, The University of Michigan, Yang Laboratory, Department of Pharmaceutics, College of Pharmacy, Ann Arbor, Michigan

Animal Models of Immune Thrombocytopenia: Application for the Development and Evaluation of New Treatments for ITP, January 2000, University at Buffalo, Department of Pharmaceutics, Buffalo, New York

Inverse Targeting: New Approaches of Enhancing the Selectivity of Ovarian Cancer Chemotherapy, August 1999, University at Buffalo, Department of Pharmaceutics, Buffalo, New York

Inverse Targeting: A New Approach for Enhancing the Selectivity of the Chemotherapy of Peritoneal Tumors, July 1999, Huntsman Cancer Institute, Salt Lake City, Utah

Understanding the Influence of Drug Formulation on PK/PD: Introduction of a New Inverse-Targeting Approach, May 1999, Lipocine Inc., Salt Lake City, Utah

Optimization of Cancer Chemotherapy, February 1999, Utah State University, Department of Chemistry, Logan, Utah

Optimization of intraperitoneal cancer chemotherapy with anti-drug antibodies: Pharmacokinetic rationale and experimental results, November 1995, American Association of Pharmaceutical Scientists, Eli Lilly Graduate Symposium, Miami, Florida

PROFESSIONAL ASSOCIATIONS (past & present membership)

American Association for the Advancement of Science
American Society of Hematology
American Association of Colleges of Pharmacy
American Association of Pharmaceutical Scientists
American Society for Pharmacology and Experimental Therapeutics

PROFESSIONAL SERVICE

Associate Editor

Journal of Pharmacokinetics and Pharmacodynamics (6/2005 – 9/2009)
Drug Metabolism and Disposition (1/2012 – 6/2014)

Member, Editorial Board

Current Clinical Pharmacology (6/2005 – present)
Journal of Pharmaceutical Sciences (1/2008 – present)
AAPS Journal (1/2009 – present)
Mabs (1/2009 – present)
CPT: Pharmacometrics & Systems Pharmacology (6/2014 – present)

Scientific Reviewer
Blood, Immunology, Pharmaceutical Research, Drug Metabolism and Disposition, Journal of Pharmaceutical Sciences, Journal of Pharmacokinetics and Pharmacodynamics, AAPS Journal, AAPS PharmSci, Biochemical Pharmacology, Journal of Controlled Release, British Journal of Clinical Pharmacology, European Journal of Pharmaceutics, Cancer Chemotherapy and Pharmacology

Symposium Organizer
- AAPS, 2005 National Biotechnology Conference, Organized symposium titled: Application of PK/PD modeling to expedite development of monoclonal antibodies, June 2005, San Francisco, California
- AAPS, 2006 National Biotechnology Conference, Organized symposium titled: Targeting the Brambell Receptor (FcRn) to modulate antibody absorption and disposition, June 2006, Boston, Massachusetts
- AAPS, 2006 National Biotechnology Conference, Organized symposium titled: Diabodies: What are they and what advantages do they provide?, June 2006, Boston, Massachusetts
- AAPS, 2009 Annual Meeting, Co-Organized symposium titled: Mechanism-based modeling: Its role in discovery and early development of biologics, November 2009, Los Angeles, California
- AAPS, 2011 National Biotechnology Conference, Organized symposium titled: Unusual and unexpected PK behavior of monoclonal antibodies, San Francisco, California
- GPEN, 2016 Conference at the University of Kansas, Organized workshop titled: Mechanistic modeling of protein therapeutics, Lawrence, Kansas
- ADME BioTx, Organized workshop at the University at Buffalo, August 2017

Symposium Moderator
- AAPS, 2005 National Biotechnology Conference, Moderated symposium titled: Application of PK/PD modeling to expedite development of monoclonal antibodies, June 2005, San Francisco, California
- AAPS, 2006 National Biotechnology Conference, Moderated symposium titled: Targeting the Brambell Receptor (FcRn) to modulate antibody absorption and disposition, June 2006, Boston, Massachusetts
- AAPS, 2006 National Biotechnology Conference, Moderated symposium titled: Diabodies: What are they and what advantages do they provide?, June 2006, Boston, Massachusetts
- AAPS, 2009 Annual Meeting, Moderated symposium titled: Mechanism-based modeling: Its role in discovery and early development of biologics, November 2009, Los Angeles, California
- AAPS, 2011 National Biotechnology Conference, Moderated symposium titled: Unusual and unexpected PK behavior of monoclonal antibodies, San Francisco, California

Committee Member, Grant Review
- University of Utah, College of Pharmacy Research Program, Review committee member, 1998 and 1999
- American Association of Colleges of Pharmacy, New Investigators Program, Committee member and Chair, Pharmaceutics 'B' Review Section, 2000
- American Association of Colleges of Pharmacy, New Investigators Program, Committee member and Chair, Pharmaceutics Review Section, 2002
- 2002 -2003: NIH Biodefense and Virology Study Section (SSS-Q)
- 6/2003: Ad Hoc Reviewer, NIH Pharmacology Study Section (PHRA)
- 2003-2004: NIH Bacterial Biodefense (IDMA) Study Section
- 10/2004: Ad Hoc Reviewer, NIH Neural Basis of Psychopathology, Addictions and Sleep Disorders (NPAS)
- 2005-2008: NIH Special Emphasis Panel (ZRG1 DIG-F 02)
- 2005-2006: NIH Cancer Development and Therapeutics (SBIR, ONC-T)
- 2/2007: Ad Hoc Reviewer, NIH Xenobiotic and Nutrient Disposition and Analysis (XNDA)
- 2/2009: Ad Hoc Reviewer, NIH Special Emphasis Panel (ZRG1DKUS-03M)
- 7/2009: Ad Hoc Reviewer, NIH Special Emphasis Panel (ZRG1 DKUS-A 58)
- 8/2010: Ad Hoc Reviewer, NIH GM Program Project Review Panel
- 6/2011: Ad Hoc Reviewer, NIH Xenobiotic and Nutrient Disposition and Analysis (XNDA)

- 10/2011: Ad Hoc Reviewer, NIH Gene and Drug Delivery (GDD)
- 2013-2015: NIH Special Emphasis Panel (ZRG1 EMNR-R(56))
- 1/2016: Ad Hoc Reviewer, NIH Nanotechnology (NANO)
- 6/2017 – 6/2021: Standing Member, NIH Gene and Drug Delivery (GDD)

Committee Member, National Associations
- American Association of Pharmaceutical Sciences PPDM Education Sub-Committee 1993 – 1994.
- American Association of Pharmaceutical Sciences Education Committee, 1993 – 1995.
- American Association of Colleges of Pharmacy, Committee for Graduate Education, Scholarship, and Research, University of Utah College of Pharmacy Liaison, 1998-1999.
- Expert Panel on Complex Activities, United States Pharmacopoeia, 2003
- AAPS, BIOTECH Section, Planning Committee, 2005 National Biotechnology Meeting (June 2005, San Francisco)
- AAPS, BIOTECH Section, Planning Committee, 2006 National Biotechnology Meeting
- AACP, Paul Dawson Biotechnology Award, Selection Committee, January 2006
- AAPS, PPDM Section, Planning Committee, 2007 National Biotechnology Meeting

UNIVERSITY SERVICE

Committee Member, College / School / Department
- University of Utah, College of Pharmacy Learning and Teaching Committee, 1997-1999
- University of Utah, College of Pharmacy Curriculum Committee, 1998-1999
- University at Buffalo, Pharmacy and Pharmaceutical Sciences, Curriculum Committee, 2000 - 2003
- University at Buffalo, Pharmaceutical Sciences, Faculty Search Committee, Chair, 2003-2004
- University at Buffalo, Pharmaceutical Sciences, Faculty Search Committee, Chair, 2005-2006
- University at Buffalo, Pharmaceutical Sciences, Chair Search Committee, Chair, 2016-present

Committee Member, University
- University of Utah, Technology Transfer Advisory Committee, 1999
- University of Utah, Clinical Institutional Review Board, 1999
- University at Buffalo, Graduate School Advisory Committee on Electronic Theses and Dissertations, 2000
- University at Buffalo, Radiation Safety Committee, member, 2000- 2005
- University at Buffalo, Radiation Safety Committee, Chair, 2004- 2005
- University at Buffalo, Radiation Safety Committee, Chair, 2012 – present
- University at Buffalo, Conflict of Interest Committee, Member, 2013 – present

AWARDS/HONORS

Western New York Science Congress, Bronze Medal
New York State Regents Scholarship
SUNY at Buffalo School of Pharmacy Scholarship
Rho-Chi Honor Society
Schering-Plough Pre-doctoral Fellowship
AFPE Pre-doctoral Fellowship
AAPS / Eli Lilly Graduate Symposium Award, 1995
SUNY at Buffalo Department of Pharmaceutics Graduate Scholar Award
AACP New Investigator Award, 1997
University of Utah, College of Pharmacy Distinguished Teaching Award, 1998
AAPS PPDM Annual Meeting Travel Award, 2001
University at Buffalo, Young Investigator Achievement Award, 2002
University at Buffalo, Top 100 Federal Grantee Award
AAPS Fellow
AAPS Outstanding Manuscript in Modeling and Simulation, 2009

UNC / Eisai Distinguished Lecturer in Drug Delivery, 2010

LICENSES

New York State Pharmacy License (040627-1)

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ReoPro®
Abciximab
For intravenous administration

DESCRIPTION:

Abciximab, ReoPro®, is the Fab fragment of the chimeric human-murine monoclonal antibody 7E3. Abciximab binds to the glycoprotein (GP) IIb/IIIa receptor of human platelets and inhibits platelet aggregation.

The chimeric 7E3 antibody is produced by continuous perfusion in mammalian cell culture. The 47,615 dalton Fab fragment is purified from cell culture supematant by a series of steps involving specific viral inactivation and removal procedures, digestion with papain and column chromatography.

ReoPro® is a clear, colorless, sterile, non-pyrogenic solution for intravenous (IV) use. Each single use vial contains 2 mg/mL of Abciximab in a buffered solution (pH 7.2) of 0.01 M sodium phosphate, 0.15 M sodium chloride and 0.00 1% polysorbate 80 in Water for Injection. No preservatives are added.

CLINICAL PHARMACOLOGY:

General

Abciximab binds to the intact platelet GPIIb/IIIa receptor, which is a member of the integrin family of adhesion receptors and the major platelet surface receptor involved in platelet aggregation. Abciximab inhibits platelet aggregation by preventing the binding of fibrinogen, von Willebrand factor, and other adhesive molecules to GPIIb/IIIa receptor sites on activated platelets. The mechanism of action is thought to involve steric hindrance and/or conformational effects to block access of large molecules to the receptor rather than direct interaction with the RGD (arginine-glycine-aspartic acid) binding site of GPIIb/IIIa.

Pre-clinical experience

Maximal inhibition of platelet aggregation was observed when ≥ 80% of GPIIb/IIIa receptors were blocked by Abciximab. In non-human primates, Abciximab bolus doses of 0.25 mg/kg generally achieved a blockade of at least 80% of platelet receptors and fully inhibited platelet aggregation. Inhibition of platelet function was temporary following a bolus dose, but receptor blockade could be sustained at ≥80% by continuous intravenous infusion. The inhibitory effects of Abciximab were substantially reversed by the transfusion of platelets in monkeys. The antithrombotic efficacy of prototype antibodies [murine 7E3 Fab and $F(ab')_2$] and Abciximab was evaluated in dog, monkey and baboon models of coronary, carotid, and femoral artery thrombosis. Doses of the murine version of 7E3 or Abciximab sufficient to produce high-grade (≥ 80%) GPIIb/IIIa receptor blockade prevented acute thrombosis and yielded lower rates of thrombosis compared with aspirin and/or heparin.

Pharmacokinetics

Following intravenous bolus administration, free plasma concentrations of Abciximab decrease rapidly with an initial half-life of less than 10 minutes and a second phase half-life of about 30 minutes, probably related to rapid binding to the platelet GPIIb/IIIa receptors. Platelet function generally recovers over the course of 48 hours (1,2), although Abciximab remains in the circulation for 15 days or more in a platelet-bound state. Intravenous administration of a 0.25 mg/kg bolus dose of Abciximab followed by continuous infusion of 10 μg/min (or a weight-adjusted infusion of 0.125 μg/kg/min to a maximum of 10 μg/min) produces approximately constant free plasma concentrations throughout the infusion. At the termination of the infusion period, free plasma concentrations fall rapidly for approximately six hours then decline at a slower rate.

Pharmacodynamics

Intravenous administration in humans of single bolus doses of Abciximab from 0.15 mg/kg to 0.30 mg/kg produced rapid dose-dependent inhibition of platelet function as measured by *ex* vivo platelet aggregation in response to adenosine diphosphate (ADP) or by prolongation of bleeding time. At the two highest doses (0.25 and 0.30 mg/kg) at two hours post injection, over 80% of the GPIIb/IIIa receptors were blocked and platelet aggregation in response to 20 μM ADP was almost abolished. The median bleeding time increased to over 30 minutes at both doses compared with a baseline value of approximately five minutes.

Intravenous administration in humans of a single bolus dose of 0.25 mg/kg followed by a continuous infusion of 10 μg/min for periods of 12 to 96 hours produced sustained high-grade GPIIb/IIIa receptor blockade (≥ 80%) and inhibition of platelet function (ex vivo platelet aggregation in response to 5 μM or 20 μM ADP less than 20% of baseline and bleeding time greater than 30 minutes) for the duration of the infusion in most patients. Similar results were obtained when a weight-adjusted infusion dose (0.125 μg/kg/min to a maximum of 10 μg/min) was used in patients weighing up to 80 kg. Results in patients who received the 0.25 mg/kg bolus followed by a 5 μg/min infusion for 24 hours showed a similar initial receptor blockade and inhibition of platelet aggregation, but the response was not maintained throughout the infusion period.

Low levels of GPIIb/IIIa receptor blockade are present for more than 10 days following cessation of the infusion. After discontinuation of Abciximab infusion, platelet function returns gradually to normal. Bleeding time returned to ≤ 12 minutes within 12 hours following the end of infusion in 15 of 20 patients (75%), and within 24 hours in 18 of 20 patients (90%). *Ex vivo* platelet aggregation in response to 5 μM ADP returned to ≥ 50% of baseline within 24 hours following the end of infusion in 11 of 32 patients (34%) and within 48 hours in 23 of 32 patients (72%). In response to 20 μM ADP, ex ***vivo*** platelet aggregation returned to ≥ 50% of baseline within 24 hours in 20 of 32 patients (62%) and within 48 hours in 28 of 32 patients (88%).

CLINICAL STUDIES:

Abciximab has been studied in three Phase 3 clinical trials, all of which evaluated the effect of Abciximab in patients undergoing percutaneous coronary intervention: in patients at high risk for abrupt closure of the treated coronary vessel (EPIC), in a broader group of patients (EPILOG), and in unstable angina patients not responding to conventional medical therapy (CAPTURE). Percutaneous intervention included balloon angioplasty, atherectomy, or stent placement. All trials involved the use of various, concomitant heparin dose regimens and, unless contraindicated, aspirin (325 mg) was administered orally two hours prior to the planned procedure and then once daily.

EPIC was a multicenter, double-blind, placebo-controlled trial of Abciximab in patients undergoing percutaneous transluminal coronary angioplasty or atherectomy (3). In the EPIC trial, 2099 patients between 26 and 83 years of age who were at high risk for abrupt closure of the treated coronary vessel were randomly allocated to one of three treatments: 1) an Abciximab bolus (0.25 mg/kg) followed by an Abciximab infusion (10 μg/min) for 12 hours (bolus plus infusion group); 2) an Abciximab bolus (0.25 mg/kg) followed by a placebo infusion (bolus group), or; 3) a placebo bolus followed by a placebo infusion (placebo group). Patients at high risk during or following percutaneous coronary intervention were defined as those with unstable angina or non-Q wave myocardial infarction (n=489), those with an acute Q-wave myocardial infarction within 12 hours of symptom onset (n=66), and those who were at high risk because of coronary morphology and/or clinical characteristics (n=1544). Treatment with study agent in each of the three arms was initiated IO-60 minutes before the onset of percutaneous coronary intervention. All patients initially received an intravenous heparin bolus (10,000 to 12,000 units) and boluses of up to 3,000 units thereafter to a maximum of 20,000 units during percutaneous coronary intervention. Heparin infusion was continued for 12 hours to maintain a therapeutic elevation of activated partial thromboplastin time (APTT,1.5-2.5 times normal).

The primary endpoint was the occurrence of any of the following events within 30 days of percutaneous coronary intervention: death, myocardial infarction (MI), or the need for urgent intervention for recurrent ischemia [i.e., urgent percutaneous transluminal coronary angioplasty, urgent coronary artery bypass graft (CABG) surgery, a coronary stent, or an intra-aortic balloon pump]. The 30-day (Kaplan-Meier) primary endpoint event rates for each treatment group by intention-to-treat analysis of all randomized patients are shown in Table 1. The 4.5% lower incidence of the primary endpoint rates in the bolus plus infusion treatment group, compared with the placebo group, was statistically significant, whereas the 1.3% lower incidence in the bolus treatment group was not. A lower incidence of the primary endpoint was observed in the bolus plus infusion treatment arm for all three high-risk subgroups: patients with unstable angina, patients presenting within 12 hours of the onset of symptoms of an acute myocardial infarction, and patients with other high-risk clinical and/or morphologic characteristics (3). The treatment effect was largest in the first two subgroups and smallest in the third subgroup.

Table 1
PRIMARY ENDPOINT EVENT RATE AT 30 DAYS - EPIC TRIAL

	Placebo (n=696)	Abcixhnab Bolus (n=695)	Abciximab Bolus + Infusion (n=708)
		Number of Patients (%)	
Death, MI, or urgent intervention[1]	89 (12.8)	79 (11.5)	59 (8.3)
p-value vs. placebo		0.428	0.008
Components of Primary Endpoint[b]			
Death	12 (1.7)	9 (1.3)	12 (1.7)
Acute myocardial infarctions in surviving patients	55 (7.9)	40 (5.8)	31 (4.4)
Urgent interventions in surviving patients without an acute myocardial infarction	22 (3.2)	30 (4.4)	16 (2.2)

[a]Patients who experienced more than one event in the first 30 days are counted only once.
[b]Patients are counted only once under the most serious component (death > acute MI > urgent intervention).

The primary endpoint event rates in the bolus plus infusion treatment group were reduced mostly in the first 48 hours and this benefit was sustained through blinded evaluations at 30 days(3), and six months(4). At the six-month follow-up visit this event rate remained lower in the bolus plus infusion arm (12.3%) than in the placebo arm (17.6%) (p=0.006 vs. placebo).

EPILOG was a randomized, double-blind, multicenter, placebo-controlled trial which evaluated Abciximab in a broad population of patients undergoing percutaneous coronary intervention (excluding patients with myocardial infarction and unstable angina meeting the EPIC high risk criteria)(5). EPILOG tested the hypothesis that use of a low-dose, weight-adjusted heparin regimen, early femoral arterial sheath removal, improved access site management and weight-adjustment of the Abciximab infusion dose could significantly lower the bleeding rate yet maintain the efficacy seen in the EPIC trial. EPILOG was a three treatment-arm trial: Abciximab plus standard dose, weight-adjusted heparin[1]; Abciximab plus low dose, weight-adjusted heparin[2]; and placebo plus standard dose, weight-adjusted heparin. The Abciximab bolus dose was the same as that used in the EPIC trial

[1] Bolus administration of 100 U/kg weight-adjusted heparin to achieve an activated clotting time (ACT) of 300 seconds (maximum initial bolus 10,000 units).
[2] Bolus administration of 70 U/kg weight-adjusted heparin to achieve an activated clotting time (ACT) of 200 seconds (maximum initial bolus 7,000 units).

(0.25 mg/kg), but the continuous infusion dose was weight adjusted in patients up to 80 kg[3] (0.125 µg/kg/min). Specific patient and access site management procedures as well as a strong recommendation for early sheath removal were also incorporated into the trial as described in PRECAUTIONS. The EPILOG trial achieved the objective of lowering the bleeding rate while maintaining efficacy: in the Abciximab treatment arms major bleeding was not significantly different from that in the placebo arm (see ADVERSE REACTIONS: Bleeding).

The primary endpoint of the EPILOG trial was the composite of death or MI occurring within 30 days of percutaneous coronary intervention. The composite of death, MI, or urgent intervention was an important secondary endpoint. As seen in the EPIC trial, the endpoint events in the Abciximab treatment group were reduced mostly in the first 48 hours and this benefit was sustained through blinded evaluations at 30 days and six months. The (Kaplan-Meier) endpoint event rates at 30 days are shown in Table 2 for each treatment group by intention-to-treat analysis of all 2792 randomized patients. At the six-month follow-up visit, the event rate for death, MI, or repeat (urgent or non-urgent) intervention remained lower in the Abciximab treatment arms (22.3% and 22.8%, respectively, for the standard- and low-dose heparin arms) than in the placebo arm (25.8%) and the event rate for death, MI, or urgent intervention was substantially lower in the Abciximab treatment arms (8.3% and 8.4%, respectively, for the standard- and low-dose heparin arms) than in the placebo arm (14.7%). The proportionate reductions in endpoint event rates were similar irrespective of the type of coronary intervention used (balloon angioplasty, atherectomy, or stent placement). Risk assessment using the American College of Cardiology/American Heart Association clinical/morphological criteria had large inter-observer variability. Consequently, a low risk subgroup could not be reproducibly identified in which to evaluate efficacy.

Table 2
ENDPOINT EVENT RATES AT 30 DAYS - EPILOG TRIAL

	Placebo + Standard Dose Heparin (n=939)	Abciximab + Standard Dose Heparin 18)-9	Abciximab + Low Dose Heparin (n=935)
		Number of Patients (%)	
Death or MI□	85 (9.1)	38 (4.2)	35 (3.8)
p-value vs. placebo		co.00 1	co.00 1
Death, MI, or urgent intervention'	109 (11.7)	49 (5.4)	48 (5.2)
p-value vs. placebo		co.00 1	co.00 1
Components of Composite Endpoints[b]			
Death	7 (0.8)	4 (0.4)	3 (0.3)
Acute myocardial infarctions in surviving patients	78 (8.4)	34 (3.7)	32 (3.4)
Urgent interventions in surviving patients without an acute myocardial infarction	24 (2.6)	11 (1.2)	13 (1.4)

[a]Patients who experienced more than one event in the first 30 days are counted only once.
[b]Patients are counted only once under the most serious component (death > acute MI > urgent intervention).

[3] Bolus administration of 0.25 mg/kg Abciximab 10 to 60 minutes before percutaneous coronary intervention immediately followed by a 0.125 µg/kg/ᵒ min infusion (maximum 10 µg/min) for 12 hours

CAPTURE was a randomized, double-blind, multicenter, placebo-controlled trial of the use of Abciximab in unstable angina patients not responding to conventional medical therapy for whom percutaneous coronary intervention was planned, but not immediately performed (6). In contrast to the EPIC and EPILOG trials, the CAPTURE trial involved the administration of placebo or Abciximab starting 18 to 24 hours prior to percutaneous coronary intervention and continuing until one hour after completion of the intervention.

Patients were assessed as having unstable angina not responding to conventional medical therapy if they had at least one episode of myocardial ischemia despite bed rest and at least two hours of therapy with intravenous heparin and oral or intravenous nitrates. These patients were enrolled into the CAPTURE trial, if during a screening angiogram, they were determined to have a coronary lesion amenable to percutaneous coronary intervention. Patients received a bolus dose and intravenous infusion of placebo or Abciximab for 18 to 24 hours. At the end of the infusion period, the intervention was performed. The Abciximab or placebo infusion was discontinued one hour following the intervention. Patients were treated with intravenous heparin and oral or intravenous nitrates throughout the 18 to 24-hour Abciximab infusion period prior to the percutaneous coronary intervention.

The Abciximab dose was a 0.25 mg/kg bolus followed by a continuous infusion at a rate of 10 μg/min. The CAPTURE trial incorporated weight adjustment of the standard heparin dose only during the performance of the intervention, but did not investigate the effect of a lower heparin dose, and arterial sheaths were left in place for approximately 40 hours. The primary endpoint of the CAPTURE trial was the occurrence of any of the following events within 30 days of percutaneous coronary intervention: death, MI, or urgent intervention. The 30-day (Kaplan-Meier) primary endpoint event rates for each treatment group by intention-to-treat analysis of all 126.5 randomized patients are shown in Table 3.

Table 3
PRIMARY ENDPOINT EVENT RATE AT 30 DAYS - CAPTURE TRIAL

	Placebo (n=635)	Abciximab (n=630)
	Number of Patients (%)	
Death, MI, or urgent intervention[a]	101 (15.9)	71 (11.3)
p-value vs. placebo		0.012
Components of Primary Endpoint[b]		
Death	8 (1.3)	6 (1.0)
MI in surviving patients	49 (7.7)	24 (3.8)
Urgent intervention in surviving patients without acute MI	44 (6.9)	41 (6.6)

[a]Patients who experienced more than one event in the first 30 days are counted only once. Urgent interventions included any unplanned percutaneous coronary intervention after the planned intervention, as well as any stent placement for immediate patency and any unplanned CABG or use of an intra-aortic balloon pump.
[b]Patients are counted only once under the most serious component (death>acute MI>urgent intervention).

The 30-day results are consistent with EPIC results, with the greatest effects on the myocardial infarction and urgent intervention components of the composite endpoint. As secondary endpoints, the components of the composite endpoint were analyzed separately for the period prior to the percutaneous coronary intervention and the period from the beginning of the intervention through Day 30. The greatest difference in MI occurred in the post-intervention period: the rates of MI were lower in the Abciximab group compared with placebo (Abciximab 3.6%, placebo 6.1%). There was also a reduction in MI occurring prior to the percutaneous coronary intervention

(Abciximab 0.6%, placebo 2.0%). An Abciximab-associated reduction in the incidence of urgent intervention occurred in the post-intervention period. No effect on mortality was observed in either period. At six months of follow up, the composite endpoint of death, MI, or repeat intervention (urgent or non-urgent) was not different between the Abciximab and placebo groups (Abciximab 3 1.0%, placebo 30.8%, p-0.77).

Mortality was uncommon in all three trials, EPIC, EPILOG and CAPTURE. Similar mortality rates were observed in all arms within each trial. In all three trials, the rates of acute MI were significantly lower in the groups treated with Abciximab. Urgent intervention rates were also lower in Abciximab-treated groups in these trials.

Anticoagulation: Due to the incidence of bleeding seen in the EPIC trial, the dosing regimens of concomitant heparin and the target levels for anticoagulation were successively varied in the CAPTURE and EPILOG trials. These modified dosing regimens combined with other measures for patient management were associated with reduced bleeding rates (see ADVERSE REACTIONS: Bleeding)

EPILOG trial: Heparin was weight adjusted in all treatment arms. A baseline ACT was determined prior to percutaneous coronary intervention. In the low-dose heparin arm of the trial, heparin was administered as follows:

The initial heparin bolus was based upon the results of the baseline ACT, according to the following regimen:

ACT < 150 seconds: administer 70 U/kg heparin
ACT 150 - 199 seconds: administer 50 U/kg heparin
ACT ≥200 seconds: administer no heparin

Additional 20 U/kg heparin boluses were given to achieve and maintain an ACT of 200 seconds during the procedure.

Discontinuation of heparin immediately after the procedure and removal of the arterial sheath within six hours were strongly recommended in the trial. If prolonged heparin therapy or delayed sheath removal was clinically indicated, heparin was adjusted to keep the APTT at a target of 60 to 85 seconds.

CAPTURE trial: Anticoagulation was initiated prior to the administration of Abciximab. Anticoagulation was initiated with an intravenous heparin infusion to achieve a target APTT of 60 to 85 seconds. The heparin infusion was not uniformly weight adjusted in this trial. The heparin infusion was maintained during the Abciximab infusion and was adjusted to achieve an ACT of 300 seconds or an APTT of 70 seconds during the percutaneous coronary intervention. Following the intervention, heparin management was as outlined above for the EPILOG trial.

INDICATIONS AND USAGE:

Abciximab is indicated as an adjunct to percutaneous coronary intervention for the prevention of cardiac ischemic complications

- in patients undergoing percutaneous coronary intervention
- in patients with unstable angina not responding to conventional medical therapy when percutaneous coronary intervention is planned within 24 hours

Abciximab use in patients not undergoing percutaneous coronary intervention has not been studied.

Abciximab is intended for use with aspirin and heparin and has been studied only in that setting, as described in CLINICAL STUDIES.

CONTRAINDICATIONS:

Because Abciximab may increase the risk of bleeding, Abciximab is contraindicated in the following clinical situations:

- Active internal bleeding
- Recent (within six weeks) gastrointestinal (GI) or genitourinary (GU) bleeding of clinical significance.
- History of cerebrovascular accident (CVA) within two years, or CVA with a significant residual neurological deficit
- Bleeding diathesis
- Administration of oral anticoagulants within seven days unless prothrombin time is ≤ 1.2 times control
- Thrombocytopenia (< 100,000 cells/μL)
- Recent (within six weeks) major surgery or trauma
- Intracranial neoplasm, arteriovenous malformation, or aneurysm
- Severe uncontrolled hypertension
- Presumed or documented history of vasculitis
- Use of intravenous dextran before percutaneous coronary intervention, or intent to use it during an intervention

Abciximab is also contraindicated in patients with known hypersensitivity to any component of this product or to murine proteins.

WARNINGS:

Abciximab has the potential to increase the risk of bleeding, particularly in the presence of anticoagulation, e.g., from heparin, other anticoagulants, or thrombolytics (see ADVERSE REACTIONS: Bleeding).

The risk of major bleeds due to Abciximab therapy may be increased in patients receiving thrombolytics and should be weighed against the anticipated benefits.

Should serious bleeding occur that is not controllable with pressure, the infusion of Abciximab and any concomitant heparin should be stopped.

PRECAUTIONS:

Bleeding Precautions

Results of the EPILOG trial show that bleeding can be reduced by the use of low-dose, weight-adjusted heparin regimens, adherence to stricter anticoagulation guidelines, early femoral arterial sheath removal, careful patient and access site management and weight-adjustment of the Abciximab infusion dose.

Therapy with Abciximab requires careful attention to all potential bleeding sites (including catheter insertion sites, arterial and venous puncture sites, cutdown sites, needle puncture sites, and gastrointestinal, genitourinary, and retroperitoneal sites).

Arterial and venous punctures, intramuscular injections, and use of urinary catheters, nasotracheal intubation, nasogastric tubes and automatic blood pressure cuffs should be minimized. When obtaining intravenous access, non-compressible sites (e.g., subclavian or jugular veins) should be avoided. Saline or heparin locks should be considered for blood drawing. Vascular puncture sites should be documented and monitored. Gentle care should be provided when removing dressings.

Femoral artery access site:

Arterial access site care is important to prevent bleeding. Care should be taken when attempting vascular access that only the anterior wall of the femoral artery is punctured, avoiding a Seldinger (through and through) technique for obtaining sheath access. Femoral vein sheath placement should be avoided unless needed. While the vascular sheath is in place, patients should be maintained on complete bed rest with the head of the bed $\leq 30°$ and the affected limb restrained in a straight position. Patients may be medicated for back/groin pain as necessary.

Discontinuation of heparin immediately upon completion of the procedure and removal of the arterial sheath within six hours is strongly recommended if APTT $\leq$ 50 sec or ACT $\leq$ 175 sec (See PRECAUTIONS: Laboratory Tests). In all circumstances, heparin should be discontinued at least two hours prior to arterial sheath removal.

Following sheath removal, pressure should be applied to the femoral artery for at least 30 minutes using either manual compression or a mechanical device for hemostasis. A pressure dressing should be applied following hemostasis. The patient should be maintained on bed rest for six to eight hours following sheath removal or discontinuation of Abciximab, or four hours following discontinuation of heparin, whichever is later. The pressure dressing should be removed prior to ambulation. The sheath insertion site and distal pulses of affected leg(s) should be frequently checked while the femoral artery sheath is in place and for six hours after femoral artery sheath removal. Any hematoma should be measured and monitored for enlargement.

The following conditions have been associated with an increased risk of bleeding and may be additive with the effect of Abciximab **in the** angioplasty setting: percutaneous coronary intervention within 12 hours of the onset of symptoms for acute myocardial infarction, prolonged percutaneous coronary intervention (lasting more than 70 minutes) and failed percutaneous coronary intervention.

Use of Thrombolytics, Anticoagulants and Other Antiplatelet Agents

In the EPIC, EPILOG and CAPTURE trials, Abciximab was used concomitantly with heparin and aspirin. For details of the anticoagulation algorithms used in these clinical trials, see CLINICAL STUDIES: Anticoagulation. Because Abciximab inhibits platelet aggregation, caution should be employed when it is used with other drugs that affect hemostasis, including thrombolytics, oral anticoagulants, non-steroidal anti-inflammatory drugs, dipyridamole, and ticlopidine.

In the EPIC trial, there was limited experience with the administration of Abciximab with low molecular weight dextran. Low molecular weight dextran was usually given for the deployment of a coronary stent, for which oral anticoagulants were also given. In the 11 patients who received low molecular weight dextran with Abciximab, five had major bleeding events and four had minor bleeding events. None of the five placebo patients treated with low molecular weight dextran had a major or minor bleeding event (see CONTRAINDICATIONS).

There are limited data on the use of Abciximab in patients receiving thrombolytic agents. Because of concern about synergistic effects on bleeding, systemic thrombolytic therapy should be used judiciously.

Thrombocytopenia

Platelet counts should be monitored prior to treatment, two to four hours following the bolus dose of Abciximab and at 24 hours or prior to discharge, whichever is first. If a patient experiences an acute platelet decrease (e.g., a platelet decrease to less than 100,000 cells/μL and a decrease of at least 25% from pre-treatment value), additional platelet counts should be determined. These platelet counts should be drawn in three separate tubes containing ethylenediaminetetraacetic acid (EDTA), citrate and heparin, respectively, to exclude pseudothrombocytopenia due to ***in vitro*** anticoagulant interaction. If true thrombocytopenia is verified, Abciximab should be immediately discontinued and the condition appropriately monitored and treated. For patients with thrombocytopenia in the clinical trials, a daily platelet count was obtained until it returned to normal. If a patient's platelet count dropped to 60,000 cells/μL, heparin and aspirin were discontinued. If a patient's platelet count dropped below 50,000 cells/μL, platelets were transfused. Most cases of severe thrombocytopenia (<50,000 cells/μL) occurred within the first 24 hours of Abciximab administration.

. Restoration of Platelet Function

In the event of serious uncontrolled bleeding or the need for emergency surgery, Abciximab should be discontinued. If platelet function does not return to normal, it may be restored, at least in part, with platelet transfusions.

Laboratory Tests

Before infusion of Abciximab, platelet count, prothrombin time, ACT and APTT should be measured to identify pre-existing hemostatic abnormalities.

Based on an integrated analysis of data from all studies, the following guidelines may be utilized to minimize the risk for bleeding:

When Abciximab is initiated 18 to 24 hours before percutaneous coronary intervention, the APTT should be maintained between 60 and 85 seconds during the Abciximab and heparin infusion period.

During percutaneous coronary intervention the ACT should be maintained between 200 and 300 seconds.

If anticoagulation is continued in these patients following percutaneous coronary intervention, the APTT should be maintained between 60 and 85 seconds.

The APTT or ACT should be checked prior to arterial sheath removal. The sheath should not be removed unless APTT ≤ 50 seconds or ACT ≤ 175 seconds.

Readministration

Administration of Abciximab may result in human anti-chimeric antibody (HACA) formation that could potentially cause allergic or hypersensitivity reactions (including anaphylaxis), thrombocytopenia or diminished benefit upon readministration of Abciximab. In the EPIC, EPILOG, and CAPTURE trials, positive HACA responses occurred in approximately 5.8% of the Abciximab-treated patients. There was no excess of hypersensitivity or allergic reactions related to Abciximab treatment.

Readministration of Abciximab to 29 healthy volunteers who had not developed a HACA response after first administration has not led to any change in Abciximab pharmacokinetics or to any reduction in antiplatelet

potency. However, results in this small group of patients suggest that the incidence of HACA response may be increased after readministration. Readministration to patients who have developed a positive HACA response after initial administration has not been evaluated in clinical trials.

Allergic Reactions

Anaphylaxis has not been reported for Abciximab-treated patients in any of the Phase 3 clinical trials. However, anaphylaxis may occur. If it does, administration of Abciximab should be immediately stopped and standard appropriate resuscitative measures should be initiated.

Drug Interactions

Although drug interactions with Abciximab have not been studied systematically, Abciximab has been administered to patients with ischemic heart disease treated concomitantly with a broad range of medications used in the treatment of angina, myocardial infarction and hypertension. These medications have included heparin, warfarin, beta-adrenergic receptor blockers, calcium channel antagonists, angiotensin converting enzyme inhibitors, intravenous and oral nitrates, and aspirin. Heparin, other anticoagulants, thrombolytics, and antiplatelet agents may be associated with an increase in bleeding. Patients with HACA titers may have allergic or hypersensitivity reactions when treated with other diagnostic or therapeutic monoclonal antibodies.

Carcinogenesis, Mutagenesis and Impairment of Fertility

In vitro and ***in vivo*** mutagenicity studies have not demonstrated any mutagenic effect. Long-term studies in animals have not been performed to evaluate the carcinogenic potential or effects on fertility in male or female animals.

Pregnancy Category C

Animal reproduction studies have not been conducted with Abciximab. It is also not known whether Abciximab can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Abciximab should be given to a pregnant woman only if clearly needed.

Nursing Mothers

It is not known whether this drug is excreted in human milk or absorbed systemically after ingestion. Because many drugs are excreted in human milk, caution should be exercised when Abciximab is administered to a nursing woman.

Pediatric Use

Safety and effectiveness in pediatric patients have not been studied.

ADVERSE REACTIONS:

Bleeding

Abciximab has the potential to increase the risk of bleeding, particularly in the presence of anticoagulation, e.g. from heparin, other anticoagulants or thrombolytics. Bleeding in the Phase 3 trials was classified as major, minor or insignificant by the criteria of the Thrombolysis in Myocardial Infarction study group(7). Major bleeding events were defined as either an intracranial hemorrhage or a decrease in hemoglobin greater than 5 g/dL. Minor bleeding events included spontaneous gross hematuria, spontaneous hematemesis, observed blood loss with a

hemoglobin decrease of more than 3 g/dL, or a decrease in hemoglobin of at least 4 g/dL without an identified bleeding site. Insignificant bleeding events were defined as a decrease in hemoglobin of less than 3 g/dL or a decrease in hemoglobin between 3-4 g/dL without observed bleeding. In patients who received transfusions, the number of units of blood lost was estimated through an adaptation of the method of Landefeld, et al.(8).

In the EPIC trial, in which a non-weight-adjusted, standard heparin dose regimen was used, the most common complication during Abciximab therapy was bleeding during the first 36 hours. The incidences of major bleeding, minor bleeding and transfusion of blood products were significantly increased. Approximately 70% of Abciximab-treated patients with major bleeding had bleeding at the arterial access site in the groin. Abciximab-treated patients also had a higher incidence of major bleeding events from gastrointestinal, genitourinary, retroperitoneal, and other sites.

Bleeding rates were reduced in the CAPTURE trial, and further reduced in the EPILOG trial by use of modified dosing regimens and specific patient management techniques. In EPILOG, using the heparin and Abciximab dosing, sheath removal and arterial access site guidelines described under PRECAUTIONS, the incidence of major bleeding in patients treated with Abciximab and low-dose, weight-adjusted heparin was not significantly different from that in patients receiving placebo.

Subgroup analyses in the EPIC and CAPTURE trials showed that non-CABG major bleeding was more common in Abciximab patients weighing ≤ 75 kg. In the EPILOG trial which used weight-adjusted heparin dosing, the non-CABG major bleeding rates for Abciximab-treated patients did not differ substantially by weight subgroup.

Although data are limited, Abciximab treatment was not associated with excess major bleeding in patients who underwent CABG surgery. (The range among all treatment arms was 3-5% in EPIC and 1-2% in the CAPTURE and EPILOG trials.) Some patients with prolonged bleeding times received platelet transfusions to correct the bleeding time prior to surgery. (See PRECAUTIONS: Restoration of Platelet Function.)

The rates of major bleeding, minor bleeding and bleeding events requiring transfusions in the EPIC, CAPTURE and EPILOG trials are shown in Table 4. The rates of insignificant bleeding events are not included in Table 4.

Table 4
NON-CABG BLEEDING IN THE EPIC, EPILOG AND CAPTURE TRIALS
Number of Patients with Bleeds (%)

EPIC:

	Placebo (n = 696)	Abciximab (Bolus + Infusion) (n = 708)
Major□	23 (3.3)	75 (10.6)
Minor	64 (9.2)	119 (16.8)
Requiring Transfusion[b]	14 (2.0)	55 (7.8)

CAPTURE:

	Placebo (n = 635)	Abciximab (n = 630)
Major□	12 (1.9)	24 (3.8)
Minor	13 (2.0)	30 (4.8)
Requiring Transfusion[b]	9 (1.4)	15 (2.4)

EPILOG:

	Placebo (n = 939)	Abciximab + Standard-dose Heparin (n = 918)	Abciximab + Low-dose Heparin (n = 935)
Major□	10 (1.1)	17 (1.9)	10 (1.1)
Minor	32 (3.4)	70 (7.6)	37 (4.0)
Requiring Transfusion[b]	10 (1.1)	7 (0.8)	6 (0.6)

□Patients who had bleeding inmore than one classification are counted only once according to the most severe classification. Patients with multiple bleeding events of the same classification are also counted once within that classification.
[b]Packed red blood cells or whole blood

Intracranial Hemorrhage and Stroke

The total incidence of intracranial hemorrhage and non-hemorrhagic stroke across all three trials was not significantly different, 7/2225 for placebo patients and 10/3 112 for Abciximab treated patients. The incidence of intracranial hemorrhage was 3/2225 for placebo patients and 6/3 112 for Abciximab patients.

Thrombocytopenia

In the clinical trials, patients treated with Abciximab were more likely than patients treated with placebo to experience decreases in platelet counts. The rates of thrombocytopenia and transfusions were lower in the subsequent CAPTURE and EPILOG trials (Table 5).

Table 5
THROMBOCYTOPENIA AND PLATELET TRANSFUSIONS[a]

	Placebo + Standard-dose Henarin	Abciximab + Standard-dose Henarin	Abciximab + Low-dose Heparin
	Total number of patients enrolled		
EPIC	n = 696	n = 708	--
CAPTURE	n = 635	n = 630	--
EPILOG	n = 939	n = 918	n = 935
Patients with decrease of platelets to <50,000 cells/μL[a]	% of patients with events		
EPIC	0.7	1.6	--
CAPTURE	0.3	1.7	--
EPILOG	0.4	0.9	0.4
Patients with decrease of platelets to <100,000 cells/μL[a]			
EPIC	3.4	5.2	--
CAPTURE	1.3	5.6	--
EPILOG	1.5	2.6	2.5
Patients who received platelet transfusions[b]			
EPIC	2.6	5.5	--
CAPTURE	0.3	2.1	--
EPILOG	1.1	1.6	0.9

[a]Patients with a platelet count of <50,000 cells/μL are also included in the category of patients with a platelet count of <100,000 cells/μL.
[b]Includes patients receiving platelet transfusions for thrombocytopenia or any other reason.

Other Adverse Reactions

Table 6 shows adverse events other than bleeding and thrombocytopenia from the combined EPIC, EPILOG and CAPTURE trials which occurred in patients in the bolus plus infusion arm at an incidence of more than 0.5% higher than in those treated with placebo.

Table 6
ADVERSE EVENTS AMONG TREATED PATIENTS IN THE EPIC, EPILOG AND CAPTURE TRIALS

Event	Placebo (n = 2226)	Bolus + Infusion (n = 3111)
	Number of Patients (%)	
Cardiovascular System		
Hypotension	230 (10.3)	447 (14.4)
Bradycardia	79 (3.5)	140 (4.5)
Gastrointestinal System		
Nausea	255 (11.5)	423 (13.6)
Vomiting	152 (6.8)	226 (7.3)
Abdominal Pain	49 (2.2)	97 (3.1)
Miscellaneous		
Back Pain	304 (13.7)	546 (17.6)
Chest Pain	208 (9.3)	356 (11.4)
Headache	122 (5.5)	200 (6.4)
Puncture Site Pain	58 (2.6)	113 (3.6)
Peripheral Edema	25 (1.1)	49 (1.6)

The following additional adverse events from the EPIC, EPILOG and CAPTURE trials were reported by investigators for patients treated with a bolus plus infusion of Abciximab at incidences which were less than 0.5% higher than for patients in the placebo arm.

Cardiovascular System-ventricular tachycardia (1.4%), pseudoaneurysm (0.8%), palpitation (0.5%), arteriovenous fistula (0.4%), incomplete AV block (0.3%), nodal arrhythmia (0.2%), complete AV block (0.1%), embolism (limb)(0.1%); thrombophlebitis (0.1%);

Gastrointestinal System—dyspepsia (2.1%), diarrhea (1 .1%), ileus (0.1%), gastroesophogeal reflux (0.1%);

Hemic and Lymphatic System-anemia (1.3%), leukocytosis (OS%), petechiae (0.2%);

Nervous System-dizziness (2.9%), anxiety (1.7%), abnormal thinking (1.3%), agitation (0.7%), hypesthesia (0.6%), confusion (0.5%) muscle contractions (0.4%), coma (0.2%), hypertonia (0.2%), diplopia (0.1%);

Respiratory System-pneumonia (0.4%), rales (0.4%), pleural effusion (0.3%), bronchitis (0.3%) bronchospasm (0.3%), pleurisy (0.2%), pulmonary embolism (0.2%), rhonchi (0.1%);

Musculoskeletal System-myalgia (0.2%);

Urogenital System-urinary retention (0.7%), dysuria (0.4%), abnormal renal function (0.4%), frequent micturition (0.1%), cystalgia (0. 1%), urinary incontinence (0. 1%), prostatitis (0.1%);

Miscellaneous—pain (5.4%), sweating increased (1 .0%), asthenia (0.7%) incisional pain (0.6%), pruritus (0.5%) abnormal vision (0.3%), edema (0.3%), wound (0.2%), abscess (0.2%), cellulitis (0.2%), peripheral coldness (0.2%), injection site pain (O.l%), dry mouth (0.1%), pallor (0.1%), diabetes mellitus (0.1%), hyperkalemia (0.1%), enlarged abdomen (0. 1%), bullous eruption (0.1%), inflammation (0. 1%), drug toxicity (0.1%).

OVERDOSAGE:

There has been no experience of overdosage in human clinical trials.

DOSAGE AND ADMINISTRATION:

The safety and efficacy of Abciximab have only been investigated with concomitant administration of heparin and aspirin as described in CLINICAL STUDIES.

In patients with failed percutaneous coronary interventions, the continuous infusion of Abciximab should be stopped because there is no evidence for Abciximab efficacy in that setting.

In the event of serious bleeding that cannot be controlled by compression, Abciximab and heparin should be discontinued immediately.

The recommended dosage of Abciximab in adults is a 0.25 mg/kg intravenous bolus administered IO-60 minutes before the start of percutaneous coronary intervention, followed by a continuous intravenous infusion of 0.125 μg/kg/min (to a maximum of 10 μg/min) for 12 hours.

Patients with unstable angina not responding to conventional medical therapy and who are planned to undergo percutaneous coronary intervention within 24 hours may be treated with an Abciximab 0.25 mg/kg intravenous bolus followed by an 18 to 24-hour intravenous infusion of 10 μg/min, concluding one hour after the percutaneous coronary intervention.

Instructions for Administration

1. Parenteral drug products should be inspected visually for particulate matter prior to administration. Preparations of Abciximab containing visibly opaque particles should NOT be used.

2. Hypersensitivity reactions should be anticipated whenever protein solutions such as Abciximab are administered. Epinephrine, dopamine, theophylline, antihistamines and corticosteroids should be available for immediate use. If symptoms of an allergic reaction or anaphylaxis appear, the infusion should be stopped and appropriate treatment given.

3. As with all parenteral drug products, aseptic procedures should be used during the administration of Abciximab.

4. Withdraw the necessary amount of Abciximab for bolus injection into a syringe. Filter the bolus injection using a sterile, non-pyrogenic, low protein-binding 0.2 or 0.22 μm filter (Millipore SLGV025LS or equivalent).

5. Withdraw the necessary amount of Abciximab for the continuous infusion into a syringe. Inject into an appropriate container of sterile 0.9% saline or 5% dextrose and infuse at the calculated rate via a continuous infusion pump. The continuous infusion should be filtered either upon admixture using a sterile, non-pyrogenic, low protein-binding 0.2 or 0.22 μm syringe filter (Millipore SLGV025LS or equivalent) or upon administration using an in-line, sterile, non-pyrogenic, low protein-binding 0.2 or 0.22 μm filter (Abbott #4524 or equivalent). Discard the unused portion at the end of the infusion.

6. No incompatibilities have been shown with intravenous infusion fluids or commonly used cardiovascular drugs. Nevertheless, Abciximab should be administered in a separate intravenous line whenever possible and not mixed with other medications.

7. No incompatibilities have been observed with glass bottles or polyvinyl chloride bags and administration sets.

HOW SUPPLIED:

Abciximab (ReoPro®) 2 mg/mL is supplied in 5 mL vials containing 10 mg (NDC 0002-7 140-O 1).

Vials should be stored at 2 to 8°C (36 to 46°F). Do not freeze. Do not shake. Do not use beyond the expiration date. Discard any unused portion left in the vial.

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The Efficacy and Safety of Rituximab in Patients With Active Rheumatoid Arthritis Despite Methotrexate Treatment

Results of a Phase IIb Randomized, Double-Blind, Placebo-Controlled, Dose-Ranging Trial

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Objective. **To examine the efficacy and safety of different rituximab doses plus methotrexate (MTX), with or without glucocorticoids, in patients with active rheumatoid arthritis (RA) resistant to disease-modifying antirheumatic drugs (DMARDs), including biologic agents.**

Methods. **A total of 465 patients were randomized into 9 treatment groups: 3 rituximab groups (placebo [n = 149], 500 mg [n = 124], or 1,000 mg [n = 192] on days 1 and 15) each also taking either placebo glucocorticoids, intravenous methylprednisolone premedication, or intravenous methylprednisolone premedication plus oral prednisone for 2 weeks. All patients received MTX (10–25 mg/week); no other DMARDs were permitted.**

Results. **Significantly more patients who received 2 500-mg or 2 1,000-mg infusions of rituximab met the American College of Rheumatology 20% improvement criteria (achieved an ACR20 response) at week 24 (55% and 54%, respectively) compared with placebo (28%; $P < 0.0001$). ACR50 responses were achieved by 33%, 34%, and 13% of patients, respectively ($P < 0.001$), and ACR70 responses were achieved by 13%, 20%, and 5% of patients ($P < 0.05$). Changes in the Disease Activity Score in 28 joints (−1.79, −2.05, −0.67; $P < 0.0001$) and moderate to good responses on the European League Against Rheumatism criteria ($P < 0.0001$) reflected the ACR criteria responses. Glucocorticoids did not contribute significantly to the primary efficacy end point, ACR20 response at 24 weeks. Intravenous glucocorticoid premedication reduced the frequency and intensity of first infusion–associated events; oral glucocorticoids conferred no additional safety benefit. Rituximab was well tolerated; the type and severity of infections was similar to those for placebo.**

Conclusion. **Both rituximab doses were effective and well tolerated when added to MTX therapy in patients with active RA. The primary end point (ACR20 response) was independent of glucocorticoids, although intravenous glucocorticoid premedication improved tolerability during the first rituximab infusion.**

Although cytokine-specific biologic agents have improved the treatment of rheumatoid arthritis (RA), some patients have an inadequate response to such therapies and, in clinical practice, most have only a

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partial response (1). The frequency of remission has remained <50% for patients with established RA, many of whom display "moderate" disease activity, according to their Disease Activity Score (DAS) (2). In order to achieve additional significant gains in RA therapy, new targets need to be identified. One such target is the B cell (3).

Rituximab is a monoclonal antibody that selectively depletes CD20+ B cells via 3 putative mechanisms: antibody-dependent cell-mediated cytotoxicity, complement-dependent cytotoxicity, and promotion of CD20+ B cell apoptosis (4–6). CD20 is not expressed on stem cells or plasma cells (7,8); this is consistent with the observation that B cell recovery and Ig production were not compromised after a single course of rituximab (9).

Rituximab has shown efficacy in the treatment of CD20+ B cell non-Hodgkin's lymphoma (NHL) (10). More recently, a role for rituximab and B cell modulation has been investigated in rheumatoid factor (RF)–positive patients with active RA. In a phase IIa study, 2 1,000-mg infusions of rituximab (combined with methotrexate [MTX] and a 2-week treatment with glucocorticoids) significantly improved RA symptoms for up to 1 year compared with MTX alone (9). Moderate to good responses according to the European League Against Rheumatism (EULAR) criteria were also evident at 2 years in 33% of the rituximab plus MTX group (11).

To date, no studies have investigated the influence of the rituximab dosage or the role of glucocorticoids in the clinical efficacy and/or tolerability of rituximab regimens in RA. The objective of this study was therefore to examine these variables in patients with active RA who had an inadequate response to disease-modifying antirheumatic drugs (DMARDs) other than MTX or biologic agents, and who currently had an inadequate clinical response to MTX.

PATIENTS AND METHODS

Role of the funding source. This study was funded by Roche, and Roche scientists contributed to the study design, collection of data, and data analysis. The manuscript was written by Dr. Emery. The agreement to submit the manuscript and the approval of the content were made jointly by the authors.

Study design. This phase IIb, randomized, double-blind, double-dummy, placebo-controlled, international multifactorial trial of 9 different treatment regimens examined 2 dosage levels (and rituximab placebo) of both rituximab and glucocorticoids in a 3 × 3 configuration (Figure 1) with concomitant MTX in patients with active RA. Patients were evaluated at 4-week intervals between baseline and week 24.

Patients. Outpatients were eligible for study if they were between 18 and 80 years of age and had presented at least 6 months prior to randomization with moderate or severe RA

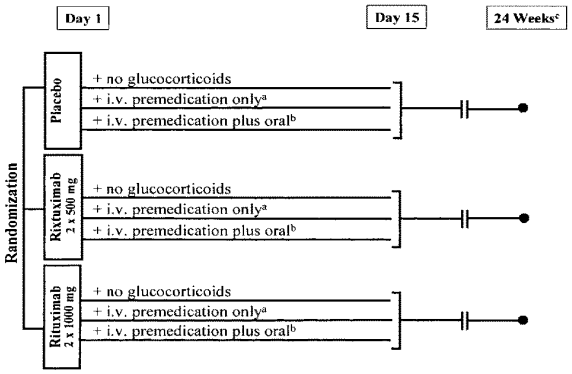


Figure 1. Study design. All groups received weekly methotrexate 10–25 mg parenterally or orally. a = 100 mg methylprednisolone administered intravenously (IV) prior to rituximab infusions on days 1 and 15. b = 100 mg methylprednisolone administered IV prior to rituximab infusions on days 1 and 15, and prednisone administered orally at 60 mg on days 2–7 and at 30 mg on days 8–14. c = primary end point: percentage of patients who met the American College of Rheumatology 20% improvement criteria at week 24.

(diagnosed according to the American College of Rheumatology [ACR; formerly, the American Rheumatism Association] revised criteria [22]) despite ongoing treatment with MTX at a dosage of 10–25 mg/week (orally or parenterally) for at least 12 weeks before randomization, with a stable dosage during the last 4 weeks. Active disease was defined as a swollen and tender joint count ≥8 and either an erythrocyte sedimentation rate ≥28 mm/hour or a C-reactive protein (CRP) serum level ≥1.5 mg/dl.

Patients must have failed prior treatment, manifesting as a lack or loss of response to treatment with at least 1 but not more than 5 DMARDs (other than MTX) and/or biologic response modifiers. Patients discontinued DMARDs (except MTX) and biologic therapy at least 4 weeks before randomization and discontinued infliximab, adalimumab, or leflunomide at least 8 weeks before randomization. Patients who were already taking oral glucocorticoids (≤10 mg/day of prednisone or equivalent) were included if the dosage had been stable for at least 4 weeks before trial entry, and patients who had received intraarticular or parenteral glucocorticoids were included, provided that the most recent administration was >4 weeks before screening. Concomitant treatment with nonsteroidal antiinflammatory drugs was permitted if the dosage had been stable for at least 2 weeks prior to entry. Concomitant treatment with any DMARD (other than MTX), anti–tumor necrosis factor α, or other biologic therapy was prohibited.

Patients were excluded if they had significant systemic involvement secondary to RA, evidence of significant other illnesses or laboratory abnormalities, a history of severe allergic or anaphylactic reactions to humanized or murine monoclonal antibodies, or previous treatment with rituximab or any lymphocyte-depleting therapies. In addition, patients were excluded if they had a history of recurrent significant infection.

The study was performed in accordance with the Declaration of Helsinki. All participating sites received ap-

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Table 1. Randomization of trial medication and disposition of patients at 24 weeks*

	Treatment†		
	Rituximab placebo, RF+/RF−	Rituximab 2 500-mg doses, RF+/RF−	Rituximab 2 1,000-mg doses, RF+/RF−
Glucocorticoid placebo	42/21	41/0	43/22
IV glucocorticoid premedication‡	42/0	41/0	42/20
IV glucocorticoid premedication + oral glucocorticoid§	44/0	42/0	43/22
Total no. of patients randomized¶	149	124	192
Patients withdrawn, no. (%)	52 (35)	11 (9)	27 (14)
Insufficient response	46 (31)	8 (6)	16 (8)
Adverse events	0	3 (2)	6 (3)
All other reasons#	6 (4)	0	5 (3)
Completed 24 weeks, no. (%)	97 (65)	113 (91)	165 (86)

* RF = rheumatoid factor; IV = intravenous.
† Rituximab or rituximab placebo was administered as 2 infusions: 1 on day 1 and 1 on day 15. All patients received 10–25 mg of methotrexate orally or parenterally on a weekly basis.
‡ Methylprednisolone 100 mg IV on days 1 and 15.
§ Methylprednisolone 100 mg IV on days 1 and 15 plus oral prednisone 60 mg on days 2–7 and 30 mg on days 8–14 (total oral prednisone dose 570 mg).
¶ Patients received at least 1 dose of rituximab.
In the rituximab placebo and rituximab groups receiving 2 1,000-mg infusions, 4 patients (3%) and 4 patients (2%), respectively, refused treatment.

proval from their governing institutional review board (or equivalent). See Appendix A for additional members of the Dose-Ranging Assessment: International Clinical Evaluation of Rituximab in Rheumatoid Arthritis (DANCER) Study Group and their locations. All patients provided written informed consent. The primary study population for efficacy analysis was composed of RF-positive patients. A limited number of RF-negative patients were also included for an initial exploratory analysis of the safety of rituximab in this group.

Study treatment. Rituximab was administered by intravenous (IV) infusion in RF-positive patients: placebo, 500 mg or 1,000 mg on days 1 and 15 (total dose 0 mg, 1,000 mg, and 2,000 mg). Glucocorticoids were administered as placebo methylprednisolone, given IV 30–60 minutes before the infusion of rituximab (or rituximab placebo) on days 1 and 15, premedication methylprednisolone 100 mg, given IV on days 1 and 15 (250 mg prednisone equivalent), or premedication methylprednisolone 100 mg, given IV on days 1 and 15 plus 60 mg of oral prednisone on days 2–7 and 30 mg on days 8–14 (total glucocorticoid dose 820 mg prednisone equivalent) (Table 1). RF-negative patients received either placebo or rituximab (2 1,000-mg infusions), with or without glucocorticoids. All patients received a weekly regimen of MTX (10–25 mg orally or parenterally) with folate (≥5 mg/week).

Trial end points. The primary end point of the trial was the proportion of RF-positive patients who met the ACR 20% improvement criteria (achieved an ACR20 response) (defined as a 20% improvement in the tender and swollen joint counts and a 20% improvement in 3 of the 5 other ACR improvement criteria) (13) at week 24. Secondary and exploratory analyses examined group differences in ACR50 and ACR70 responses, DAS 28-joint assessment for swelling and tenderness (DAS28) (14), EULAR responses (15), fatigue, as measured by the Functional Assessment of Chronic Illness Therapy–Fatigue (FACIT-F) subscale (16), and the effect on individual parameters of the ACR improvement criteria. The level of physical functioning and disability was assessed using the disability index (DI) of the Health Assessment Questionnaire (HAQ) (17).

Safety outcomes were expressed as the incidence of adverse events, graded according to National Cancer Institute (NCI) Common Toxicity Criteria (CTC), version 2.0 (18). CD19+ counts, measured by fluorescence-activated cell sorting, were used as a marker of the onset and duration of peripheral B cell depletion (7). Effects on Ig levels, protective antibody titers, and human antichimeric antibody (HACA) levels were also evaluated.

Statistical analysis. The trial was planned to recruit 440 patients: 40 RF-positive patients per group (a total of 360 patients), who constituted the primary efficacy population, and 20 RF-negative patients in 4 of the 9 groups (a total of 80 patients), for exploratory analysis. Using a 2-sided 5% significance level, it was calculated that a sample size of 360 RF-positive patients would provide at least 80% power to detect differences between the proportions of patients who achieved an ACR20 response in the rituximab 2 1,000-mg and 2 500-mg infusion groups, as well as dose-response trends across the 3 pooled rituximab groups. Randomization was stratified by region (US versus non-US) and by RF status. Steroids were included in the main effects model of ACR20 response at 24 weeks to determine whether a significant effect of the steroid regimen had been achieved. The study was powered to test for an association between ACR20 response at week 24 and rituximab treatment while controlling for corticosteroid usage and region (US or non-US) for RF-positive patients, using a logistic regression model (main effects model). It was not powered to determine any individual effects of these controlling factors.

Primary analyses were based on intent-to-treat (ITT), with nonresponders imputed for all categorical end points (e.g., ACR and EULAR responses), and the last observation was carried forward for continuous variables (e.g., ACR improvement criteria and DAS28). All patients who dropped out of the study were considered nonresponders for purposes of categorical end points. A main-effect logistic regression model

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Table 2. Patient demographics and baseline disease characteristics*

	Rituximab placebo	Rituximab 2 500-mg infusions	Rituximab 2 1,000-mg infusions
Safety population, no.	149	124	192
Demographics			
Female, %	80	83	80
Age, mean years	51.1	51.4	51.1
Duration of RA, mean years	9.3	11.1	10.8
Previous DMARDs (other than MTX), mean no.	2.2	2.5	2.5
Prior anti-TNFα treatment, %	26	33	28
MTX dose, mean mg/week	15.6	16.0	14.9
US patients, %	33	33	34
Primary ITT population, no.	122	123	122
Disease characteristics			
Swollen joint count, mean no.	21	22	22
Tender joint count, mean no.	35	33	32
Baseline HAQ DI score, mean	1.7	1.8	1.7
RF, mean IU/liter	437	421	437
ESR, mean mm/hour	40	45	41
Serum CRP, mean mg/dl	3.3	3.2	3.0
DAS28, mean	6.8	6.8	6.7

* RA = rheumatoid arthritis; DMARDs = disease-modifying antirheumatic drugs; MTX = methotrexate; TNFα = tumor necrosis factor α; ITT = intent to treat; HAQ = Health Assessment Questionnaire; DI = Disability Index; RF = rheumatoid factor; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DAS28 = Disease Activity Score in 28 joints.

was used to evaluate ACR20 response at week 24 in the primary efficacy population across all study groups, with terms for rituximab, glucocorticoid usage, and region (US or non-US). This model also studied any effect of adding RF-negative patients to the analysis population. Contrast tests were conducted for rituximab 2 1,000-mg infusions versus placebo, and rituximab 2 500-mg infusions versus rituximab 2 1,000-mg infusions, in addition to a dose-response relationship in the ACR20 response across the upper- and lower-dose rituximab and placebo groups. No comparisons between the rituximab 2 500-mg infusions and placebo were originally planned.

RESULTS

Patient demographics and characteristics. A total of 465 patients were treated, 380 RF-positive patients and 85 RF-negative patients; this constituted the safety population (Table 1). Patients in each of the rituximab groups were generally well matched in terms of demographic parameters and region, baseline disease characteristics, and previous DMARD/biologic treatments (Table 2). Antimalarial agents had been taken by 38–68% of patients across treatment groups, sulfasalazine by 38–68% and immunosuppressive agents (cyclosporine, azathioprine, and/or leflunomide) by 24–44%. Overall, 29% of patients had previously taken biologic agents (infliximab, adalimumab, etanercept, or anakinra), with no significant differences between the randomized groups.

Of the 465 patients, 65% in the placebo group, 91% in the rituximab 2 500-mg infusions group, and 86% in the rituximab 2 1,000-mg infusions group completed the 24-week primary analysis period (Table 1). Fewer patients discontinued rituximab than placebo as a result of insufficient therapeutic response, but rituximab was associated with more withdrawals because of adverse events. Nine patients (all in rituximab groups) withdrew for safety reasons (mainly as a result of infusion-related events). One patient in the 2 500-mg rituximab infusions plus IV glucocorticoid group died due to a cerebrovascular accident.

The primary ITT efficacy population was composed of 367 RF-positive patients; the remaining RF-positive patients were excluded from this analysis for reasons related to accidental unblinding, drug dispensing errors, and unverifiable data.

Efficacy outcomes. The proportion of patients in the primary ITT efficacy population who achieved an ACR20 response at 24 weeks, the primary end point of the trial, was significantly greater in each of the rituximab groups than in the placebo group ($P < 0.0001$) (Figure 2A). At week 24, 55% of the patients in the rituximab 2 500-mg infusions group and 54% of those in the 2 1,000-mg infusions group had achieved an ACR20 response, compared with 28% in the placebo group. There was no statistically significant difference in the odds of achieving an ACR20 response between the groups taking 2 1,000-mg infusions of rituximab versus 2 500-mg infusions of rituximab (odds ratio 0.93, $P = 0.768$). Analyses of the proportions of patients who achieved an ACR50 or ACR70 response showed a similar pattern. Compared with placebo, a significantly greater proportion of patients treated with either dosage

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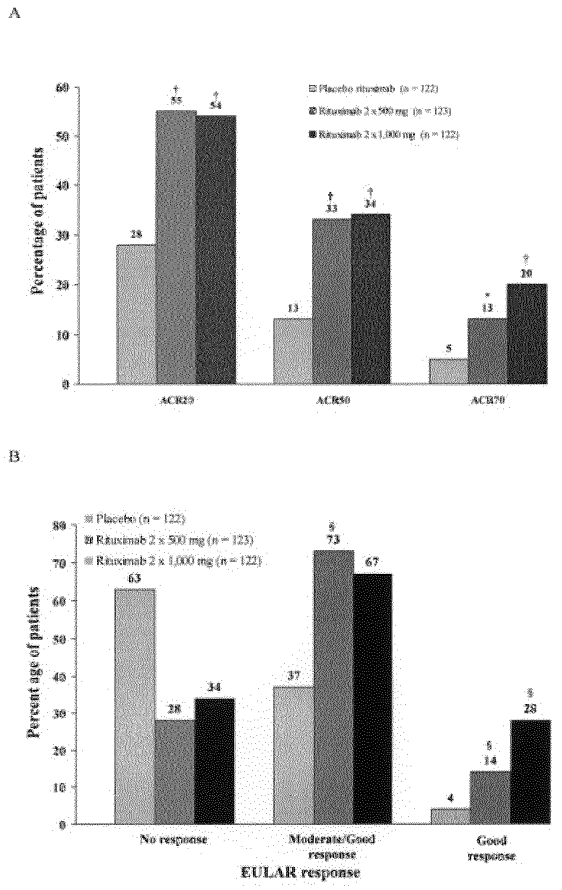


Figure 2. Patients who experienced responses according to **A,** the American College of Rheumatology 20% (ACR20), 50% (ACR50), and 70% (ACR70) improvement criteria and **B,** the European League Against Rheumatism (EULAR) criteria at 24 weeks. Results are expressed using pooled data for glucocorticoid dose. Patients who withdrew or who had insufficient data from which to calculate an ACR score were classified as nonresponders. * = $P = 0.029$; † = $P \leq 0.001$; § = $P < 0.0001$, versus placebo.

of rituximab achieved an ACR50 response ($P \leq 0.001$ versus placebo in each rituximab dosage group) and an ACR70 response ($P = 0.029$ for rituximab 2 500-mg infusions group and $P \leq 0.001$ for rituximab 2 1,000-mg infusions group). Although ACR20 and ACR50 responses were similar for both rituximab dosages, ACR70 responses were numerically more frequent in the rituximab 2 1,000-mg infusions group than in the 2 500-mg infusions group (Figure 2A).

Primary analysis within the main-effect model showed that glucocorticoid treatment had no significant effect on ACR20 response ($P = 0.166$). Dose-contrast tests between individual glucocorticoid treatments showed that the probability of achieving an ACR20 response in any of the main treatment groups was not significantly different for glucocorticoid placebo versus IV glucocorticoids ($P = 0.62$) or IV and oral glucocorticoids ($P = 0.18$). Patients who received glucocorticoids were, however, more likely to achieve an ACR20 response by week 4 than those who received no glucocorticoids. Consequently, since glucocorticoid treatment was not a prerequisite for the efficacy of rituximab, efficacy results are expressed using pooled glucocorticoid dose cohorts within each rituximab group.

The time course for achieving an ACR response showed early separation of rituximab from placebo. The proportion of patients who achieved an ACR20 response in the placebo group leveled off at ~30% at week 4, while in the rituximab groups, it continued to increase, leveling off at ~55% at 12 weeks. The same was essentially true for ACR50 and ACR70 responses, except in the rituximab 2 1,000-mg infusions group, in which ACR70 was seen in 10% of patients at week 12 and increased to 20% at week 24, suggesting a time-related effect on achievement of higher-level responses at this dose.

Changes in the DAS28 at week 24 reflected ACR response findings. Analysis of variance showed the adjusted mean change in the DAS28 from baseline to be significantly greater in patients treated with rituximab 2 500-mg and 2 1,000-mg infusions (−1.79 and −2.05, respectively) than in patients treated with placebo (−0.67; $P < 0.0001$). Similarly, EULAR responses in patients treated with rituximab at either dosage were significantly greater than those seen in patients who received placebo (Figure 2B). Compared with placebo, moderate or good EULAR responses were achieved in a significantly greater proportion of patients treated with rituximab 2 500-mg infusions ($P < 0.0001$) and rituximab 2 1,000-mg infusions ($P < 0.0001$), with a trend toward a higher proportion of patients achieving a good EULAR response with 2 1,000-mg infusions of rituximab.

CRP levels fell by a mean of 1.7 mg/dl between baseline and week 24 in patients treated with the active rituximab regimens. The corresponding mean change in CRP in the rituximab placebo group was 0.1 mg/dl. These differences in favor of the active rituximab groups were observed after week 4 and continued to week 24. In patients who were RF-positive at baseline, the proportion with RF titers below the level of detection by week 24 was 1.6% in the placebo group and 12% and 9% in the rituximab 2 500-mg infusions and 2 1,000-mg infu-

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Table 3. Most frequently reported adverse events in the safety population (n = 465)*

Adverse events	Rituximab placebo (n = 149)	Rituximab 2 500-mg infusions (n = 124)	Rituximab 2 1,000-mg infusions (n = 192)
All events	105 (70)	100 (81)	164 (85)
Exacerbation of RA	44 (30)	21 (17)	27 (14)
Headache	19 (13)	14 (11)	21 (11)
Nausea	13 (9)	8 (6)	19 (10)
URI	9 (6)	10 (8)	12 (6)
Nasopharyngitis	8 (5)	7 (6)	10 (5)
Arthralgia	5 (3)	5 (4)	11 (6)
Diarrhea	8 (5)	7 (6)	6 (3)
Fatigue	8 (5)	5 (4)	8 (4)
Hypertension	4 (3)	5 (4)	12 (6)
Rigors	3 (2)	5 (4)	13 (7)
Dizziness	6 (4)	4 (3)	10 (5)
Serious noninfection adverse events	2 (1)†	9 (7)‡	9 (5)§
Serious infections	2 (1)	0	4 (2)

* All adverse events affecting ≥5% of patients in any one group are shown. Values are the number (%). RA = rheumatoid arthritis; URI = upper respiratory tract infection.
† Food allergy and exacerbation of RA in 1 patient each.
‡ Cerebral infarction, fatal cerebrovascular accident unrelated to study treatment, convulsion, myocardial infarction, supraventricular tachycardia, drug hypersensitivity (occurring within 24 hours of rituximab infusion), failure of implant, lower limb fracture, and interstitial lung disease in 1 patient each.
§ Epilepsy, serotonin syndrome, chest pain, generalized edema (occurring within 24 hours of rituximab infusion), exacerbation of RA, status asthmaticus, intestinal obstruction, metrorrhagia, and thromboangiitis obliterans in 1 patient each.

sions groups, respectively. This reflected a mean increase in total RF titers in the placebo groups (ranging from 7.1% to 37.4% among placebo groups) compared with a mean decrease in the active rituximab groups (ranging from 11.5% to 47.9% among rituximab groups).

Patient-reported outcomes mirrored the improvements in clinical and laboratory findings in the primary ITT population. Changes in the mean DI scores of the HAQ between baseline and week 24 were −0.43 and −0.49 in the rituximab 2 500-mg and 2 1,000-mg infusions groups, respectively, compared with −0.16 in the rituximab placebo group. The proportions of patients with an improvement of −0.22 from baseline (minimum clinically important difference [MCID] [19]) were 63%, 67%, and 34%, respectively in these 3 groups. Furthermore, changes in fatigue, measured as the percentage improvement in the FACIT-F score between baseline and week 24, were 20% and 28% in the rituximab 2 500-mg and 2 1,000-mg infusions groups, respectively, compared with 4% in the placebo group. In both rituximab groups, the mean change from baseline in the FACIT-F score exceeded 4 points, which is the MCID (16). This was not the case in the placebo group.

The inclusion of data from RF-negative patients in the analyses did not alter the primary outcome. In the combined RF-positive and RF-negative population, 52% of the rituximab 2 1,000-mg doses group achieved an ACR20 response at week 24 compared with 32% of the placebo group. An exploratory analysis of RF-negative patients alone proved inconclusive, since it was confounded by an unusually high placebo response (52% versus 48% in the rituximab group) and a relatively small sample size (21 and 63 patients, respectively).

There was a significant difference at week 24 between the absolute proportions of US and non-US patients who achieved an ACR20 response across treatment groups, including placebo ($P = 0.002$), with an ACR20 response being more frequent in the non-US region. Between regions, the efficacy of rituximab relative to placebo (i.e., the difference between placebo and active drug responses) was similar, and consequently, there was no significant interaction between rituximab and region ($P = 0.797$). An exploratory analysis of the 2 regions showed that the only major baseline difference between the US and non-US populations was the proportion of patients previously exposed to biologic therapy (55% of US versus 23% of non-US patients). While there was no identified interaction between prior use of biologic therapies and rituximab treatment, ACR20 response rates were generally lower in patients with prior exposure to biologic therapies.

Safety outcomes. Overall, 70% of patients treated with placebo reported at least 1 adverse event at any time during the study period, compared with 81% and 85% of patients in the rituximab 2 500-mg and 2

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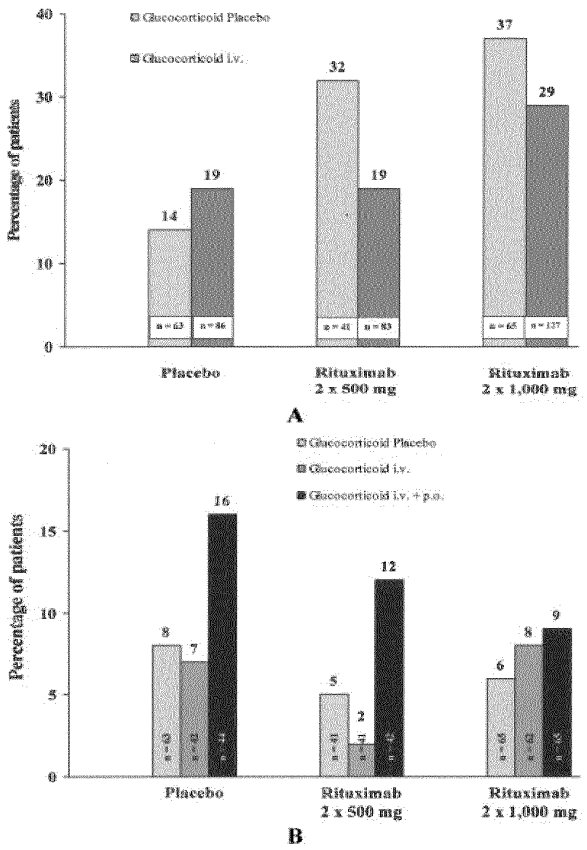


Figure 3. Effect of glucocorticoids on the frequency of acute infusion reactions after **A,** the first infusion and **B,** the second infusion. IV = intravenous; PO = by mouth.

1,000-mg infusions groups, respectively. The majority (82%) of events in each group were mild to moderate in severity (NCI CTC grades 1–2). A similar proportion of patients reported severe (NCI CTC grade 3) events in each group (18% of patients receiving placebo and 17% and 18% of patients in the rituximab 2 500-mg and 2 1,000-mg infusions groups, respectively). The most frequently reported adverse events in each treatment group are shown in Table 3 and were mainly associated with the first infusion. Exacerbation of RA, reported as an adverse event, distinguished rituximab placebo from both doses of active rituximab, reflecting the efficacy of rituximab relative to placebo. There were no unexpected adverse events in the RF-negative subgroup.

Infusion-associated events. Infusion-associated events (defined as any adverse event that occurred during or within 24 hours of rituximab infusion) occurred with the first infusion in 18% of patients who received placebo and in 31% and 38% of patients who received rituximab 2 500-mg and 2 1,000-mg infusions, respectively.

Acute infusion reactions. Symptoms or signs suggesting an acute infusion reaction (pruritus, fever, urticaria/rash, chills, pyrexia, rigors, sneezing, angioneurotic edema, throat irritation, cough and bronchospasm, hypotension, or hypertension) were more commonly associated with the first infusion of rituximab compared with placebo. Overall, acute infusion reactions were reported at the first infusion in 17% of placebo-treated patients compared with 23% and 32% of patients who received 2 500-mg or 2 1,000-mg rituximab, respectively. However, premedication with IV glucocorticoid significantly reduced both the incidence and severity of acute infusion reactions. Without glucocorticoid premedication, these events were reported by 14% of the placebo group, 32% of the rituximab 2 500-mg infusions group, and 37% of the rituximab 2 1,000-mg infusions group, as compared with 19%, 19%, and 29% of these groups, respectively, following IV glucocorticoid premedication (Figure 3A).

The number of patients who experienced these symptoms following the second infusion of the treatment course was substantially lower in all groups, irrespective of glucocorticoid premedication (Figure 3B). The addition of 2 weeks of oral prednisone treatment did not appear to provide any further reduction of acute infusion reactions with the second infusion.

Two adverse events occurred within the first 24 hours of the first infusion: 1 case of drug hypersensitivity reaction occurred in the rituximab 2 500-mg infusions group, and 1 case of generalized edema occurred in the rituximab 2 1,000-mg infusions group. Both events occurred in patients who had not received IV glucocorticoid premedication. Five patients (2.6%) in the 2 1,000-mg infusions rituximab group were withdrawn from treatment because of acute infusion-associated events following the first infusion. Four of these patients had not received glucocorticoid premedication.

Infection-related adverse events. Adverse events classed as infections and infestations were reported in 28% of the placebo group and in 35% of each of the active rituximab groups. The type and severity of infections were similar across the placebo and active rituximab groups (the most common being respiratory tract infections, urinary tract infections, and nasopharyngitis),

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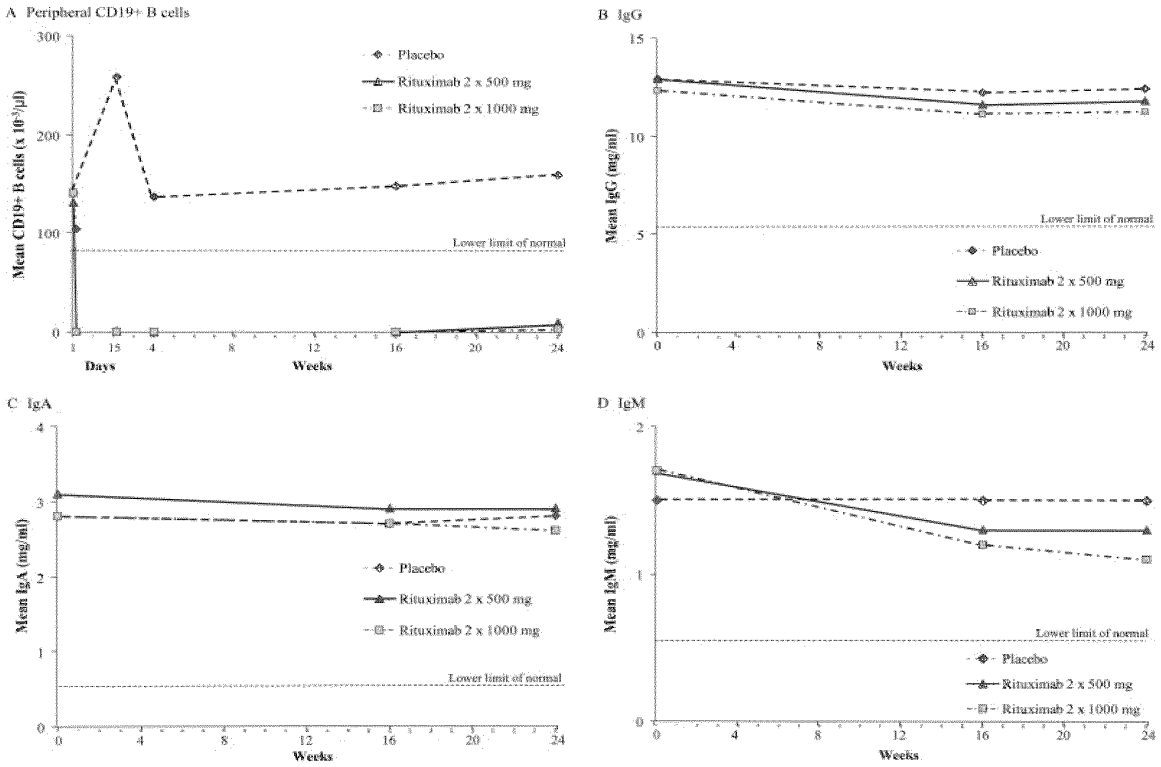


Figure 4. Levels of **A,** peripheral blood CD19+ B cells, **B,** IgG, **C,** IgA, and **D,** IgM during the 24-week treatment period.

so the pattern of infections did not distinguish between placebo- and rituximab-treated patients.

Six serious infections were reported. Two (1%)occurred in the placebo group (pneumonia and respiratory tract infection) and 4 (2%) in the rituximab 2 1,000-mg infusions group (2 cases of pyelonephritis, and 1 each of bronchitis and epiglottitis). These events resolved without sequelae. No serious infections occurred in the rituximab 2 500-mg infusions group. The rate of serious infections per 100 patient-years was 3.19 for the rituximab placebo group, 0 for the 2 500-mg infusions of rituximab group, and 4.74 for the 2 1,000-mg infusions of rituximab group.

Serious adverse events. A total of 26 serious adverse events were reported, 4 (3%) in the placebo group (3%), 9 in the rituximab 2 500-mg infusions group (7%), and 13 in the rituximab 2 1,000-mg infusions group (7%), with no consistent pattern or signal. Of these, 20 were noninfection events in 2, 9, and 9 patients in the placebo, rituximab 2 500-mg, and rituximab 2 1,000-mg infusions groups (1%, 7%, and 5%, respectively) (Table 3). Five events were nervous system disorders, while others were distributed evenly across body systems in no discernible pattern. There was 1 fatal event, which occurred in the rituximab 2 500-mg infusions group. A 73-year-old woman died of a cerebral infarction 167 days after the first infusion of rituximab. This patient had a history of atrial fibrillation, hypertension, diabetes mellitus, and hyperlipidemia; at the time of enrollment into the trial, she was receiving concomitant celecoxib, MTX, folic acid, lisinopril, atorvastatin, and sertraline.

Pharmacodynamics. In the placebo group, peripheral B cell levels remained stable around baseline levels throughout the study period, after an initial transient increase during the 2-week glucocorticoid dosing period between rituximab placebo infusions. Treatment with rituximab was associated with nearly complete

depletion of peripheral blood B cells, which persisted throughout the 24-week study period. Peripheral blood B cell recovery began after week 16 (Figure 4A).

Although peripheral blood B cell levels were depleted in rituximab-treated patients, mean and median Ig levels (IgG, IgA, and IgM) remained above the laboratory lower limit of normal at all times; the greatest change from baseline occurred in IgM (Figures 4B–D). Anti-tetanus titers remained stable.

Antibodies to the test agent, i.e., HACA (analyzed up to and including week 24), were detectable in 0.7%, 4.2%, and 2.7% of the placebo, rituximab 2 500-mg, and rituximab 2 1,000-mg infusions groups, respectively. There were no serious adverse events in the group of patients with HACAs. Moreover, 6 of the 10 rituximab-treated patients with positive HACAs at week 24 achieved ACR20 responses at that time.

DISCUSSION

Results of the DANCER trial have confirmed that a single course of rituximab, given as 2 infusions 2 weeks apart, is highly effective over 24 weeks in the treatment of active RA in patients who have shown an incomplete response to MTX. Followup of these patients beyond the primary end point is ongoing, and radiologic outcomes (not measured in this study) will be addressed in future studies. Rituximab has a novel mode of action that results in the depletion of B cells, and it is therefore distinct from other biologic therapies for RA that target T cells and their related cytokines. The present study corroborates earlier studies that support a role for B cells in the inflammatory processes that underlie RA (9,10,20–24).

Both rituximab dosages were effective, with 54–55% of patients achieving an ACR20 response by week 24, and a high proportion achieving ACR50 or ACR70 responses. Importantly, clinical improvements in rituximab-treated patients were reflected in patient- and physician-reported outcome measures, as well as in changes in laboratory parameters. Moreover, the beneficial effects of rituximab were often evident early in the course of treatment.

In general, the efficacy results from the DANCER study broadly mirror those from a phase IIa study of rituximab in 161 RF-positive RA patients (9), although in the latter, a higher ACR20 response rate was observed. A possible explanation for this discrepancy is the prior use of biologic therapies at baseline (an indication of refractory disease). In the phase IIa study, only ~10% of patients had been exposed to a biologic agent, whereas in the DANCER trial, the proportion was 30–40% in the active rituximab groups. A subanalysis of DANCER trial patients who received active rituximab with no prior exposure to biologic therapies determined an ACR20 response rate of 62% for this group, which is closer to previously reported rates (9). Previous use of biologic agents may also be a contributory factor to the lower absolute ACR response observed in US patients compared with non-US patients in this study. A greater proportion of US patients had received such treatment and may therefore have constituted a more refractory population. Nevertheless, rituximab was similarly effective relative to placebo both in patients who had received prior biologics and those naive to these therapies.

No dose-response relationship was established, based on the ACR20 criteria, for the 2 rituximab doses studied. There were, however, trends to indicate that the dose may influence the achievement of high-level response (i.e., ACR70 response and good EULAR response), and further evaluation of both rituximab doses is warranted.

The use of concomitant glucocorticoids with rituximab was based on the successful application of similar regimens in patients with NHL (10) and the initial trials in patients with RA (9,20,21). This trial has, however, established that glucocorticoids play no significant role in determining the primary end point of an ACR20 response at 24 weeks in RA patients treated with rituximab, although they may enhance the early response (up to 4 weeks) by virtue of their antiinflammatory properties. Consequently, the use of glucocorticoids does not appear to be a prerequisite for a clinical response to rituximab.

Both dosages of rituximab were generally well tolerated, with a low incidence of serious adverse events, including serious infections. The majority of adverse events were infusion associated, mild to moderate in intensity, and easily managed. Premedication with IV glucocorticoids did, however, reduce the incidence and severity of infusion-associated events following the first infusion of rituximab. The incidence of reactions following the second infusion was low in all groups, with 2 weeks of oral glucocorticoid treatment providing no additional safety benefit over IV glucocorticoid premedication alone. Overall, the incidence of infusion-related adverse events in RA patients appears to be lower than that observed in NHL patients (who may be prone to such events because of elevated levels of white blood cells or tumor lysis) (10,25).

All patients had depleted B cell levels following rituximab treatment, with peripheral blood B cell levels falling to below the lower limit of normal. Mean and

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median Ig levels stayed within the normal range, but there was a downward trend specifically with IgM; however, there was no significant change in acquired immunity. Overall, the incidence of immunogenicity (HACAs) was low and had no apparent clinical relevance over the primary 24-week course of this trial, but patients continue to be monitored for this, together with all other pharmacodynamic parameters, including CD19 and Ig levels.

The DANCER study is a novel design for the study of RA: 9 treatment groups with a logistic regression main-effects model as its primary means of analysis. The complexity of the study was driven by a need to understand the effects of different components of a potential therapeutic regimen (i.e., rituximab dose and effects of glucocorticoids). Both objectives were met, but several factors must be taken into account when interpreting the results. First, extrapolation of these data to the larger RA population must be done with care, given the relatively small number of patients involved. In particular, specific comparison between any of the individual 9 groups is inappropriate; however, valuable guidance concerning both the use of glucocorticoids and the dosage of rituximab has been obtained. Second, it should be acknowledged that the present data represent just 24 weeks of followup; longer-term followup will be required to better characterize safety and, possibly, to identify potential differences between rituximab dosages. Last, with regard to the subset of RF-negative patients, small numbers of patients and a large placebo effect prevent the drawing of firm conclusions about the safety and efficacy of rituximab in this population.

In conclusion, both dosages of rituximab were highly effective and generally well tolerated when given with continuing MTX to patients with active RA. The study has also shown that concomitant glucocorticoids are not a prerequisite for the efficacy of rituximab in RA. However, premedication with IV glucocorticoids does appear to ameliorate acute infusion reactions. Both dosages of rituximab explored in this study warrant further differential exploration and longer-term followup.

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BY ONLINE FILING

The European Patent Office
Bayerstrasse 34
(entrance via Zollstrasse 3)
80335 Munich
Germany

22 March 2018

Dear Sirs

Opposition to European Patent No. 2359834
(Application No. 11001632.6)
ALEXION PHARMACEUTICALS, INC.
Our Ref: C411599EP PXT/ALS

In response to the Communication dated 13 September 2017, I attach the Patentee's response to the two Oppositions. Attached are:

- the Response;
- documents D33 to D38 as listed in section 2 of the response; and
- a consolidated list of documents D1 to D38.

The Patentee's requests are set out in section 1 of the response. Specifically, it is requested that the Opposition Division maintains the patent as granted. If the Opposition Division is minded to refuse this request, I request that oral proceedings be appointed. If oral proceedings are appointed, I shall speak in English and shall require interpretation into English should any other party speak in a language other than English.

Yours faithfully

Electronically Signed
DR PAMELA M TUXWORTH
ASSOCIATION NO. 811

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22 March 2018

Our Ref: C411599EP

RESPONSE TO NOTICE OF OPPOSITION

Patent No:	2359834
Application No:	11001632.6
Patentee:	Alexion Pharmaceuticals, Inc.
Opponents:	(1) Amgen Inc. (2) Hoffmann Eitle

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1 REQUESTS

1.1 **Main Request**

1.1.1 The Patent Proprietor requests that the Opposition Division maintain the Patent as granted.

1.1.2 If the Opposition Division contemplates any decision other than the maintenance of the Patent as granted, then Oral Proceedings are requested. If Oral Proceedings are appointed, I shall speak in English. I shall require interpretation into English should any other party speak in a language other than English.

1.2 **Auxiliary Requests**

1.2.1 The Patent Proprietor requests the opportunity to file further claim requests if it is appropriate to do so during these Opposition Proceedings.

2 DOCUMENTS

2.1 The following documents are cited:

D33 Declaration of Dr Miano

D34 Rouet *et al*; FEBS Letters 588 (2014) 269-277

D35 Summary of antibodies listed in D20/D21 Tables

D36 Graphical representation of D20 Table 2

D37 Summary of antibody products approved by EMA since the filing date

D38 Extracts from Kabat *et al.*, Sequences of Proteins of Immunological Interest, US Dept, Health and Human Services (1991)

2.2 We also enclose a consolidated list of all of the documents cited by both Opponents, and including the additional documents mentioned above.

3 No Added Subject Matter

3.1 Opponent 1 (O1) argues that claim 1 includes added subject matter compared with the application as filed. O1's reasoning is that claim 1 of the patent as granted does not include the term "single unit dosage form" which is found both in the passage on page 5, lines 3 to 9 and that on page 22, lines 7 to 14 of the application as filed. O1 argues that this is presented as an essential element of the pharmaceutical composition and

therefore its omission from claim 1 leads to inadmissible subject matter. Notably, Opponent 2 (O2) has not raised the same objection and makes no argument that the claim as granted includes added subject matter.

3.2 The Patentee disagrees with O1's position. Basis for the wording used in claim 1 of the patent as granted is provided at the following passages of the application as filed:

- Page 5, lines 9 to 10: *"In certain embodiments, the 300mg single-use dosage form comprises 30ml of a 10mg/ml sterile, preservative free solution."*
- Page 6, lines 21 to 23: *"In certain embodiments, the pharmaceutical composition comprises a 300mg single-use formulation of 30ml of a 10mg/ml sterile, preservative free solution."*
- Page 22, lines 11 to 13: *"In certain embodiments, the pharmaceutical composition comprises a 300mg single-use formulation of 30ml of a 10mg/ml sterile, preservative free solution."*

3.3 As is apparent from the above extracts from the application as filed, in none of these passages is the term "single-unit dosage form" presented as an essential feature. Accordingly, these passages are sufficient to provide basis for the wording used in claim 1 of the present patent. There is no addition of subject matter in contravention of Article 123 (2) EPC.

3.4 For completeness, it is noted that the Patentee disagrees that D10 supports O1's argument. D10 does not define either the terms "single-use" or "single-unit dosage form". For at least this reason, D10 is therefore not relevant to the interpretation of these terms.

4 Inventive Step: State of the Art

4.1 The inventive step of the claimed invention will be discussed with reference to the problem and solution approach in section 5 below. It is useful, however, firstly to provide a discussion of the situation in the art at the time the invention was made, in particular the general knowledge of the skilled person in the art in relation to eculizumab and formulating antibodies in general.

4.2 *Eculizumab and treatment of PNH*

4.2.1 Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematologic disease which results in PNH blood cells which have a partial or complete deficiency of GPI-anchored proteins. Anemia is a significant problem for PNH patients leading to an impaired quality of life which is disproportionate to the degree of anemia. Many PNH patients depend on blood transfusions to maintain adequate haemoglobin levels.

4.2.2 The nature of PNH is such that patients, once identified, need to start on treatment very quickly. Thus, the required drug, in the present case

eculizumab, must be available and ready for use as soon as possible. It is therefore stored on site or at a location nearby (within a certain radius) such that it is essentially ready for use when a patient is identified.

4.2.3 PNH is, however, an ultra-rare disease, so the frequency with which patients are diagnosed is low. The person of skill in the art of PNH treatment would therefore be aware of the need for a long shelf-life product, in order that it can be stored where it is needed for a long period of time without loss of drug activity.

4.2.4 Eculizumab has been identified as a useful treatment for PNH and is the first antibody of its kind to be brought to market. Prior to eculizumab, the vast majority of antibodies brought to market were of IgG1 type, or occasionally IgG2 or IgG4. Eculizumab is none of these IgG types. Instead, appended to eculizumab's variable region is a unique, non-naturally occurring CH1-hinge-CH2-CH3 sequence. No analogously-engineered antibodies were even in development at the filing date of the patent and certainly none had been approved for clinical use. Even today there are no antibodies on the market with the same uniquely-engineered CH1-hinge-CH2-CH3 sequence as eculizumab.

4.3 *Formulation of Antibodies is a Complex Challenge*

4.3.1 A number of antibody drugs were in development at the time the present invention was made, and a small number had already reached the market. However, the issue of the formulation of antibody drugs was at the time regarded as a highly complex and unpredictable issue. As with other protein drugs, it is very important to maintain the integrity of the antibody and to minimize any possible degradation pathways. Therefore, in order to maintain activity of the antibody, both in storage and for administration, a balance is required between maintaining functionality of the antibody and minimizing the inevitable processes of protein degradation that occur in solution. Providing formulations in which antibodies maintain good stability, however, had proved to be a challenging problem.

4.3.2 These issues are discussed in D20 and D21, review articles published in 2007 and 2006 respectively. As Wang *et al* put it in the abstract of D20:

> *"Although antibodies share certain structural similarities, development of commercially viable antibody pharmaceuticals has not been straightforward because of their unique and somewhat unpredictable solution behaviour."*

4.3.3 The various different degradation pathways to which antibodies may be susceptible are described in the section beginning at page 8 onwards of D20. These include denaturation, aggregation, surface adsorption, disulphide bond formation/exchange, non-reducible cross-linking, deamidation, isomerisation, oxidation, formation of acidic or basic species, C-terminal clipping, fragmentation and Maillard reaction. The skilled person looking to formulate an antibody therefore has a multitude of

different problems to consider, each of which will apply in different ways to each individual antibody.

4.3.4 Of course, it is not the case that only one degradation pathway will necessarily dominate for any one antibody. In the right-hand column, top paragraph of D20, Wang *et al* states that:

> *"In many cases, multiple degradation pathways can occur at the same time and the degradation mechanism may change depending on the stress conditions."*

4.3.5 Moreover, the strategies available to minimise the various degradation pathways may conflict with one another. For instance, deamidation, a major degradation pathway in antibodies, has been shown to have a strong pH-dependence, with less deamidation occurring at pH 4 compared to pH 7 (D20, page 12, right-hand column penultimate paragraph). In contrast, antibody precipitation can be a significant problem at low pH values. D20 reports (page 11, left-hand column first paragraph) that incubation of two mouse monoclonal antibodies (IgG2a and IgG1) at 37°C for 32 days caused precipitation at pH 3 and pH 4 solutions for both antibodies. It is therefore difficult to create a stable antibody formulation due to the numerous degradation pathways possible, and the potentially conflicting strategies available to the formulation chemist for dealing with each of them in the context of each individual antibody.

4.3.6 This issue is also highlighted in D21. In the passage set out below, the authors comment not only on this issue but also on the critical need to devise a formulation that remains compatible with delivery:

> *"In general, **successful antibody formulation designs are simply the best compromise of minimizing several competing degradation pathways**. One approach is to find a pH where the minimal amount of degradation events such as deamidation and oxidation will occur. Another is to identify a buffer system that can stabilize the formulation at that pH without damaging the antibody. One common tactic taken to reduce the rate of many of these chemical degradation events is to prepare lyophilized or freeze-dried antibody preparation but even these can be compromised by aggregation issues due to thermodynamic stress that occurs during freezing, drying and reconstitution. A number of protective excipients, mostly carbohydrates, can reduce the propensity of antibodies to aggregate during these stressful events. **Another issue is to produce a formulation that is compatible with delivery — neither hypotonic — nor hypertonic solutions are well-tolerated upon injection.**"* (D21, page 701 right-hand column, first full paragraph emphasis added)

4.4 *Each Antibody is Unique*

4.4.1 Despite the various structural similarities between some of the different antibody drugs available in 2007, different antibodies have significantly

different behaviours and each requires completely separate study in terms of its formulation. Daugherty et al put it like this in D21:

> *" 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 relating to its requirements for stability****; a phenomenon derived from the fact that the small differences between these antibodies are focused on surface-exposed amino acid differences that stipulate antigen specificity. Thus, the interfacial surface of each antibody drug is unique and thus requires specific formulation components to provide maximal stability and retention of activity."* (D21, page 690, right-hand column, first paragraph, emphasis added)

4.4.2 This need to treat even very similar antibodies individually in terms of their stability characteristics is illustrated at page 693, left-hand column of D21. Here the authors describe a recombinant monoclonal antibody that recognises vascular endothelial growth factor (rhMab-VEGF) which was studied for its kinetic and thermodynamic aspects of antibody solution dimerization. The results are reported by Daugherty et al as follows:

> *"It was found that for this IgG1 class antibody, aggregation rates were greater in slightly alkaline (pH 7.5-8.5) compared to slightly acidic (pH 6.5-7.5) conditions. A high salt environment (1 M NaCl) also enhanced dimerization. …. One of the most striking aspects of these dimerization studies on rhMab-VEGF is that trastuzumab, another genetically engineered antibody with the same IgG1 framework and a 92% sequence homology with rhMab- VEGF, fails to demonstrate aggregation events under the same conditions."* (D21, page 693, left-hand column)

4.4.3 This example therefore illustrates that antibodies having very close structural similarity can behave in very different ways in terms of their degradation pathways. Strategies to provide stable antibody formulations therefore must be considered on a case by case basis for each individual antibody.

4.4.4 It is also notable that the vast majority of the discussion of both D20 and D21 relates to IgG1 antibodies. There is a brief discussion in the first paragraph of page 689 of D21 of engineered antibody molecules. D21 notes that such structurally-different antibodies could have very different properties. D21 goes on to state that:

> *"as these new approaches gain prominence, more information will be acquired about their unique requirements for successful formulation and delivery. At present, however, there is little information on formulation approaches for antibodies produced using these production processes."*

4.4.5 Therefore, the authors of D21 acknowledge that experience gained in relation to IgG1 antibodies may well not be applicable at all to structurally different antibodies, for example the non-natural, uniquely-engineered structure of eculizumab.

4.5 *No Single Technique Provides Stability*

4.5.1 It can be seen from the discussion above that the formulation of antibody therapeutics was a highly complex field, multiple degradation pathways needed to be taken into account and potentially conflicting strategies were available to the skilled person for dealing with these degradation pathways. Further, each antibody must be individually studied, since small differences in antibody structure were known to lead to significant differences in antibody stability and degradation behaviour. It follows, therefore, that no single technique, no single group of excipients, and no single formulation strategy was available to the skilled person to provide a stable antibody formulation. This is evident from the variety of strategies discussed in the art. Not only do D20 and D21 themselves describe a wide variety of different strategies for effecting antibody stability, but this is also apparent from other prior art documents cited by the Opponents. For example:

- D28 describes a technique for providing stable lyophilised protein formulations using a lyoprotectant such as sucrose or trehalose (see paragraph 8). This is exemplified only in two specific antibodies, an anti-HER2 antibody and an anti-IGE antibody.

- In D29 a very different strategy is used in which the antibodies are not subject to lyophilisation but particular formulation pH and excipients are considered. This strategy was applied to two specific antibodies, an anti-CD18 antibody and an anti-CD20 antibody. Notably, D29 provides an entirely separate study of the suitable pH, buffer and excipients for each of these two separate antibodies.

4.6 *Antibody Concentrations Used in Earlier Formulations*

4.6.1 The Opponents have argued that Table 1 of D20 and Table 1 of D21 demonstrate that antibodies were commonly formulated at 10mg/ml in the art and that therefore this would be an obvious choice of formulation concentration for eculizumab. That 10mg/ml is not the obvious choice for eculizumab is explained in detail below with reference to the problem and solution approach. However, it is useful here to consider what, if any, information the skilled person could derive from the Tables of D20 and D21 when considering suitable formulations for any future antibody drug.

4.6.2 The enclosed table D35 lists each antibody of Table 1 of D20 and Table 1 of D21. The type of antibody is listed, as well as the antibody concentration (in the form in which the product is stored), together with details of the formulation. D35 therefore summarises the information provided in Table 1 of D20 and Table 1 of D21, whilst additionally providing the antibody type. IgG2 and IgG4 antibodies are highlighted in bold.

4.6.3 A number of observations can be made based on D35 regarding the antibody products on the market before the filing date of the patent:

- The concentrations of products provided in liquid form range from 1 to 50mg/ml with a wide variety of different concentrations being used for different products.
- Those products that can be stored in solution form include five products having a concentration from 1 to 2 mg/ml; four products having a concentration of 10 mg/ml; and three products having a concentration of 20 mg/ml or above.
- Eight products are provided in lyophilised form.
- Half of the antibodies provided in solution form were formulated at concentrations of 5 mg/ml or less.
- The vast majority of antibody drugs which had reached the market before the filing date are IgG1 antibodies.

4.6.4 It is difficult, if not impossible, to derive any pattern in terms of antibody formulation based on the wide variety of formulations used for marketed drugs. The only possible conclusion is that since each antibody is unique for its binding specificity to an antigen, a unique formulation must be developed individually for each antibody. It certainly cannot be concluded that 10 mg/ml is a "typical" concentration for an antibody formulation. Some commercial antibodies were formulated at 10 mg/ml, but the majority were not and it is not possible to determine from the information in Table 1 of D20 and Table 1 of D22 why each formulation was selected. There is no indication as to whether other future antibodies might or might not be suitable for formulation at 10 mg/ml.

4.6.5 D35 also shows that the largest group of antibodies are lyophilised. Again, there is no indication in the Tables of D20 and D21 as to why these particular products are lyophilised for storage. However, the first paragraph of the left-hand column of page 18 of D20 hints that this could be due to instabilities:

> "*Like most proteins, some antibodies are not stable enough in a liquid form and lyophilized dosage forms will have to be considered*".

4.6.6 The wide variety of different formulation types, concentrations and the varying use of lyophilisation which is apparent from D35 demonstrates the unpredictability of antibody formulation. Each antibody needs separate consideration, and the behaviour of other antibodies clearly has not in the past led to any consistency in the way that these products are formulated. The same is true for any future antibody product: the evidence of Table 1 of D20 and Table 1 of D21 is no guide to a suitable formulation for eculizumab.

4.6.7 This is particularly the case given that eculizumab is a non-natural, uniquely-engineered antibody, whilst the vast majority of those antibodies which had reached the market by the filing date were IgG1 type

antibodies. There are only five examples of non-IgG1 antibodies on the market before the filing date. These were formulated at 1 mg/ml, 1.1 mg/ml, 14 mg/ml, 20 mg/ml and in lyophilised form. The skilled person simply cannot predict from this information a suitable formulation for any future non-IgG1 product. It is certainly not possible to predict from this information a suitable formulation type for a product such as eculizumab. No antibody of this type had reached the market before the filing date.

4.6.8 As well as providing information on the formulation of marketed antibodies, D20 also provides information on antibody stability, including aggregation, in Table 2. Table 2 lists five antibodies where the percent aggregates following storage of a liquid formulation is provided. In D36, the Patentee has plotted the concentration of the formulation against the percent aggregates for these five examples. It is notable that there is no clear trend evident from this graph. Aggregate formation does not vary linearly as antibody concentration changes. There is no direct relationship between these two parameters and thus there are other factors at play in determining the degree of aggregation. In particular, the tendency towards aggregation is very different for different antibodies.

4.6.9 In summary, formulation of antibody drugs remains to this day a challenging topic. There is no single technique or set of excipients and buffer/pH conditions, which formulation experts have at their disposal, which is guaranteed to lead to stability of any particular antibody product. Rather, each antibody has a unique biochemical behaviour. The process of developing a suitable formulation for an antibody, which maintains its biological integrity and activity over time, is one which needs to be carried out individually for each new antibody product. This is particularly the case for an antibody having a novel type of structure, such as the uniquely-engineered structure of eculizumab.

5 Inventive Step: Problem and Solution Approach

5.1 Closest Prior Art

5.1.1 Both Opponents have suggested that D1 is a suitable prior art document. The problem and solution analysis below is accordingly based on D1 as the closest prior art.

5.2 Difference from D1

5.2.1 The claims of the patent differ from the disclosure of D1 in that:

- The formulation is a single use 300mg dosage form
- The concentration is 10 mg/ml
- The volume is 30ml
- The solution is preservative free

5.3 *Technical Problem to be solved*

5.3.1 The patent aims to provide treatments for PNH. Any such treatment must, to ensure efficacy, be provided in a form which maintains its biological integrity and activity during any period of storage. This is clearly important so that the drug remains an effective treatment for PNH. The treatment must also be in a suitable form for a clinician to administer to a patient.

5.3.2 The specific pharmaceutical composition of eculizumab set out in claim 1 of the patent provides the ideal balance of these properties. The composition is easy for the clinician to use: it is a liquid formulation so there is no need for reconstitution which brings inherent inconvenience for clinical staff and risks of incomplete dissolution. Further, the composition provides a balance of (a) excellent long-term storage stability without loss of biological integrity, (b) minimising the bulk of the stored product, and (c) a suitable concentration for use to provide convenient administration in an acceptable infusion time. All of these factors make the product an excellent treatment for PNH, which has led to the approval of the claimed composition as a treatment for PNH by the regulatory bodies.

5.3.3 It is, of course, critical that the antibody maintains its biological integrity and activity in the period between manufacture and use. Aggregation is a common indicator of antibody stability, and accordingly, any formulation for the treatment of PNH needs to have a low incidence of aggregation on long-term storage. The enclosed declaration by Doctor Miano (D33) provides evidence of the long-term stability of eculizumab 10 mg/ml formulations, and the improved stability of these formulations compared with 30 mg/ml eculizumab formulations.

5.3.4 In paragraph 2 and Exhibits 2 and 3 of D33, Dr Miano provides evidence demonstrating that the eculizumab formulation at 10 mg/ml (i.e. the commercial formulation, Soliris®) is stable for a total period of 48 months. The product is stable for 18 months when stored as the drug substance, i.e. in bulk, and for a further 30 months when stored as the drug product in individual vials. Even over this long storage period, the product meets the commercial requirement that at least 98% monomer antibody remains.

5.3.5 Dr Miano also provides data demonstrating the improved stability of the 10 mg/ml formulation compared with a formulation which is identical in all respects, except that the eculizumab concentration is 30 mg/ml. The studies were carried out at 2 to 8°C and also at 23 to 27°C. As Dr Miano explains at paragraph 5 of D33, these higher temperature stability studies provide a good indication of effects which might be seen at 2 to 8°C over a longer period of time.

5.3.6 As is apparent from Figure 1 of D33, after only six months storage at 23 to 27°C the 30 mg/ml concentration led to an unacceptable level of aggregate formation, with the % monomer dropping to below the threshold of 98% which is required for release. In contrast, the 10 mg/ml concentration maintained over 99% monomer under the same storage conditions.

5.3.7 Similar trends are seen in the studies at 2 to 8°C, where the % monomer retained after storage is significantly higher for the 10 mg/ml concentration compared with the 30 mg/ml concentration. Based on this data, Dr Miano concludes that the 30 mg/ml concentration would not meet the release standards after 36 months of storage.

5.3.8 Dr Miano's evidence therefore demonstrates that the 10 mg/ml composition of eculizumab provides extremely good long-term stability such that the antibody maintains its integrity and biological activity. It also demonstrates that the 10 mg/ml concentration of eculizumab provides improved stability compared with other concentrations.

5.3.9 The 10 mg/ml concentration combines this enhanced stability with ease of use for the clinician due to the low-volume liquid formulation. Firstly, because it is a liquid formulation, there is no need for reconstitution. Secondly, simple dilution of product required by the label conveniently provides the required 600 or 900 mg administration dosages. Thirdly, the 600 or 900 mg doses required by the patient can be administered via IV infusion of only 120 or 180 ml of solution (following dilution for administration). This relatively low volume enables the drug to be administered by IV infusion over relatively short periods of time and without the need for high infusion volumes which are undesirable for the patient. Finally, the low volume of product in the storage vials (30 mL per vial) minimises the volume which must be stored on site at each clinic.

5.3.10 The technical problem which is solved by the invention is therefore the provision of a composition of eculizumab which demonstrates the ideal balance of properties for use in the treatment of PNH, including enhanced maintenance of biological integrity following prolonged storage in the absence of preservative, whilst also providing a low-volume liquid formulation having acceptable infusion times and which is convenient to store.

5.3.11 The solution to this problem is the 300 mg single-use dosage form comprising 30 mL of a 10mg/ml preservative-free solution.

5.4 *Response to Opponents' Comments on Technical Solution*

5.4.1 The Opponents argue that the 10 mg/ml concentration of the formulation is an arbitrary selection. This is not the case, for the reasons set out above: the 10 mg/ml formulation shows enhanced maintenance of biological integrity following long-term storage, combined with low volumes for convenient dilution, administration and storage. The formulation as claimed is therefore not only extremely effective but is improved over alternative formulations of eculizumab.

5.4.2 Opponent 1 has filed a declaration by Dr Treuheit (D23) which is said to show the equivalence of differing concentrations of eculizumab. The evidence provided by Dr Treuheit does not show the equivalence of the different concentrations for the following reasons:

- Dr Treuheit tested stability only over a 13 week period. This is significantly less than the 48-month stability demonstrated for the 10 mg/ml eculizumab product in D33. As the Opposition Division will clearly appreciate, where stability tests are carried out for a very short period of time, differences between the samples tested may not be detected, or may be very small.

- Even at the short period of time over which Dr Treuheit's test was conducted, an increase in formation of aggregates can be seen at elevated temperature for both the 30 mg/ml and 50 mg/ml formulations. These trends support the 30 mg/mL data presented in Dr. Miano's declaration on behalf of the Patentee.

5.4.3 As Dr Treuheit explains in paragraph 5 of his declaration, aggregation of the antibody was studied by size-exclusion ultra high-performance liquid chromatography (SE-UHPLC). The results are shown in Appendix A to his declaration. The lower panel of Appendix A shows that the 30 mg/ml and 50 mg/ml formulations resulted in a reduced percentage of intact antibody after 13 weeks. In both cases, the percent antibody remaining after this period of time was lower than at base line. However, the 10 mg/ml formulation did not show any reduction in percent antibody after 13 weeks even after storage at 25°C.

5.4.4 This result is in accordance with the longer 6 month study carried out by Dr Miano where the 30 mg/ml concentration had a reduced proportion of intact antibody as storage progressed compared with the 10 mg/ml concentration.

5.4.5 The SEC data in Appendix A of Dr Treuheit's declaration shows that the 30 mg/ml and 50 mg/ml formulations had close to 98% antibody remaining after 13 weeks of storage at 25°C. This antibody content falls below the release parameter for commercial lots of eculizumab, indicating that this drug would not be sufficiently stable over this period of time for clinical use.

5.4.6 Opponent 1 also comments on D25 which discloses formulations of anti-C5 antibodies, including eculizumab, of various concentrations. Opponent 1 highlights formulations of eculizumab at various concentrations which were found to be stable. However, the evidence of D25 does not provide any indication of the relative stability of these high concentration formulations compared to the claimed 10 mg/ml formulation. As is clear from the formulation flow chart depicted in the abstract of D25, the formulation is changed on moving from the 10 mg/ml phosphate-based solution to the higher concentration solutions. Thus, it is not possible to directly compare the 10 mg/ml solutions with the higher concentration solutions of D25. Indeed, the skilled person might assume that the higher concentration solutions of eculizumab needed particular treatment, with variation of buffers and excipients, since simply increasing the concentration of the 10 mg/ml solution did not lead to sufficient stability.

5.4.7 In summary, in light of D1,

- The claimed subject matter provides a specific formulation for eculizumab which is a 300 mg single use dosage form comprising 30 mL of a 10 mg/ml preservative free solution.
- This formulation has extremely good storage stability (48 months at 2-8°C) which means that clinics can store the product for long periods of time and still rely on the biological activity of the product being suitable for treatment of PNH.
- The 10 mg/ml concentration has an *improved* storage stability compared with 30 mg/ml as demonstrated by Dr Miano (D33).
- The volume of the formulation is only 30 ml, such that the volume of product which needs to be stored and administered is low. This has benefits in terms of acceptable infusion volumes and times for the treatment of PNH as well as providing the ability to store eculizumab at various sites to ensure that PNH patients are able to quickly receive this critical therapy.
- The product can be stored in liquid form, avoiding risks of incomplete/incorrect reconstitution by a clinician and providing convenience of use in the treatment of patients having PNH.

5.4.8 The claimed subject matter therefore solves the technical problem of providing a composition of eculizumab which demonstrates the ideal balance of enhanced maintenance of biological integrity following prolonged storage in the absence of preservative, whilst also providing a low volume liquid formulation.

5.5 *The Solution is Not Obvious From the Art: Overview*

5.5.1 As is set out in the Guidelines for Examination, the consideration of whether or not a claimed invention would have been obvious involves asking:

> *"whether there is any teaching in the prior art as a whole that **would** (not simply could, but would) have prompted the skilled person, faced with the objective technical problem, to modify or adapt the closest prior art while taking account of that teaching, thereby arriving at something falling within the terms of the claims, and thus achieving what the invention achieves (see **G-VII, 4**).*
>
> *In other words, the point is not whether the skilled person could have arrived at the invention by adapting or modifying the closest prior art, but whether he **would have done** so because the prior art incited him to do so in the hope of solving the objective technical problem or in expectation of some improvement or advantage (see **T 2/83**)."*

5.5.2 It is therefore necessary in the present case to consider whether the skilled person *would* have arrived at the claimed 300 mg preservative-free dosage form containing 30ml of a 10 mg/ml eculizumab solution, with a reasonable expectation that such a composition would provide the ideal balance of maintaining biological integrity of the antibody following storage in a low-

volume liquid formulation (not merely whether he/she *could* have done so).

5.5.3 The Boards of Appeal have further held that it is necessary to consider whether a skilled person would have followed a particular course of action with a reasonable expectation of successfully solving the technical problem. A reasonable expectation of success has been distinguished from a "hope to succeed" or from a situation where it is "obvious to try" (T296/93). Rather, what is required is that the skilled person can predict, before starting a project, whether or not there is a realistic expectation of a successful conclusion to a project (i.e. of solving the technical problem.)

5.5.4 The Boards of Appeal have, in a few occasions, denied an inventive step on the basis that the skilled person is in a "try and see" situation, as mentioned by Opponent 1 (T333/97). However, a "try and see" situation is considered by the Boards to be limited to particular situations, i.e. where the prior art clearly envisages a specific feature, and the skilled person merely has to determine by routine tests whether such feature does indeed provide a desired effect. In T333/97, for instance, the skilled person is faced with the problem of providing a method for blocking in plants a given pathogenic target RNA strand activity. A specific, suitable method was known in E coli and all parties agreed that it would be obvious to try this specific method in plants. However, it was not known whether or not the method would succeed. A lack of inventive step was found since a single course of action, agreed to be the route obvious to the skilled person, merely had to be tested to see if it worked.

5.5.5 In the present case, as will be discussed further below, none of the documents cited by the Opponents gives the skilled person a reasonable expectation of achieving, in a formulation of eculizumab, good stability and low liquid volume via *any* type of formulation. There is certainly no suggestion that a liquid formulation of 30 ml at 10 mg/ml would provide the ideal balance of properties. It is also clear that the present situation is not a "try and see" situation. The prior art does not provide an indication of a single suitable formulation which could be tested to see if it is effective. Rather, a wide variety of different formulations are used for different antibodies. To determine a suitable composition for the uniquely- engineered eculizumab antibody, the skilled person would have to conduct a significant research program to fully investigate the stability and biological integrity of the antibody, as well as the various factors (and their interplay) affecting each.

5.5.6 Indeed, the Opponents largely seek to rely on references which relate to formulations of other antibodies. As discussed in detail above in Section 4, each antibody has individual properties in terms of its stability, even where structure is very similar. In the present case, eculizumab is a non-naturally occurring, non-IgG1 isotype and is therefore *not* structurally similar to the largely IgG1 antibodies referred to by the Opponents. No conclusions can therefore be drawn from prior art which refers to suitable formulations of different antibodies.

5.6 *Claimed Subject Matter is Not Obvious over D1 combined with D19-D21*

5.6.1 In light of D1, the technical problem to be solved is to provide a composition for eculizumab which has the right balance of properties providing biological integrity following storage and a low volume liquid formulation for ease of storage and administration. The question in relation to inventive step is: would the skilled person have chosen a 30ml single use formulation of 10 mg/ml eculizumab in a preservative free solution, based on the teaching in D19 to D21, with a reasonable expectation of solving this problem?

5.6.2 In Section 4 above we discussed the knowledge in the art in relation to antibody formulations in D20 and D21. This can be summarised as follows:

- Formulation of antibodies was a complex area with a wide array of different stability considerations to be taken into account for each individual antibody.
- Each antibody is unique and needed separate consideration in terms of determining a suitable composition, even where antibodies were structurally similar.
- There was no single "common" formulation, or antibody concentration, which the skilled person would turn to first.
- The knowledge in the art was largely in relation to IgG1 antibodies. Non-IgG1 antibodies with non-naturally occurring, engineered constant regions were known to act differently, but little work had been done on formulation of such antibodies.
- A wide array of different formulations had been used for various antibodies on the market, giving no clear guidance as to preferable or even suitable formulations for future therapeutic molecules.

5.6.3 It is clear from the above, therefore, that the skilled person would not have known which concentration to use in a pharmaceutical composition of eculizumab. The skilled person would not have had any awareness as to how eculizumab would be affected by the wide variety of potential stability issues to consider. The skilled person would not have had any awareness of which of the many tools available to the formulation scientist (pH, excipients, antibody concentration, lyophilisation) would be effective in addressing the as yet unknown stability issues. The skilled person would certainly not have any indication that 30ml of a 10 mg/ml liquid composition of eculizumab would provide the combined benefits of improved biological integrity of the antibody on storage at up to 48 months and low volume liquid formulation for improved administration. Rather, a full research project would be needed to arrive at such a suitable and ideal composition.

5.6.4 Importantly, the skilled person would be aware that the knowledge about formulation in D20 and D21 largely relates to IgG1 isotype antibodies. However, as set out in D21, little information was available about engineered antibodies:

> *"A number of engineered antibody-like molecules are now being examined for therapeutic applications. Some engineered fragments,*

such as diabodies and minibodies, are much smaller than intact, full length IgG1 and have very different properties of systemic distribution and clearance. Additionally, methods to prepare other antibody types such as dimeric secretory IgA and methods to prepare antibodies using non-mammalian cell systems, such as plants, from the eggs of chimeric chickens and from transgenic animals, are now being explored by academic and industrial groups. As these new approaches gain prominence, more information will be acquired about their unique requirements for successful formulation and delivery. At present, however, there is little information on formulation approaches for antibodies produced using these production processes." (D21, p689, LH column)

5.6.5 Eculizumab is an example of an engineered antibody having a unique, non-naturally-occurring CH1-hinge-CH2-CH3 structure. Given the unpredictability of behaviour even between IgG1-type antibodies, the skilled person would not have been able to use the information about IgG1 antibodies in the art to predict the behaviour of eculizumab. Indeed, the skilled person would not have been able to predict whether a liquid formulation would have been possible at all for this new type of antibody. There is certainly no reasonable expectation of successfully providing a formulation which maintains the biological integrity of the antibody, in a low volume liquid formulation, over a period of 48 months. The claimed subject matter is therefore not obvious from D1 in combination with D20 and D21.

5.6.6 D19 is also relied on by O1 to show that it is desirable to exclude preservatives for regulatory reasons. However, there is no suggestion in D19 that a 30 ml eculizumab formulation at a concentration of 10 mg/ml would have beneficial properties. D19 is not therefore relevant to solving the objective technical problem.

5.7 *Antibody Concentrations After The Filing Date*

5.7.1 The Opponents have indicated that 10 mg/ml was a typical antibody concentration and it would have been obvious for a skilled person developing a new antibody to use this concentration based on the common knowledge. If the Opponents are right in their assertion, it could be expected that antibodies developed after the filing date would generally have a concentration of 10 mg/ml. However, this is not the case.

5.7.2 D37 summarises data available from the European Medicines Association (EMA) website (http://www.ema.europa.eu). D37 provides a list of active antibodies (excluding biosimilars) which have been given EMA authorisation after the filing date of the patent. The nature of the product is also indicated, including the product concentration, where the product is provided as a solution.

5.7.3 As is evident from D37, only six out of 44 antibody products were approved having a formulation of a 10 mg/ml solution. It is quite clear, therefore, that other factors are considered by developers when selecting an

appropriate concentration for a formulation of any new drug. 10 mg/ml is not an automatic choice. Indeed, as for antibodies formulated before the filing date, the largest group of products were provided in lyophilised form.

5.7.4 A recent review article on antibody formulation by Rouet *et al* (D34) confirms that, even well after the filing date of the present patent, the issue of providing a stable formulation for an antibody product remains a challenging problem. Even in 2014, Rouet considered that:

> "*Despite recent advances, the identification of suitable formulation conditions for a specific monoclonal antibody remains challenging and cannot be determined from its amino acid sequence. Indeed, a considerable proportion of human monoclonal antibody candidates fail formulation studies, often at relatively late pre-clinical stage, placing heavy burdens onto drug development pipelines.*" (Page 271, right-hand column, second paragraph).

5.7.5 In the first paragraph of the right-hand column of page 271, D34 gives the example of therapeutic monoclonal antibody cetuximab (Erbitux). Even the initial suggested formulation conditions of 2 mg/ml in phosphate buffer at pH 6.0 led to visible aggregates under mechanical stress and had to be reformulated. Notably, the new formulation was at a higher concentration – 5 mg/mL – but at a different pH and using different excipients.

5.8 *Claimed Subject Matter is Not Obvious over D1 Combined with D22*

5.8.1 O1 relies on D22 which relates to a nebulisable formulation. A critical factor in considering a suitable formulation of any drug is the manner in which it is administered. Clearly, a topically administered drug will be formulated very differently from an orally or parenterally administered drug. Similarly, the skilled person looking for a suitable formulation for the D1 treatments, which are administered by IV, would not consider nebulized formulations. Thus, the skilled person would not consider D22 when seeking suitable formulations in light of the D1 disclosure.

5.8.2 Moreover, even if the skilled person had considered D22, this would not have led him to the claimed solution. As O1 points out, D22 exemplifies at para [170] to [172] formulations described as eculizumab formulations at 30 mg/ml. However, this example would, if anything, lead the skilled person *away* from the claimed 10 mg/ml concentration. There is no hint or suggestion in D22 that 10 mg/ml would provide improved biological integrity of eculizumab following storage compared with 30 mg/ml. Indeed, there is no information whatsoever regarding stability in D22.

5.8.3 Moreover, a skilled person seeking a dosage form of eculizumab having a *low volume* would not consider reducing the concentration of the 30 mg/ml solution as this would inherently *increase* the volume of solution needed to provide the same drug content. This document would not, therefore, have led the skilled person to the claimed invention starting from D1.

5.9 *Claimed Subject Matter is Not Obvious over D1 combined with D29*

5.9.1 Starting from D1, the skilled person is seeking a suitable formulation for a unique antibody having a non-naturally-occurring, engineered heavy chain. D29 would not be of assistance to the skilled person since it relates to very different antibodies, which the skilled person would understand to likely have very different stability properties.

5.9.2 D29 is primarily related to formulations for anti-CD18 and anti-CD20 antibodies. This is clear, for example, from column 2, lines 19 to 22 which state that: *"There is a need in the art for a stable aqueous pharmaceutical formulation comprising an antibody, such as an anti-CD18 or anti-CD20 antibody, which is suitable for therapeutic use"*. The only examples of specific formulations given in D29 are for anti-CD18 and anti-CD20 antibodies. Specifically, Example 1 relates to rhuMAb CD18 which is a $F(ab')_2$ molecule (column 24, line 42) and Example 2 relates to rhuMAb CD20.

5.9.3 Eculizumab, on the other hand, is not at all similar to the antibodies exemplified in D29. Eculizumab has a uniquely engineered CH1-hinge-CH2-CH3 sequence and is structurally remote from the antibodies described in D29. The skilled person would have no expectation that the formulation behaviour and stability of the anti-CD18 and anti-CD20 antibodies described in D29 would shed any light at all on a composition for eculizumab. The skilled person would not therefore consider combining D29 with D1.

5.9.4 Further, even if the skilled person had considered D29, the focus of the document is on the excipients used in the formulation. As set out in the summary of the invention at column 2, beginning at line 25, the invention of D29 suggests that a buffer providing a pH in the range 4.5 to 6.0, a surfactant and a polyol are useful in providing a formulation which is "*preferably* […] stable at a temperature of 2-8°C for at least one year, and/or is stable at a temperature of about 30°C for at least one month" (emphasis added). Had the skilled person followed the suggestions of D29, they would have considered primarily the excipients in the formulation to achieve stability. The concentration is not mentioned in the summary of the invention at all. Further, they would not have expected to be able to achieve stability (of a very different antibody) for as long as 48 months, given D29's suggestion of 12 months.

5.9.5 D29 does make some reference to antibody concentration at column 22, as pointed out by O2. However, even D29 itself makes clear that in the actual selection of antibody concentration to use, other factors need to be taken into account. At column 22, line 10 onwards, D29 explains, for example, that the amount of antibody is determined by taking into account the desired dose volumes and mode(s) of administration. The actual concentrations used in the Examples of D1 are very different for the two antibodies: Example 1 uses 1 mg/ml, whereas Example 2 uses 40 mg/ml. Notably, neither is within the narrowest range of 2 to 10 mg/ml which O2 suggests would be the obvious choice of concentration for *any* antibody formulation.

5.9.6 Based on this information, there is no hint that 10 mg/ml is an obvious choice for a skilled person. Indeed, a skilled person could just as easily consider 1 mg/ml, 40 mg/ml or any other concentration in the range of 1 to 50 mg/ml. There is certainly no suggestion that the specific concentration of 10 mg/ml, when used for an eculizumab formulation, would provide an ideal balance of maintaining biological integrity on storage and low volume liquid formulation.

5.9.7 In summary, D29 gives the skilled person no additional information than they would already be aware of from his common general knowledge with regard to antibody concentration. The skilled person knows that many antibodies (where liquid formulation is possible) may be formulated in the range of 1 mg/ml to 50 mg/ml, and that some of those are formulated in the range of 2 mg/ml to 10 mg/ml. But, the skilled person also knows that various considerations must be taken into account when selecting a suitable concentration for any particular antibody. The skilled person also knows that each antibody requires its own individual study into suitable formulations: concentrations of antibody in a given formulation that lead to stability for that one antibody may not do so for another antibody. This is borne out by the contrast between the concentrations used in Examples 1 and 2 of D29. D29 therefore does not lead the skilled person any closer to the present invention.

5.9.8 Finally, it is noted that O2 draws comparison between the commercial formulation of rituximab, an anti-CD20 antibody, and D29. This does not have any bearing on the scope of D29 itself, which suggests a different concentration of 40 mg/ml in Example 2. Moreover, given the significant structural differences between rituximab and eculizumab, the skilled person would have had no expectation that a formulation for rituximab would prove useful for eculizumab, let alone that it would provide a storage stable composition for at least 48 months.

5.10 *Definition of the technical problem*

5.10.1 The discussion above does, of course, make reference to stability data for eculizumab. The Guidelines for Examination in the European Patent Office confirm that any technical effect derivable from the application as filed can be used as a basis to reformulate the objective technical problem. The relevant passage in the Guidelines reads as follows (G-VII-5.2).

> *"As a matter of principle any effect provided by the invention may be used as a basis for the reformulation of the technical problem, as long as said effect is derivable from the application as filed (see T386/89).* ***It is also possible to rely on new effects submitted subsequently during the proceedings by the applicant, provided that the skilled person would recognise these effects as implied by or related to the technical problem initially suggested*** *(see G-VII, 11 and T184/82)".* (Emphasis added.)

5.10.2 The technical effects at issue in the present case are in the maintenance of the biological integrity of the antibody following storage, and suitable volumes for storage and administration to a patient. These properties are clearly intimately connected with the underlying problem of providing a useful treatment for PNH. Unless the biological integrity of the antibody is maintained, the treatment cannot be expected to be efficacious. Similarly, the ability of the clinician to safely and effectively administer the drug to a patient is clearly connected to the ability to treat PNH.

5.10.3 As noted in the passage of the Guidelines set out above, effects which are related to the technical problem set out in the application as filed may be relied upon in the formulation of the technical problem. The relevant passage is set out at G-VII-11:

> *"Subsequently, the applicant submits new evidence which shows that the claimed composition exhibits an unexpected advantage in terms of low toxicity. In this case, it is allowable to reformulate the technical problem by including the aspect of toxicity,* ***since pharmaceutical activity and toxicity are related in the sense that the skilled person would always contemplate the two aspects together****".* (Emphasis added.)

5.10.4 It is therefore clearly acceptable to rely on the evidence set out above in the formulation of the objective technical problem for the purposes of a problem/solution analysis for inventive step.

5.11 *Inventive Step: Summary*

5.11.1 In summary, starting from D1, the technical problem which is solved by the present invention is the provision of a composition of eculizumab which demonstrates the ideal balance of properties for use in the treatment of PNH, including enhanced stability and thus maintenance of biological integrity following prolonged storage in the absence of preservative, whilst also providing a low-volume liquid formulation having acceptable infusion times and which is convenient to store.

5.11.2 Based on D20 and D21, the skilled person would have been aware that formulation of antibodies is a complex area and that each antibody needs to be treated as its own unique formulation project. The skilled person would also have treated with scepticism any information in the prior art that was based on IgG1 antibodies given the non-IgG1, non-naturally occurring, engineered structure of eculizumab: the skilled person would not have any expectation that formulations which had been successful with previous antibodies would be useful in the case of eculizumab.

5.11.3 Moreover, based on D20 and D21, the skilled person would see that there is no clear starting place for considering antibody formulation and that a separate research program would be needed to find the ideal formulation for eculizumab. The result of that research program, carried out by the inventors, was the finding that 30 ml of a 10 mg/ml liquid formulation of eculizumab is surprisingly stable over a period of 48 months, having

superior maintenance of biological integrity compared with other concentrations of eculizumab, whilst also providing a low volume of liquid formulation. The skilled person could not have had any reasonable expectation of this result in advance of carrying out the research.

5.11.4 Moreover, neither D22 nor D29 cited by the Opponents provides any assistance. D22 relates to a different type of formulation from D1 so would not be considered relevant. In any case, it directs the skilled person to inferior 30 mg/ml formulations. D29 relates primarily to the excipients used in antibody formulation, in particular anti-CD18 and anti-CD20 formulations. Again, it would give the skilled person no guidance for suitable compositions for eculizumab.

5.11.5 The claimed subject matter is therefore inventive over the art.

6 Sufficiency

6.1 The skilled person would be able to carry out the invention without undue burden.

6.2 There is no confusion as to what the heavy and light chain amino acid sequences of eculizumab are. The patent discloses the variable heavy and variable light chain regions of eculizumab (SEQ ID NO: 1 and SEQ ID NO: 3) and the full heavy chain and light chain sequences of eculizumab (SEQ ID NO: 2 and SEQ ID NO: 4).

6.3 The skilled person would immediately recognise, based on his common general knowledge, that SEQ ID NO: 4 (and also the light chain variable region sequence of SEQ ID NO: 3) includes the N-terminal leader sequence which is not present in the mature antibody.

6.4 It was well known in the art at the filing date of the application that a signal peptide is present at the N-terminus of immature heavy and light chain polypeptides. Kabat *et al.*, Sequences of Proteins of Immunological Interest, US Dept, Health and Human Services (1991) (D38), which is a database of biological sequences containing sequence alignments and is evidence of the skilled person's common general knowledge, shows the signal peptides of various human light chains.

6.5 The attached page 1 from Kabat *et al.* shows the 22-residue signal peptides of human kappa light chains. The first three residues are predominantly Met Asp Met and the last two residues are predominantly Arg Cys. In SEQ ID NO: 4 of the patent, the first three residues are Met Asp Met and residues 21 and 22 are Arg Cys. Therefore, the skilled person would recognise that SEQ ID NO: 4 is the amino acid sequence of the immature light chain that contains a signal peptide that is not present in the mature eculizumab antibody.

6.6 Furthermore, the skilled person would know that the framework of human kappa light chains commonly starts with the residues Asp Ile Gln Met as shown in the Table at pages 103 to 108 of Kabat *et al*. Residues 23 to 26 of SEQ ID NO: 4 are Asp Ile Gln Met, which the skilled person would recognise as being the start of the mature light chain of eculizumab.

6.7 The existence of post-published D25 that includes the mature sequence of SEQ ID NO: 4 has no bearing on whether the disclosure of the patent is sufficient. The date for determining sufficiency of disclosure is the filing date of the patent. At any rate, D25 merely confirms what the skilled person would know from reading the patent in light of his common general knowledge, i.e. that SEQ ID NO: 4 contains the light chain signal peptide as well as the light chain sequence present in the mature antibody.

6.8 It should also be pointed out that the skilled person would produce eculizumab using cell-based recombinant methods, and not by artificially synthesising the polypeptide chains. The cells used to make eculizumab would process the light chain polypeptide sequence to remove the signal sequence during antibody production in the cells. Therefore, contrary to the assertion of O1, the skilled person would not use an antibody that is not eculizumab to treat PNH because he would not be able make an antibody that contains the whole sequence of SEQ ID NO:4.

6.9 Thus, the skilled person seeking to reproduce the claimed invention based on the disclosure in the patent of SEQ ID NOs: 2 and SEQ ID NO: 4 would be able to make and formulate eculizumab as required by claim 1 using routine methods known in the art.

6.10 The claimed subject matter is therefore sufficiently disclosed in the patent.

7 Summary

7.1 For the reasons discussed above, the patent does not contain added matter, is inventive over the art and is sufficient. The oppositions should therefore be dismissed and the patent maintained as granted.

Signed

A SIMONS P TUXWORTH

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Date
05.07.2018

Reference	Application No./Patent No.
AMG17203OP OPPO 01	11001632.6 - 1112 / 2359834
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.

The matters to be discussed are set out in the communication accompanying this summons (EPO Form 2906).

The oral proceedings, which will be public, will take place before the opposition division

on 21.01.19 at 09.00 hrs in Room 1653 at the EPO,
Grasserstr. 2, PschorrHöfe, D-80335 München

No changes to the date of the oral proceedings can be made, except on serious grounds (see OJ EPO 1/2009, 68). If you do not appear as summoned, the oral proceedings may continue without you (R. 115(2) EPC).

Your attention is drawn to Rule 4 EPC, regarding the language of the oral proceedings, and to the Special edition No. 3 OJ EPO 2007, 128, 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 21.11.18.

You are requested to report in good time beforehand to the porter in the EPO foyer. Room 1657 is available as waiting room.

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 the porters' lodge in the main foyer "Bayerstrasse", the parking ticket will be revoked.

1st Examiner:	2nd Examiner:	Chairman:
Trifilieff-Riolo S	Durrenberger A	Albayrak T

For the Opposition Division

Annexes:
Confirmation of receipt (Form 2936)
Rule 4 EPC (EPC Form 2043)
Communication (EPO Form 2906)

Registered letter with advice of delivery to EPO postal service: 28.06.18

Europäisches Patentamt
European Patent Office
Office européen des brevets

European Patent Office
80298 MUNICH
GERMANY
Tel: +49 89 2399 0
Fax: +49 89 2399 4465

Application No.: 11 001 632.6

Patent No.: EP-B-2 359 834

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 21.01.2019 Time 09:00

Archibus

1653

Conference Room booked

ORAL 01, 02, 03 and 05 coded

Date Initials

md 28.05.18

2.1 If no room is available, notify the division on Form 2088

2.2 Parties' submissions in preparation for the oral proceedings, if any, should be made no later than

2 month(s)

before the date of the oral proceedings
(transfer to Form 2008.1 /2310.1)

2.3 Encode ORAL(04) coded

md 28.05.18

Date Initials

2.4 Dispatch Form 2008.7 /2310.7 to division

md 28.06.18

Date Initials

3. ☐ Arrange for the following special equipment to be provided in the conference room:

.....
Date Initials

4. Return the dossier to primary examiner with Form 2041 (15 days before the oral proceedings)

.....
Date Initials

21/06/18
.....
Date

.....	.....	.....	.....
Albayrak, Tibur	Durrenberger, Anne	Trifilieff-Biolo, S	
Chairman	2nd examiner	1st examiner	Legal member

Enclosure(s):

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1. European patent 2 359 834 having the title "TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT" is based upon European patent application No. 11 001 632.6 which is a divisional application of European patent application 07 753 249.7 filed on 15.03.2007. It claims priority of US 783070 filed on 15.03.2006.

Proprietor of the patent is : Alexion Pharmaceuticals Inc., Cheshire, CT 06410, US.

2. Notices of opposition have been filed by:

Amgen Inc., One Amgen Center Drive, Thousand Oaks, CA 91320-1799, US (O1) on 04.08.2017 and by

Hoffmann Eitle, Patent- und Rechtsanwälte PartmbB, Arabellastrasse 30, 81925 München
DE (O2) on 09.08.2017.

The opponents request revocation of the patent in its entirety as follows:

O1 contends that the subject-matter extends beyond the content of the application as filed (A. 100(c) ; A. 76(1) and A. 123(2)) and that the invention is not sufficiently disclosed (A. 100(b); A. 83).

O1 and O2 dispute the validity of the claimed priority (A. 87) in relation to the determination of the proper prior art documents.

O1 and O2 both contend that the claimed subject-matter does not meet the requirements for inventive step (A. 100(a), A. 56).

3. The proprietor (P) requests the rejection of the oppositions and maintenance of the patent as granted.

4. All parties further request oral proceedings which are hereby summoned.

5. The present communication represents the provisional and non binding opinion of the opposition division on the above topics.

The numbering of the documents referred to hereinafter is that filed by P with his letter of 22.03.2018. It will be adhered to in the rest of the procedure.

6. A. 100(c); A 76(1), A. 123(2):

The wording of granted claim 1 concerning the expression "300 mg eculizumab single-**use** dosage form comprising 30 ml of a 10 mg eculizumab/ml sterile, preservative free solution" is found in the original description on p. 5, l. 8-9, p. 6, l. 22-23 and p. 22, l. 12-13. In these passages, there is no mandatory link between "a single-**use** dosage form" and "a single-**unit** dosage form". Both are cited as separate possible embodiments.

Hence it seems that the requirements of A. 76(1) would be met.

7. Validity of the priority (A. 87):

The priority document US 783070 does not disclose a single-use dosage form of a liquid and sterile composition comprising 30 ml of a 10 mg eculizumab/ml and no preservative, let alone its use to treat PNH.

Thus the actual filing date of the present patent appears to be the 15.03.2007.

8. A. 100(a), A. 83:

The claimed invention is the therapeutic use of a specific composition comprising a **known** product, namely eculizumab.

As acknowledged by O1, this product was well known and used at the filing date of the patent. The example of the patent (§74) also indicates a commercial source for eculizumab. This constatation alone would appear sufficient to consider that the skilled person would have had no difficulty in carrying out the invention as claimed which is to provide a single-use dosage form of a sterile and preservative free solution comprising 30 ml of a 10 mg eculizumab /ml and to use it to treat PNH patients.

The fact that a post-published document (D25) describes the **mature** sequence of SEQ ID NO: 4 (as mentioned in the patent) whereas the patent describes the corresponding **immature** sequence does not bear any consequence on the above reasoning.

Therefore it seems that the requirements of A. 83 would be met.

9. A. 100(a), A. 56, inventive step:

All parties consider that D1 can represent the closest prior art document.

The opposition division sees no reason to differ.

D1 (§82) describes a study wherein PNH patients are treated with a weekly 600 mg intravenous infusion of eculizumab during 4 weeks, followed by 900 mg of eculizumab 1 week later and then 900 mg on a bi-weekly basis. The study was extended for a total of 2 years for 10 out of the 11 original patients.

The effects of this treatment on different physiological parameters and symptoms involved in PNH are analyzed in D1 and show that the treatment is successful.

The differences between the disclosure of D1 and the subject-matter of granted claim 1 are: eculizumab is in a single-use dosage form of a solution comprising a total amount of 300 mg of eculizumab

the eculizumab concentration is 10 mg/ml

the volume of the dosage form is 30 ml

the solution is free of preservative

The feature concerning the sterility can be regarded as implicitly disclosed by D1 since D1 describes an intravenous infusion.

According to O1 and O2, no technical effect would result from any of these differences so that the problem with respect to D1 would be the provision of a pharmaceutical composition comprising eculizumab for use in the clinical study described in D1.

More specifically, the total amount of 300 mg of eculizumab would be obvious in view of D1 itself, the feature concerning the lack of preservative would be obvious in view of common general knowledge or in view of regulatory aspects (O1; D19) or mention of such a possibility in other prior art documents (O2; D29).

In O2's view, the claimed concentration of 10 mg/ml would be part of the common general knowledge in the field of proteins/ antibodies (D20, D29 both summarized in D31 and D32).

In O1's view the concentration of 10 mg/ml would be an arbitrary choice in the context of routine experimentation when considering the teaching of D5, D20, D21 and D22. This would be emphasized by D23 which depicts assays assessing the stability of eculizumab solutions at 2, 5, 10, 30 and 50 mg/ml and shows that they are all equally stable for 13 weeks at 4°C and 25°C.

Finally, it would follow from the obvious total amount (300 mg) and from the obvious concentration (10 mg/ml) that a volume of 300 ml would also be obvious.

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According to the patent proprietor (P) the differences would lead to a special technical effect which is that the solution demonstrates the ideal balance of properties for its use to treat PNH, including enhanced maintenance of biological integrity upon prolonged storage in the absence of preservative and low-volume liquid formulation. This effect would be demonstrated in post filed D33 which compares the stabilities of a 10 mg/ml and a 30 mg/ml solution of eculizumab and shows a superior stability for the 10 mg/ml solution.

P asserts that although this effect is not mentioned in the original application, it would be intimately related to the technical problem set out in the original application which is to provide a treatment for PNH because it is mandatory for any medicament, including antibodies, to have a good stability so that the post filed data of D33 would have to be taken into account in assessing inventive step.

Hence the problem in view of D1 would lie in the provision of a eculizumab dosage form having the properties mentioned above.

The skilled person would be aware in view of D20 and D21 that the formulation of antibodies is a complex area. None of D20, D21 or any other piece of prior art such as D22 or D29 would suggest to use an eculizumab solution with a concentration of 10 mg/ml, free of preservative and comprising 300 mg eculizumab to solve the problem above.

The arguments of the parties will have to be discussed at the oral proceedings.

It will have to be examined whether the technical effect demonstrated in D33 can be taken into account for assessing inventive step. The opposition division would tend to give a positive opinion on this point.

The discussion will also have to address the issues of the claimed amount and concentration of eculizumab in relation to the prior art.

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Wichtige Hinweise zur mündlichen Verhandlung

Das Europäische Patentamt verfügt über keine eigenen Dolmetscher. Diese müssen im Bedarfsfall von außerhalb, teilweise sogar aus anderen Ländern, beigezogen werden, was mit einem hohen Aufwand an Kosten und organisatorischen Vorbereitungen verbunden ist. Muss ein Verhandlungstermin kurzfristig abberaumt werden, können Kosten für bestellte Dolmetscher nicht mehr vermieden werden.

Es wird daher gebeten, eine Simultanübersetzung nur bei wirklichem Bedarf in Anspruch zu nehmen. Es wäre wünschenswert, wenn sich die Beteiligten (zweckmäßigerweise gleichzeitig mit der Terminabstimmung) auf die Benutzung einer Amtssprache einigen könnten. Bei Verständigungsschwierigkeiten sind die Mitglieder der Einspruchsabteilung bereit zu helfen.

Die von den Verfahrensbeteiligten bevorzugte (abgestimmte) Verhandlungssprache und ggf. eine notwendige Simultanübersetzung sind dem Amt möglichst vor der in Regel 4(1) EPÜ angegebenen Frist mitzuteilen.

☐ Verfahrenssprache ist **Deutsch**

Von der/dem/den Einsprechenden wurde

☐ Englisch

☐ Französisch benutzt.

Es wird um eilige Mitteilung - möglichst per Telefax an den zuständigen Formalprüfer - gebeten,

möglichst bis

Datum **19.11.2018**

1. welche Sprache(n) Sie in der mündlichen Verhandlung verwenden (**Sprechen**)
2. aus welcher Sprache Sie eine Simultanübersetzung benötigen (**Hören**).

Important information concerning oral proceedings

The European Patent Office has no interpreters of its own. When interpreters are needed they have to be brought in from outside, sometimes even from other countries, which is costly and involves considerable organisation. If oral proceedings have to be cancelled at short notice, the cost of interpreters already engaged still has to be borne.

Please therefore make use of simultaneous interpreting facilities only where strictly necessary. If possible the parties should agree on an official language for the proceedings, preferably at the time when they arrange a date. The members of the Opposition Division will be willing to help should any communication problems arise.

The EPO should be told if possible before the period mentioned in Rule 4 (1) EPC which language the parties prefer (agree on) and whether simultaneous interpreting facilities are required.

☒ Language of the proceedings is **English**

The language used by the opponent/s was

☐ German

☐ French.

Please inform us urgently - where possible by fax addressed to the formalities officer concerned -

if possible by

Date **19.11.2018**

1. which language(s) you intend to use during the oral proceedings (**Speaking**)
2. from which language you need simultaneous interpretation (**Listening**).

Très important Procédure orale

L'Office européen des brevets ne dispose pas de son propre service d'interprètes. Aussi faut-il appel le cas échéant à des interprètes de l'extérieur, qui viennent même parfois de l'étranger, ce qui occasionne de frais élevés et demande un grand travail d'organisation. Si la date d'une procédure orale doit être annulée au dernier moment, il n'est plus possible d'éviter les frais d'interprètes.

Les parties à une procédure sont donc priées de ne demander une traduction simultanée qu'en cas de réel besoin. Il serait souhaitable qu'elles puissent se mettre d'accord en même temps qu'elles conviennent de la date sur l'utilisation d'une langue officielle comme langue des débats. Si les parties éprouvent des difficultés de compréhension lors des débats, les membres de la division d'opposition sont disposés à leur prêter leur assistance.

L'Office doit être avisé si possible avant le début du délai mentionné dans la règle 4(1) CBE de la langue préférée par les parties pour le déroulement des débat (et sur laquelle elles se sont préalablement mises d'accord) et de la nécessité éventuelle d'une traduction simultanée.

☐ La langue de la procédure est le **français**

La langue utilisée par l'opposant/les opposants était

☐ l'allemand

☐ l'anglais.

Prière d'indiquer d'urgence à l'agent des formalités compétent si possible par téléfax

si possible jusqu'au

Date **19.11.2018**

1. quelle(s) langue(s) vous utiliserez au cours de la procédure orale (**pour parler**)
2. à partir de quelle langue vous aurez besoin d'une traduction simultanée (**pour écouter**).

Sollten Sie Ihren Antrag auf mündliche Verhandlung zurückziehen oder zum anberaumten Verhandlungstermin nicht erscheinen wollen bzw. aus wichtigem Grund daran gehindert sein, werden Sie gebeten,

- **unverzüglich das Amt - möglichst per Telefax - davon zu benachrichtigen, wobei das Schriftstück mit einem deutlichen Vermerk "Dringend, mündliche Verhandlung am ..." oder sinngemäß gekennzeichnet sein sollte;**
- **in dringenden Fällen** (weniger als 1 Monat vor dem Verhandlungstermin) zusätzlich **auch dem/die anderen Verfahrensbeteiligten** bzw. ihre(n) Vertreter auf schnellstem Weg **direkt zu unterrichten.**

In jedem solchen Fall obliegt der Einspruchsabteilung die Entscheidung, ob die Verhandlung durchgeführt oder abberaumt wird. Es wird jedoch darauf hingewiesen, dass einem Verfahrensbeteiligten, der eine nicht rechtzeitige oder unterbliebene Benachrichtigung zu verantworten hat, die dadurch den anderen Beteiligten verursachten Kosten auferlegt werden können (Art. 104 EPÜ).

Hinweis auf Regel 4 EPÜ

Regel 4
Sprache im mündlichen Verfahren

(1) Jeder an einem mündlichen Verfahren vor dem Europäischen Patentamt Beteiligte kann sich anstelle der Verfahrenssprache einer anderen Amtssprache des Europäischen Patentamts bedienen, sofern er dies dem Europäischen Patentamt spätestens einen Monat vor dem angesetzten Termin mitgeteilt hat oder selbst für die Übersetzung in die Verfahrenssprache sorgt. Jeder Beteiligte kann sich einer Amtssprache eines Vertragsstaats bedienen, sofern er selbst für die Übersetzung in die Verfahrenssprache sorgt. Von diesen Vorschriften kann das Europäische Patentamt Ausnahmen zulassen.

Should you decide to withdraw your request for oral proceedings or not wish to attend on the date set, or if for some special reason you are unable to do so, you are requested

- **to notify the EPO immediately, where possible by fax, marking the document clearly with the words "Urgent, oral proceedings on ..." or similar;**
- **in urgent cases** (less than one month before the date set for the proceedings), additionally to **notify the other party/parties** and/or their representative(s) **direct as rapidly as possible.**

In all such cases the Opposition Division will decide whether the proceedings are to go ahead or be cancelled. You should however note that costs incurred by the other parties may be charged to a party who either fails to notify them or does not do so in good time (Article 104 EPC).

Attention is drawn to Rule 4 EPC

Rule 4
Language in oral proceedings

(1) Any party to oral proceedings before the European Patent Office may use an official language of the European Patent Office other than the language of the proceedings, if such party gives notice to the European Patent Office at least one month before the date of such oral proceedings or provides for interpretation into the language of the proceedings. Any party may use an official language of a Contracting State, if he provides for interpretation into the language of the proceedings. The European Patent Office may permit derogations from these provisions.

Si vous retirez votre requête tendant à recourir à la procédure orale ou si vous ne souhaitez pas vous présenter à la date fixée pour la procédure orale ou ne pouvez vous y présenter pour une raison sérieuse, veuillez

- **en faire avis sans retard à l'Office, si possible par téléfax, en partant sur votre communication clairement la mention "Urgent, procédure orale le ..." ou une indication similaire;**
- **dans les cas urgents** (moins d'un mois avant la date fixée pour la procédure orale) **en faire avis également directement** pas la voie la plus rapide à **l'autre/aux autres partie(s)** ou bien à son/leurs mandataire(s).

Il appartient alors à la division d'opposition de décider si la procédure orale aura lieu ou non. Il est néanmoins souligné que les frais causés aux autres parties par une partie qui est responsable de l'omission d'un tel avis ou de ce que cet avis n'a pas été fait en temps utile peuvent être mis à la charge de cette partie (art. 104 CBE).

Rappel de la Règle 4 CBE

Règle 4
Langues admissibles lors de la procédure orale

(1) Toute partie à une procédure orale devant l'Office européen des brevets peut utiliser une langue officielle de l'Office européen des brevets autre que la langue de la procédure, à condition soit d'en aviser l'Office européen des brevets un mois au moins avant la date de la procédure orale, soit d'assurer l'interprétation dans la langue de la procédure. Toute partie peut utiliser une langue officielle de l'un des Etats contractants à condition d'assurer l'interprétation dans la langue de la procédure. L'Office européen des brevets peut autoriser des dérogations aux présentes dispositions.

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(2) Die Bediensteten des Europäischen Patentamts können sich im mündlichen Verfahren anstelle der Verfahrenssprache einer anderen Amtssprache des Europäischen Patentamts bedienen.

(2) In the course of oral proceedings, employees of the European Patent Office may use an official language of the European Patent Office other than the language of the proceedings.

(2) Au cours de la procédure orale, les agents de l'Office européen des brevets peuvent utiliser une langue officielle de l'Office européen des brevets autre que la langue de la procédure.

(3) In der Beweisaufnahme können sich die zu vernehmenden Beteiligten, Zeugen oder Sachverständigen, die sich in einer Amtssprache des Europäischen Patentamts oder eines Vertragsstaats nicht hinlänglich ausdrücken können, einer anderen Sprache bedienen. Erfolgt die Beweisaufnahme auf Antrag eines Beteiligten, so werden die Beteiligten, Zeugen oder Sachverständigen mit Erklärungen, die sie in einer anderen Sprache als in einer Amtssprache des Europäischen Patentamts abgeben, nur gehört, sofern dieser Beteiligte selbst für die Übersetzung in die Verfahrenssprache sorgt. Das Europäische Patentamt kann jedoch die Übersetzung in eine seiner anderen Amtssprachen zulassen.

(3) Where evidence is taken, any party, witness or expert to be heard who is unable to express himself adequately in an official language of the European Patent Office or of a Contracting State may use another language. Where evidence is taken upon request of a party, parties, witnesses or experts expressing themselves in a language other than an official language of the European Patent Office shall be heard only if that party provides for interpretation into the language of the proceedings. The European Patent Office may, however, permit interpretation into one of its other official languages.

(3) Lors de l'instruction, les parties, témoins ou experts appelés à être entendus, qui ne possèdent pas une maîtrise suffisante d'une langue officielle de l'Office européen des brevets ou d'un Etat contractant, peuvent utiliser une autre langue. Si la mesure d'instruction est ordonnée sur requête d'une partie, les parties, témoins ou experts qui s'expriment dans une langue autre qu'une langue officielle de l'Office européen des brevets ne sont entendus que si cette partie assure l'interprétation dans la langue de la procédure. L'Office européen des brevets peut toutefois autoriser l'interprétation dans l'une de ses autres langues officielles.

(4) Mit Einverständnis aller Beteiligten und des Europäischen Patentamts kann jede Sprache verwendet werden.

(4) If the parties and the European Patent Office agree, any language may be used.

(4) Sous réserve de l'accord des parties et de l'Office européen des brevets, toute langue peut être utilisée.

(5) Das Europäische Patentamt übernimmt, soweit erforderlich, auf seine Kosten die Übersetzung in die Verfahrenssprache und gegebenenfalls in seine anderen Amtssprachen, sofern ein Beteiligter nicht selbst für die Übersetzung zu sorgen hat.

(5) The European Patent Office shall, if necessary, provide at its own expense interpretation into the language of the proceedings, or, where appropriate, into its other official languages, unless such interpretation is the responsibility of one of the parties.

(5) L'Office européen des brevets assure à ses frais, en tant que de besoin, l'interprétation dans la langue de la procédure, ou, le cas échéant, dans ses autres langues officielles, à moins que cette interprétation ne doive être assurée par l'une des parties.

(6) Erklärungen von Bediensteten des Europäischen Patentamts, Beteiligten, Zeugen und Sachverständigen, die in einer Amtssprache des Europäischen Patentamts abgegeben werden, werden in dieser Sprache in die Niederschrift aufgenommen. Erklärungen in einer anderen Sprache werden in der Amtssprache aufgenommen, in die sie übersetzt worden sind. Änderungen einer europäischen Patentanmeldung oder eines europäischen Patents werden in der Verfahrenssprache in die Niederschrift aufgenommen.

(6) Statements by employees of the European Patent Office, parties, witnesses or experts, made in an official language of the European Patent Office, shall be entered in the minutes in that language. Statements made in any other language shall be entered in the official language into which they are translated. Amendments to a European patent application or European patent shall be entered in the minutes in the language of the proceedings.

(6) Les interventions des agents de l'Office européen des brevets, des parties, témoins et experts faites dans une langue officielle de l'Office européen des brevets sont consignées au procès-verbal dans cette langue. Les interventions faites dans une autre langue sont consignées dans la langue officielle dans laquelle elles sont traduites. Les modifications apportées à une demande de brevet européen ou à un brevet européen sont consignées au procès verbal dans la langue de la procédure.

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J A • K E M P

BY ONLINE FILING

The European Patent Office
Bayerstrasse 34
(entrance via Zollstrasse 3)
80335 Munich
Germany

14 January 2019

URGENT ORAL PROCEEDINGS 21 JANUARY 2019

Dear Sirs

Opposition to European Patent No. 2359834
(Application No. 11001632.6)
ALEXION PHARMACEUTICALS, INC.
Our Ref: C411599EP PXT/ALS/hdb

I write with reference to the oral proceedings scheduled for 21 January 2019 and request admission of a new document (referred to herein as D54) into the proceedings. This letter and the enclosed document are also being sent directly to the two Opponents.

D54 is a second declaration of Dr Miano which provides the results of an assay to determine the potency of aggregated eculizumab. This declaration is filed in direct response to Opponent 1's submission of 21 November 2018 which stated in paragraph 18 that:

> *"there is no evidence of a correlation between aggregation and loss-of-activity for this specific antibody!".*

It is the proprietor's position that the skilled person in the art would generally be aware that aggregation is detrimental to the activity of biologic drugs. For instance, aggregation can affect the availability of binding sites. It is therefore generally accepted in the art that aggregation should be avoided. Eculizumab is no exception to this general principle.

However, in case there is any doubt over this matter, Dr Miano reports in D54 the result of a study into the potency of aggregated eculizumab. This confirms that the aggregated form has 50% of the C5-binding activity of eculizumab itself.

We appreciate that this new document is being filed after expiry of the Rule 116 deadline. However, we request the Opposition Division's discretion in admitting this document into proceedings.

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In particular, this document directly responds to a point raised in the Rule 116 submission of Opponent 1. Dr Miano's report provides a straight-forward and clear demonstration of the relative potency of the aggregate and eculizumab. It is therefore prima facie relevant to the question raised by the Opponent, namely the relevance of aggregation to eculizumab activity.

Yours faithfully

Electronically Signed
AMANDA L SIMONS
ASSOCIATION NO. 811

J A • K E M P

21 November 2018

Our Ref: C411599EP

SUBMISSION UNDER R116

Patent No:	2359834
Application No:	11001632.6
Patentee:	Alexion Pharmaceuticals, Inc.
Opponents:	(1) Amgen Inc. (2) Hoffmann Eitle

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1 REQUESTS

1.1 The Patent Proprietor requests that the Opposition Division maintains the Patent as granted.

1.2 If the Opposition Division is not able to grant the Main Request, even after discussion at Oral Proceedings, then the Patent Proprietor requests that the patent is maintained on the basis of one of Auxiliary Requests 1 or 2 enclosed herewith. Also enclosed, to assist the Opposition Division, are tracked copies of Auxiliary Requests 1 and 2 showing the amendments which have been made compared with the Main Request.

1.3 It is further requested that, should any minor alterations be required to these Auxiliary Requests, an opportunity be provided to make such amendments at the Oral Proceedings.

1.4 We shall speak in English at the Oral Proceedings. We shall require interpretation into English should any other party speak in a language other than English.

1.5 We shall be accompanied at the hearing by Todd Spalding and Tara Rahemba of Alexion Pharmaceuticals, Inc and Dr Dino Miano, also of Alexion Pharmaceuticals, Inc and author of declaration D33. I request permission for Dr Miano to speak at the oral proceedings, should this be appropriate. Dr Miano's qualifications are set out in D33. Dr Miano may speak in relation to the commercial product, Soliris. Dr Miano may also discuss the subject matter contained in his declaration, in particular the stability data presented therein, as well as the content of Dr Treuheit's declaration D23.

2 DOCUMENTS

2.1 The following additional documents are cited and enclosed herewith:

D39 Declaration of Dr Alexander Klibanov.

D40 Rational Design of Stable Protein Formulations, Pharmaceutical Biotechnology Volume 13, 2002 Kluwer Academic/Plenium Publishers; Chapters 1, 5 and 8.

D41 Alberts *et al*, Molecular Biology of the Cell, Third Edition, 1994, page 585.

2.2 We also enclose a consolidated list of all the documents cited by both Opponents and the Patent Proprietor, including the additional documents mentioned above.

3 Declaration of Dr Klibanov and Inventive Step

3.1 Dr Klibanov is an expert in the formulation of therapeutic proteins, including antibodies. In his declaration, Dr Klibanov provides his expertise on the issues which the skilled person in this field would have faced around 2007, the filing date of the present patent. Dr Klibanov particularly comments on the complexities and challenges which the skilled person would have faced in 2007, when developing a therapeutic antibody formulation.

3.2 In the Summons, the Opposition Division summarise the arguments of the Patent Proprietor in the submission of 22 March 2018 and comment briefly on the Proprietor's position that formulating antibodies was a complex area. It is the Proprietor's position that the difficulties in antibody formulation in 2007 cannot be overestimated and it is considered important that the significance of these issues is fully appreciated. Accordingly, Dr Klibanov, who is an expert in this field and was working in this area in 2007, has been asked to provide his expert opinion.

3.3 The relevance of Dr Klibanov's declaration to the Patent Proprietor's position on inventive step will be explained further below with reference to the problem and solution approach:

3.4 The Opposition Division set out their view that D1 can be taken as the closest prior art document. The difference between the claimed subject matter and that of D1 is at least the following:

- The formulation is a single use 300mg dosage form;
- The concentration is 10 mg/ml;
- The volume is 30ml;
- The solution is preservative free.

3.5 As explained in detail in section 5.3 of the 22 March 2018 submission, the technical effect of the claimed composition is extremely good long term stability, such that the antibody maintains its integrity and biological activity in the composition. Further, the 10 mg/ml concentration provides this enhanced stability in a low-volume liquid formulation which is easy for the clinician to store and use.

3.6 Accordingly, the objective technical problem to be solved over D1 is to provide a composition of eculizumab, an antibody having a unique, engineered structure, which provides the ideal and necessary balance of properties for use in the treatment of PNH, including enhanced maintenance of biological integrity following prolonged storage, whilst also providing a low-volume liquid formulation.

3.7 The solution to this problem is the 300 mg single-use dosage form of eculizumab, comprising 30 ml of a 10 mg/ml preservative free solution.

3.8 The skilled person could not have predicted, at the filing date of the patent, that the 10 mg/ml composition would provide the required balance of low volume formulation combined with high stability. Indeed, as Dr Klibanov explains in his declaration at paragraph 4, achieving long term stability was a major challenge at the time:

"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."

3.9 Dr Klibanov comments that a skilled person faced with the task of formulating a new therapeutic protein would not have expected to be able to develop a liquid composition at all, due to these potential instabilities (see paragraph 6, D39). Dr Klibanov goes on to discuss the difficulties of balancing the various causes of protein instability, concluding in paragraph 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."

3.10 Dr Klibanov's view therefore confirms the unpredictability of the field. The Opponents suggest that the composition of eculizumab described in claim 1 would have been obvious, based on disclosures relating to formulations of different antibodies, in particular D20 and D21. Dr Klibanov's experience, however, demonstrates that this is not the case: as he confirms, in fact, each antibody needs to be considered individually. This requires a uniquely-designed, detailed research program to determine the ideal composition for a given antibody. A person skilled in the art would not have had any confidence that applying a formulation found to be successful for a different antibody would also be successful with eculizumab.

3.11 In particular, in paragraphs 8 and 9 of his declaration, Dr Klibanov explains that each of D20 and D21 referred to by the Opponents highlight the uniqueness of every antibody and the unpredictable nature of formulating a stable antibody composition. D20 teaches that *"each antibody seems to have a unique personality related to its requirements for stability"* (see

paragraph 9 of D39), and D21 stresses *"that one formulation excipient stabilizing a specific antibody may not be suitable for another because of the difference in their sequence"* (see paragraph 9 of D39).

3.12 Further, eculizumab itself would have presented particular difficulties to the skilled person in developing a suitable formulation. As set out in paragraph 3.6 above, the objective technical problem relates to providing a suitable formulation for eculizumab, which has a unique, engineered structure. Dr Klibanov explains at paragraphs 7 to 9 of D39 that even small structural differences between antibodies can lead to significant differences in behaviour in liquid compositions. For instance, at paragraph 7, Dr Klibanov refers to a 2002 text book:

"*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."*

3.13 The sensitivity of protein stability to primary sequence, which Dr Klibanov here refers to, would have been particularly relevant to eculizumab, given its uniquely-engineered structure.

3.14 Dr Klibanov's declaration therefore demonstrates that the skilled person in the art in 2007, faced with the issue of developing a pharmaceutical composition for eculizumab which provides the right balance of a low volume formulation with high stability, would not have been able to predict whether a liquid composition would be possible at all, let alone be able to predict, with a reasonable expectation of success, that a 10 mg/ml formulation in particular would provide the required balance of properties. For these reasons, the Proprietor maintains that the claimed subject matter is in accordance with Article 56 EPC.

4 Insufficiency

4.1 The skilled person would have had no difficulty in carrying out the invention as claimed, which is to provide a single-use dosage form of a sterile and preservative free solution comprising 30ml of a 10mg eculizumab/ml solution and to use it to treat PNH patients. This is because the full heavy and light chain sequences of eculizumab are disclosed in SEQ ID NOs: 2 and 4 in the patent, enabling the skilled person to produce eculizumab.

4.2 Although SEQ ID NO: 4 in the patent is the *immature* light chain sequence of eculizumab and thus contains the signal peptide, the signal peptide would be cleaved off during antibody production in the cell, and this would have been known to the skilled person as part of his common general

knowledge at the effective filing date (see, for example, D41, an extract from the 1994 textbook, Alberts *et al*). Therefore, the skilled person would have been able to make (the mature form of) eculizumab, and hence a single-use dosage form of a sterile and preservative free solution comprising 30ml of a 10mg eculizumab/ml solution, without an undue burden in 2007.

5 Auxiliary Requests

5.1 Auxiliary Request 1 specifies that administration is by intravenous infusion. Basis for this amendment can be found on page 22 of the application as filed. For instance, page 22 lines 18 to 20 indicate that intravenous administration is the most preferable route of administration, and page 22, line 32 also specifies intravenous infusion.

5.2 Auxiliary Request 2 specifies that administration is via IV infusion and that the composition is diluted to a final concentration of 5 mg/ml prior to administration. Basis for this amendment can be found at page 22, lines 27 to 29 of the application as filed.

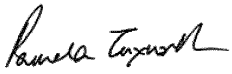
5.3 Each of these Auxiliary Requests is filed in order to address the arguments raised by the Opponents in relation to inventive step.

6 Summary

6.1 For the reasons discussed above and in our previous response, the Proprietor requests that the patent is maintained as granted and the oppositions are dismissed.

Signed





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Date
27.03.2019

Reference	Application No./Patent No.
	16172335.8 - 1112 / 3124029

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 05.09.19 at 13.00 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 05.07.19.

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:	Legally qualified member:
Collura, Alessandra	Rodríguez-Palmero, M	Orlando, Michele	Kley, Britta

Registered letter with advice of delivery
EPO Form 2008 09.18 [ORAL03=9999] (20/03/19)

to EPO postal service: 20.03.19

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For the Examining Division

Annexes:
Confirmation of receipt (Form 2936)
Communication (EPO Form 2906)

Samsung Bioepis Exhibit No. 1002
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The examination is being carried out on the **following application documents**

Description, Pages

1-4, 6-55	as originally filed	
5	filed in electronic form on	01-02-2019

Sequence listings, SEQ ID NO

1-10	as originally filed	

Claims, Numbers

1-11	filed in electronic form on	01-02-2019

Drawings, Sheets

1/4-4/4	as originally filed	

Reference is made to the documents already cited in the course of the procedure.

The applicant should also be aware of documents D8 and D9 filed by Third Parties on 12.12.2017 as well as of WO2005/074607 (D10) which is the publication pamphlet of EP1720571, referred to in D9.

Moreover, two further documents (D11 and D12), are referred to in the summons and will be discussed at the oral proceedings. A copy of the documents will be annexed to the present communication.

D1: US2005/191298

D2: Hillmen et al. (2003)

D3: Hillmen et al. (2004)

D4: Harding (2004)

D5: US2005/0221382

D6: Database Registry

D7: Thomas et al. (1996)

D8: Table of Alexion European patents and patent applications on eculizumab

D9: Submission of Alexion dated December 30, 2013 in opposition proceedings against EP1720571

D10: WO2005/074607

D11: US6355245 (cited in the application and by the applicant in his letter dated 01.02.2019)

D12: US2005/0271660 (cited in the opposition proceedings of granted EP2359834, based on EP 11 001 632.6)

All these documents as well as the submissions of the applicant made in the course of the procedure may form the basis of the discussion during the oral proceedings.

Due to the underlying discussion relating to the disclosure of the sequence for *eculizumab* the examining division has been enlarged under Article 18(2) EPC by a legally qualified member on 5 December 2018. The so enlarged examining division has decided to combine the present proceedings for reasons of procedural economy with the proceedings in the parallel case relating to EP 16 195 355.9 for the purposes of the oral proceedings. Both applications are divisional applications from EP 11 001 632.6, both examination proceedings are conducted by the same representatives for the same applicant before identically staffed examining divisions. In both proceedings the same fundamental problem arises which is decisive for both proceedings, namely the extent to which the prior art disclosing eculizumab has an impact on the patentability of the two applications pending before the examining division. After discussion of this fundamental issue, the proceedings will be dealt with successively, if necessary, during the same oral proceedings on the same day.

1. Articles 76(1) and 123(2) EPC

It has to be noted by the applicant that the first set of claims filed for the present application has been filed after the accorded date of filing and therefore they cannot be taken into account for providing basis for following amendments under Article 123(2) EPC.

Since the description of the invention is identical to the one of the parent application, the objections raised for Article 76(1) EPC in respect of the claimed subject-matter lead to analogous objections under Article 123(2) EPC.

The present application does not meet the requirements of Article 76(1) EPC and 123(2) for the following reasons.

1.1 With the amendments filed on 01.02.2019, the applicant amended page 5 of the description of the 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 the course of the procedure (see letters dated 01.02.2019 and 28.08.2018), 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 23.11.17, 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, neither in the original nor in the parent application a 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.

1.2 Present claim 7, depending also on claim 6, relates to the pharmaceutical composition for use according to claim 1 which comprises an emulsifier. Basis for this claim has been indicated in the application as originally filed (and in the parent application) on page 24, line 20. In the passage indicated by the applicant, the emulsifier is selected from a list of other excipients, such as wetting agents, lubricants, coloring agents and so on. In claim 6 the pH of the pharmaceutical composition is indicated as being 7.0. Basis for this value is indicated to be on page 24, lines 10-11. This feature as well derives from a selection of different pH values (at least the two ending points of the given pH range) for the claimed pharmaceutical

composition. Therefore, the subject-matter of claim 7 derives from the selection from two lists and is considered to violate the requirements of Articles 76(1) and 123(2) EPC.

1.3 Present claim 10 has been amended by the applicant to replace the term "eculizumab" with the expression "the antibody" in order to bring the claim in conformity with the subject-matter of independent claim 1. Basis for this amendment has been indicated to be on page 30, lines 22-24 of the application as originally filed. At the oral proceedings it will be discussed if the antibody claimed in independent claim 1 and eculizumab as referred to in the present application (see reference to D11) actually represent the same entity.

2. Novelty

The objections raised under point 1 above notwithstanding, the subject-matter of the present claims appears not to have been anticipated by any of the available prior art documents.

3. Inventive step

As already pointed out in the course of the procedure, D1-D4 all disclose eculizumab for use in the intravenous treatment of PNH in which patients are treated with a weekly 600 mg intravenous infusion for four weeks followed by 900 mg of the compound one week later and then 900 mg on a bi-weekly basis (see e.g. D1, paragraph 82; D2, left-hand column; D3, under the heading "Methods", and D4, page 675, left-hand column second full paragraph).

3.1 In his letter dated 01.02.2019, the applicant stated that D1 did not disclose the structure of eculizumab and that it referred to US6335245 (D11) and Thomas et al. (D7) for methods of its preparation. From D11 however it was not clear which of the humanized variables of 5G1.1 sequences should be attributable to eculizumab. The disclosure of D7 would lead the skilled person to use an IgG4 isotype antibody which however was not the antibody included in the claimed pharmaceutical composition.

In respect of this argument of the applicant the discussion at the oral proceedings will be associated to the discussion of the parallel case EP 16 195 355.9.

In particular the examining division has considered the following:

- Since D1-D4 are all publications referring to eculizumab and since in none of these documents it is 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 has 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 is made to D7, which discloses most of the sequence of the light and heavy chains of 5G1.1 (which is eculizumab, see D4) and explains how the sequence was found.

- 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 (see D9 relating to the opposition to 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.

- With a letter dated 28.08.2018, 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 argued 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 16.03.2007, 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.

Moreover, it has to be noted by the applicant that none of the documents cited in respect of the present application relate to clinical trials and therefore the list of the confidentiality agreements filed by the applicant with the declaration of Laural Boone does not apply to the present case.

For the reasons given above, the examining division concludes that the structure of eculizumab was available to the public at the filing date of the present application.

3.2 Should the applicant be able to prove to the examining division that the amendments carried out on the application in respect to the sequence of eculizumab represented the correction of an obvious mistake and should he succeed with the argument that the full correct sequence of eculizumab was not disclosed to the public prior to 16 March 2007, the correction of the sequence of eculizumab in CAS would still not be inventive compared to the available documents of the prior art disclosing eculizumab.

The difference between the disclosure of the prior art relating to eculizumab (e.g. D1-D4) and the present invention would lie in the sequence of the claimed compound. Apart from that, the present patent application and the the cited documents of the prior art are directed to the same purpose.

In this respect the applicant contends – and provides evidence – that “a sequence for eculizumab was submitted to Chemical Abstract Services (CAS) and entered into their STN database on 14 February 1999” under the CAS entry No. 219685-50-4. At the same time the applicant points out that the submission to CAS of 14 February 1999 was incorrect, even though it was made in good faith. It was not until 20 April

2009 that the applicant learnt of these errors in the sequence listing and requested correction of CAS Registry Number 219685-50-4 by providing complete and accurate amino acid sequences for eculizumab.

In detail, the initially submitted sequence contained a heavy chain which was longer than the actual one (i.e. 650 amino acids vs 448 amino acids) and the light chain contained one single error in the sequence, i.e. the inclusion of an arginine (R) residue at position 38 of the light chain sequence instead of a glutamine (Q).

It follows from this contention that the technical problem to be solved by the present application is the provision of the corrected sequence known to be associated to the name eculizumab in the cited prior art documents, e.g. D1-D4. However, the correction of an error in a registered sequence listing can hardly qualify as progress of technology or contribution to the art that does not already follow logically from the closest prior art. Rather, it remains to be a correction without own inventive character. As a matter of fact, at the filing date of the present application the skilled person had the knowledge to carry out the sequence of eculizumab (see teaching of D7).

The now not improved but simply corrected sequence is the contribution to technology that had already been available to the public for long before the filing date of the present application. Without additional technological process, there can be no finding of inventive step (see Guidelines Chapter VII-4) and eventually no monopoly right.

3.3 Having arrived at the above-mentioned conclusion on the availability of eculizumab to the public before the filing date of the present application, the difference between the subject-matter of present claim 1 and the disclosure of D1-D4 is represented by the pharmaceutical formulation specified in the claims and in particular in the following features:

- the pharmaceutical form is a single use dosage form comprising 300 mg of the antibody (i.e. eculizumab),

- the concentration of the antibody (i.e. eculizumab) is 10 mg/ml of the solution,

- the single-unit dosage comprises 30 ml of the solution, and

- the solution is preservative-free.

The fact that the solution is sterile is disclosed in D1, paragraph 62 which specifies that formulations for injection must be sterile and non-pyrogenic. The same paragraph teaches that the formulations may contain preservatives, thereby implying that their presence is optional.

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Page 3132

Concerning the dosage of the antibody in the dosage form, it is considered that since the dosages of eculizumab to be administered are 600 mg or 900 mg, it would be obvious for the skilled person to manufacture a formulation with 300 mg eculizumab, which would be the most practical to administer for achieving the indicated amounts.

Concerning the feature relating to the concentration of the antibody in the solution, it has to be noted by the applicant that (i) no specific technical effect has been shown to be linked to this feature of the pharmaceutical composition, and (ii) no document of the prior art addresses the problem of manufacturing eculizumab in a solution with a specific concentration.

Therefore, it is considered that the identification of a suitable concentration of an active agent in a solution would represent a basic skill of the person working in the field.

In addition to that, it has to be noted that D12 already describes solutions in which eculizumab is present in the indicated concentration. Figures 10 and 12 in relation to paragraphs 170 and 171, although relating to solutions for nebulization, describe formulations of eculizumab at concentrations between 1 and 30 mg/ml.

In the absence of any special technical effect of the claimed concentration and in the absence of any prejudice in the art to the formulation of eculizumab at the specific concentration claimed, the skilled person, faced with the problem of finding a unit dosage form of eculizumab with a suitable concentration, would have considered obvious to turn to the disclosure of D12 and would have arrived at the subject-matter of the present invention without any exercise of an inventive skill.

3.4 Taking into consideration all the above-mentioned arguments, the examining division is of the opinion that the presently claimed invention is not inventive (Article 56 EPC).

4. Article 83 EPC

The attention of the applicant is drawn to the fact that in case a positive conclusion on Article 56 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 01.02.2019, 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 arguments of the applicant, since (i) the sequences filed with the original application were not those identifying the structure of eculizumab, and (ii) 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. Double patenting

The attention of the applicant is drawn to the fact that EP 2 359 834 has been granted for subject-matter which is very similar to the one presently claimed. EP 2 359 834 has been revoked in the opposition procedure, but the decision of the opposition division is still appealable.

Should the applicant proceed to appeal with the above-mentioned related file and succeed, his attention is drawn to the Guidelines G-IV, 5.4 relating to double patenting.

6. 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.

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Date
27.03.2019

Reference	Application No./Patent No.
	16172335.8 - 1112 / 3124029
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

EPA/EPO/OEB Formblatt/Form/Formulaire : 2008

Empfangsbescheinigung über den Zugang des vorstehend bezeichneten Schriftstücks
Acknowledgement of receipt of the document specified above
Récépissé du document spécifié ci-dessus

Unter Bezugnahme auf die Mitteilung im ABl EPA 7/2010, 377 wird gebeten, die Empfangsbescheinigung mit Empfangsdatum und Unterschrift zu versehen und **umgehend** an das EPA zurückzusenden:

With reference to the Notice in OJ EPO 7/2010, 377, you are requested to date and sign the acknowledgement of receipt and return it to the EPO **immediately**:

Conformément au communiqué paru au JO OEB 7/2010, 377, vous êtes prié d'indiquer sur le récépissé la date de réception du document, de signer le récépissé et de le renvoyer **sans délai** à l' OEB:

- **über die Online-Dienste des EPA** (als Anlage zu EPA Form 1038) / **through EPO Online Services** (as annex to EPO Form 1038) / **par les services en ligne de l'OEB** (en tant que pièce jointe au formulaire OEB 1038),
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European Patent Office

80298 M U N I C H

EP 16 19 5355.9 – 1466
Alexion Pharmaceuticals, Inc.
Vossius Ref.: P2913 EP/3

München, July 10, 2017
WA/FLG/SOJ

I. COMMUNICATION PURSUANT TO RULE 69 EPC

Payment of the examination fee and the designation fee for all contracting states is effected with the enclosed sheet of Payment of fees and expenses (EPO Form 1010). Examination is herewith requested.

II. REPLY TO THE EESR PURSUANT TO RULE 70a(1) EPC

This is in reply to the extended European search report issued in the above-mentioned case on March 17, 2017.

1. INADMISSIBLE BROADENING (Article 76(1) EPC)

1.1. The Examiner is of the view that the subject-matter of claim 1 cannot be clearly and unambiguously derived from the parent application as filed.

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Germany: Partnerschaftsregister AG München PR 89
Switzerland: HR Firmennummer CH-270.9.001.271-9

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1.2. Claim 1 is directed to an antibody comprising the complete heavy chain of eculizumab (i.e. SEQ ID NO: 2) and the three light chain CDRs of eculizumab (i.e. SEQ ID NOs 8, 9 and 10).

1.3. The basis for pending claim 1 in the parent application as filed can be found on page 9, last paragraph of the parent application as filed which reads:

> "In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region comprises one or more CDR regions having an amino acid sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7, and wherein the light chain variable region comprises one or more CDR regions having an amino acid sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10. [...] In certain embodiments, the antibody that binds C5 or an active antibody 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."

On pages 44 and 45 of the apparent application as filed SEQ ID NOs NOs 1 to 10 are shown and it is described that all of them characterize the antibody eculizumab. Hence, in order arrive at claim 1 it is required to combine features of a broader embodiment and a narrower embodiment both characterizing the structure of eculizumab. As the Examining Division is aware, this is generally seen in the EPO as resulting in a so-called "intermediate generalisation".

In T 461/05 the Board of Appeal ruled that an intermediate generalisation has to be held distinct from a simple generalisation, since in the former case a definition of the invention in general terms forms part of the original disclosure. In accordance with the pertinent case law an intermediate generalisation can be accepted under Articles 123(2) and 76(1) EPC in the absence of any clearly recognisable functional or structural relationship among the features of the specific combination (see, for example, T 1067/97 and T 25/03), or if the extracted feature is not inextricably linked with those features (see T 714/00).

Moreover, the general "gold standard" (see G 2/10) for assessing compliance with Articles 123(2) and 76(1) EPC is that amendments to the claims can only be made within the limits of what a skilled person would derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of these documents as filed.

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1.4. In the present case the skilled person could be an immunologist. The structure of an antibody is part of the common general knowledge of an immunologist. As the Examining Division is aware, an antibody (monomer) is a "Y"-shaped molecular structure that consists of four polypeptide chains; two identical heavy chains and two identical light chains connected by disulfide bonds. Three variable loops each on the light and heavy chains are responsible for binding to the antigen. These loops are referred to as the complementarity determining regions (CDRs).

Thus, the skilled person knows that an antibody is composed of two different sequences, the light and heavy chain. These two chains are connected by disulfide bonds. The skilled person also knows that while the complete chains exhibit binding specificity for a particular antigen, the binding specificity is determined by the unique amino acid sequences found in the three hypervariable complementarity determining regions known as the CDRs.

Hence, the skilled person on the basis of the above-quoted last paragraph on page 9 of the parent application as filed arrives at the antibody of claim 1 comprising the heavy chain of eculizumab (i.e. SEQ ID NO: 2) and the three light chain CDRs of eculizumab (i.e. SEQ ID NOs 8, 9 and 10) without the requirement of extracting a feature being inextricably linked with other features. In line with T 714/00 this constitutes an allowable intermediate generalisation. It is of further note that since the light and heavy chain are separate there is no functional or structural relationship among these sequences that distracts a skilled person from directly and unambiguously deriving the antibody of claim 1. Thus, also in line with T 1067/97 and T 25/03 claim 1 constitutes an allowable intermediate generalisation.

In summary, it is our firm position that the claims find a clear and unambiguous basis in the parent application as filed as well as the application as filed.

2. NOVELTY AND INVENTIVE STEP (Articles 54 and 56 EPC)

2.1. The Examiner objects to the novelty of claims 1 to 3 in view of documents D1 to D5. The Examiner states that each of these documents describes eculizumab.

In view of the alleged lack of novelty the Examiner also contests inventive step of claims 1 to 3.

2.2. Because the use of eculizumab for the treatment of PNH is described in D1 to D5, the Examiner apparently assumes that eculizumab must have been publically available before the filing date of the present application.

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We respectfully note that this assumption is not correct because the mere naming of a drug such as eculizumab in a publication describing a confidential clinical trial does not place the biochemical structure in the prior art or in public use. As is evident from the enclosed declaration from the corresponding US proceedings by the inventor of the present application, founder and CEO of the Applicant Alexion Pharmaceuticals, Inc. Dr. Leonard Bell, prior to the March 15, 2007 effective filing date of the present application, the complete structure of eculizumab was not disclosed in the prior art. Also, eculizumab was never provided to a member of the public absent strict confidentiality and storage restrictions before March 15, 2007, as described in the enclosed declaration from Dr. Laural Boone that was submitted in the corresponding US proceedings..

Moreover, D1 to D5 fail to teach or suggest any part of the sequence of eculizumab, and in particular the unique, non-naturally occurring, protein-engineered full heavy chain of eculizumab (including the CH1-hinge-CH2-CH3 regions) of SEQ ID NO: 2. This engineered full heavy chain confers important and unexpected functional effects to eculizumab, as described in the declaration of Dr. Bell.

In more detail and as evidenced by the sequence alignment within the declaration, the heavy chain constant regions of eculizumab and a naturally-occurring IgG4 heavy chain differ in fourteen amino acid residues (i.e., four different residues in the CH1 domain, six different residues in the hinge region, and four different residues in the CH2 domain). Furthermore and as also discussed in the accompanying declaration, these structural differences confer eculizumab with advantageous functional features, including decreased effector function, reduced immunogenicity and increased half-life.

In sum, the complete structure of eculizumab as currently claimed – being an antibody characterized by the heavy of SEQ ID NOs 2 and the light chain CDRs of SEQ ID NOs 8, 9 and 10 – was not disclosed or otherwise made available to the public prior to the March 15, 2007 effective filing date of the present application. Therefore, prior to the effective filing date of the present application the complete structure of eculizumab as currently claimed in the present application was neither taught nor suggested in the cited prior art; nor was it obtainable by way of public availability.

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We therefore submit that the new claims are novel over D1 to D5. In view of the above-discussed unexpected and advantageous technical properties being associated with the complete structure of eculizumab, we submit that the claims are also inventive.

3. CORRECTION OF AN OBVIOUS ERROR (Rule 139 EPC)

Enclosed herewith is corrected page 44 of the application as filed and a corrected sequence listing, wherein an obvious error in the sense of Rule 139 EPC has been corrected in SEQ ID NOs 3 and 4. In more detail, the light chain of eculizumab as shown in SEQ ID NO: 4 as well as the corresponding shorter V_L sequence of eculizumab as shown in SEQ ID NO: 3 as originally filed erroneously comprise the N-terminal 22-amino acids long leader peptide which cannot be found in the mature light chain as comprised with the mature antibody. In the corrected SEQ ID NOs 3 and 4 the leader peptide has been removed.

We submit that the requested correction of SEQ ID NOs 3 and 4 complies with the prerequisites of Rule 139 EPC, because the skilled person in the field of antibodies immediately recognizes

(I) that SEQ ID NOs 3 and 4 are not correct, and

(II) that the correct SEQ ID NOs 3 and 4 are nothing else than SEQ ID NOs 3 and 4 without the N-terminal 22-amino acids long leader peptide; see Guidelines for Examination in the EPO, Part H – Chapter VI-2.2.

In this respect we refer the Examining Division to the enclosed declaration from the technical expert in the field of antibodies Dr. Bruce Andrien (Director, Early Assay Development, Alexion Pharmaceuticals, Inc.).

Regarding prerequisite (I), it can be taken from section 8 of the declaration that the skilled person immediately recognizes that SEQ ID NO: 4 is not correct, because (i) SEQ ID NO: 4 starts with methionine (M), (ii) M is followed by a stretch of 21 amino acids being mostly hydrophobic, and (iii) SEQ ID NO: 4 is with 236 amino acids considerable longer than typical mature antibody light chains of 211 to 217 amino acids. It was common textbook knowledge at the priority date in 2006 that the start codon of the genetic code (ATG) codes for the amino acid M and therefore most unprocessed protein sequences start with M. It

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was also commonly known that antibody light chains originally contain a leader peptide beginning with a methionine and followed by a hydrophobic core section (known as leader or signal petide) that is removed during protein processing and maturation. As regards the length of 211 to 217 amino acids of mature antibody light chains the declaration references the textbook Janeway et al., Immunology, 5th ed. (2001). The contents of textbook as well as handbooks, monographs, encyclopaedias and reference books establish the common general knowledge of the skilled person; see 8th ed. Of the Case Law book, section I.C.2.8.1, second paragraph. Hence, it is immediately recognisable for the skilled person, using common general knowledge, from the originally-filed application documents taken by themselves that SEQ ID NOs 3 and 4 are erroneous because they contain the signal peptide that is not present in the mature protein.

Prerequisite (II) is considered to be met if the correction is within the limits of what a skilled person derives directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the originally-filed application documents. As can be taken from sections 9 to 12 of the declaration of Dr. Andrien, the skilled person immediately recognizes that the correct SEQ ID NOs 3 and 4 are nothing else than SEQ ID NOs 3 and 4 without the N-terminal 22 amino acids long leader peptide, when considering the common general knowledge of the skilled person as being evidenced by the contents of the Kabat database in the last version as a textbook. The Kabat Database was first published as textbook in 1976 and the last edition in textbook form was published in 1991. Since then the Kabat database is accessible online. Since its establishment until today the Kabat database is extensively and foremost used by people skilled in the field of antibodies in order to derive structural and functional information on primary sequences of antibodies. As of its establishment, it was the most complete compilation of data in the antibody field. In the antibody field it had an equally high importance as the Maniatis and later Sambrook, "Molecular Cloning" textbook in the field of molecular cloning. It follows that the contents of the Kabat database and in particular the early contents as published in the format of a textbook are to be considered common general knowledge of the skilled person in the field of antibodies. Hence, it was common general knowledge of the skilled person at the priority date in 2006 that (i) leader peptides of kappa antibody light chains are usually 22 amino acids long

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and start with the amino acids "MDM" (see section 11 of the declaration), and (ii) that the mature form of kappa antibody light chains usually starts with the amino acids "DIQM" (see section 11 of the declaration), noting that also the leader peptide of eculizumab is 22 amino acids long and starts with the amino acids "MDM" and that also the mature antibody light chain of eculizumab starts with the amino acids "DIQM".

In summary, the requested correction of the antibody light chain of SEQ ID NO: 4 and consequently also the corresponding V_L sequence of SEQ ID NO: 3 are within the limits of what a skilled person can derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of the documents as filed; see also G 3/89 and G 11/91. We therefore submit that our request for the correction of SEQ ID NOs 3 and 4 under Rule 139 EPC is fully justified.

4. REQUESTS

With the above amendments and explanations it is submitted that the applicant has met the requirements as set forth in the EESR. It is requested that the adaptation of the description of the present application be deferred until an allowable set of claims is agreed upon.

If, however, the Examining Division does not agree to the above, it is requested that either a Communication pursuant to Article 94(3) EPC or a summons to attend oral proceedings according to Article 116(1) EPC be issued. If deemed expedient an informal interview is requested. The undersigned is prepared to discuss minor amendments over the phone.

Dr. Joachim Wachenfeld
European Patent Attorney

Enclosures:
Bell Declaration
Andrien Declaration (including Exhibits)
Boone Declaration
EPO Form 1010
Corrected Page 44
Corrected sequence listing (also on CD-ROM)

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Date
17.03.17

Reference	Application No./Patent No.
P2913 EP/3	16195355.9 - 1466
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Communication

The extended European search report is enclosed.

The extended European search report includes, pursuant to Rule 62 EPC, the European search report (R. 61 EPC) or the partial European search report/ declaration of no search (R. 63 EPC) and the European search opinion.

Copies of documents cited in the European search report are attached.

☑ 0 additional set(s) of copies of such documents is (are) enclosed as well.

The following have been approved:

☑ Abstract ☑ Title

☐ The Abstract was modified and the definitive text is attached to this communication.

The following figure(s) will be published together with the abstract:

Refund of search fee
If applicable under Article 9 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.

Should you wish to further prosecute this application in the examination phase, your attention is drawn to the provisions of Rule 70a EPC. An invitation to respond to the extended European search report will be issued once the date of publication of the European search report has been mentioned in the European Patent Bulletin (R. 69(1), R. 70(2) EPC).

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The examination is being carried out on the **following application documents**

Description, Pages

1-55 as originally filed

Sequence listings, SEQ ID NO

1-10 as originally filed

Claims, Numbers

1-3 as originally filed

Drawings, Sheets

1/4-4/4 as originally filed

Reference is made to the following documents; the numbering will be adhered to in the rest of the procedure.

D1 US 2005/191298 A1 (BELL LEONARD [US] ET AL) 1 September 2005 (2005-09-01)

D2 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, WILEY-BLACKWELL PUBLISHING LTD, GB,
vol. 121, no. Suppl.1, 1 May 2003 (2003-05-01), page 87, XP009144758,
ISSN: 0007-1048

D3 HILLMEN P ET AL: "EFFECT OF ECULIZUMAB ON HEMOLYSIS AND TRANSFUSION REQUIREMENTS IN PATIENTS WITH PAROXYSMAL NOCTRURNAL HEMAGLOBINURIA",
NEW ENGLAND JOURNAL OF MEDICINE, MASSACHUSETTS MEDICAL SOCIETY, BOSTON, MA, US,
vol. 350, no. 6, 5 February 2004 (2004-02-05), pages 552-559, XP009057948,
ISSN: 1533-4406, DOI: DOI:10.1056/NEJMOA031688

D4 HARDING J: "Eculizumab",
DRUGS OF THE FUTURE, PROUS SCIENCE, ES,
vol. 29, no. 7, 1 July 2004 (2004-07-01), pages 673-676, XP008113000,
ISSN: 0377-8282, DOI: DOI:10.1358/DOF.2004.029.07.819330

D5 HILL ANITA: "Eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria",
CLINICAL ADVANCES IN HEMATOLOGY & ONCOLOGY, MILLENNIUM MEDICAL PUB., NEW YORK, NY, US,
vol. 3, no. 11, 1 November 2005 (2005-11-01), pages 849-850, XP001538426,
ISSN: 1543-0790

D6 DATABASE REGISTRY [Online]
CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; 14 February 1999 (1999-02-14),
retrieved from STN accession no. 219685-50-4
Database accession no. 219685-50-4

Fro what concerns the most relevant passages of the above-mentioned documents, please see citations in the European Search Report, unless otherwise indicated.

1. Article 76(1) EPC

The present application does not meet the requirements of Article 76(1) EPC, because it relates to subject-matter which extends beyond the parent application as originally filed.

The applicant indicated page 5, last paragraph as basis for the claims of the application.

However, said paragraph puts into relation sequence 2 with sequence 4 and not with the specific sequences 8, 9 and 10. These last sequences are explicitly mentioned only in relation with specific sequences 5, 6 or 7.

2. Novelty

The present application does not meet the requirements of Article 54 EPC, because the subject-matter of the claims 1-3 is not novel.

The attention of the applicant is drawn to the fact that the present claims are directed to a product and are not limited in scope by any other features than those relating to the product itself.

The sequences indicated in the claims are told to be the sequences of the heavy and light chains of eculizuman or part of them.

Therefore, any document disclosing eculizumab published before the filing date of the present application obviously destroys the novelty of the present claims.

All documents cited in the search report refer to eculizumab and to the fact that it is an antibody which binds C5. D1-D5 also describe pharmaceutical compositions of the compound.

3. Inventive step

The present application does not meet the requirements of Article 56 EPC, because the subject-matter of the claims 1-3 is not inventive.

As a matter of fact, claims which are not novel cannot be considered to involve any exercise of an inventive activity.

4. Procedure

The applicant is invited to file new claims which take account of the above comments.

In order to facilitate the examination of the conformity of the amended application with the requirements of Article 123(2) EPC, the applicant should clearly identify the amendments carried out, irrespective of whether they concern amendments by addition, replacement or deletion, and to indicate the passages of the application as filed on which these amendments are based (see Guidelines E-II, 1).

The attention of the applicant is drawn to the fact that the application may not be amended in such a way that it contains subject-matter which extends beyond the content of the application as filed (Article 123(2) EPC).

Europäisches Patentamt
European Patent Office
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EUROPEAN SEARCH REPORT

Application Number
EP 16 19 5355

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	US 2005/191298 A1 (BELL LEONARD [US] ET AL) 1 September 2005 (2005-09-01) * the whole document * * paragraphs [0036], [0037], [0082] *	1-3	INV. A61K31/7105 A61K38/00 A61K39/395 A61P7/06
X	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, WILEY-BLACKWELL PUBLISHING LTD, GB, vol. 121, no. Suppl.1, 1 May 2003 (2003-05-01), page 87, XP009144758, ISSN: 0007-1048 * the whole document *	1-3	C07K16/18 A61K9/00
X,D	HILLMEN P ET AL: "EFFECT OF ECULIZUMAB ON HEMOLYSIS AND TRANSFUSION REQUIREMENTS IN PATIENTS WITH PAROXYSMAL NOCTURNAL HEMAGLOBINURIA", NEW ENGLAND JOURNAL OF MEDICINE, MASSACHUSETTS MEDICAL SOCIETY, BOSTON, MA, US, vol. 350, no. 6, 5 February 2004 (2004-02-05), pages 552-559, XP009057948, ISSN: 1533-4406, DOI: DOI:10.1056/NEJMOA031688 * abstract *	1-3	

-/--

TECHNICAL FIELDS SEARCHED (IPC)
A61K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	6 March 2017	Collura, Alessandra

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

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EUROPEAN SEARCH REPORT

Application Number
EP 16 19 5355

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	HARDING J: "Eculizumab", DRUGS OF THE FUTURE, PROUS SCIENCE, ES, vol. 29, no. 7, 1 July 2004 (2004-07-01), pages 673-676, XP008113000, ISSN: 0377-8282, DOI: DOI:10.1358/DOF.2004.029.07.819330 * the whole document * * page 674, left-hand column, paragraph 3 *	1-3	
X	HILL ANITA: "Eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria", CLINICAL ADVANCES IN HEMATOLOGY & ONCOLOGY, MILLENNIUM MEDICAL PUB., NEW YORK, NY, US, vol. 3, no. 11, 1 November 2005 (2005-11-01), pages 849-850, XP001538426, ISSN: 1543-0790 * the whole document *	1-3	
X	DATABASE REGISTRY [Online] CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; 14 February 1999 (1999-02-14), XP002767828, retrieved from STN accession no. 219685-50-4 Database accession no. 219685-50-4 * abstract *	1,2	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	6 March 2017	Collura, Alessandra

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

1

EPO FORM 1503 03.82 (P04C01)

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ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 16 19 5355

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.

06-03-2017

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2005191298 A1	01-09-2005	AU 2005209854 A1	18-08-2005
		CA 2554632 A1	18-08-2005
		CN 1938047 A	28-03-2007
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		SI 1720571 T1	30-10-2012
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		US 2012308559 A1	06-12-2012
		WO 2005074607 A2	18-08-2005

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

Information on Search Strategy - Pilot phase (see OJ 2015, A86)
The type of information contained in this sheet may change during the pilot for improving the usefulness of this new service.

Application Number
EP 16 19 5355

TITLE: TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

APPLICANT: Alexion Pharmaceuticals, Inc.

IPC CLASSIFICATION: A61K31/7105, A61K38/00, A61K39/395, A61P7/06, C07K16/18, A61K9/00

EXAMINER: Collura, Alessandra

CONSULTED DATABASES: WPI, EMBASE, MEDLINE, REG, CA

CLASSIFICATION SYMBOLS DEFINING EXTENT OF THE SEARCH:

IPC:

CPC:

FI/F-TERMS:

KEYWORDS OR OTHER ELEMENTS FEATURING THE INVENTION:
antibody that binds to C5 having the indicated heavy and light chain

EPO FORM P04A42

J A • K E M P

BY ONLINE FILING

The European Patent Office
Bayerstrasse 34
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80335 Munich
Germany

16 January 2018

Dear Sirs

European Patent Application No. 16195355.9
ALEXION PHARMACEUTICALS, INC.
Our Ref: N411599EP-B PXT

In response to the Communication pursuant to Article 94(3) EPC dated 17 November 2017, please replace the claims currently on file with the new claims attached herewith. Please also replace description page 5 with new description page 5 attached herewith.

To assist the Examiner, a tracked changes version of the claims and a tracked changes version of description page 5 showing the amendments being made are also attached.

Also attached are: (i) a copy of a letter from the Department of Health and Human Services to Alexion Pharmaceuticals dated 16 March 2007; (ii) a copy of a press release made by Alexion Pharmaceuticals on 16 March 2007; (iii) a Declaration of Stephen Saxe; and (iv) an Experimental Report.

Amendments

Claims 1 and 2 have been combined as new claim 1. An obvious error in referring to the whole of SEQ ID NO: 4 in the definition of the light chain of the antibody has been corrected.

A corresponding correction has been made at page 5 of the description.

Correction of Obvious Error

In the response of 10 July 2017 to the Search Opinion, correction of the sequence listing was requested. In the corrected sequence listing, the leader peptide was deleted from SEQ ID NO: 3 and SEQ ID NO: 4. The Examining Division has determined

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that this correction of the sequence listing is not allowable under Rule 139 EPC because it considers that there is no indication in the original application that SEQ ID NO: 3 and SEQ ID NO: 4 did not have to include the leader peptide.

Rather than correcting the sequence listing, we now request correction of the statements in the description that:

> "*In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region consists of SEQ ID NO: 1 and the light chain variable region consists of SEQ ID NO: 3*"; and
>
> "*In certain embodiments, the antibody that binds C5 or an active antibody 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*".

It would be immediately evident to the skilled person that an antibody light chain variable region could not consist of SEQ ID NO: 3 and that the light chain of an antibody could not consist of SEQ ID NO: 4.

As established by the Enlarged Board of Appeal in G3/89 and G11/91:

> "T*he part of a European patent application or of a European patent relating to the disclosure (the description, claims and drawings) may be corrected under Rule 88, second sentence, EPC [now Rule 139, second sentence, EPC] only within the limit of what a skilled person would derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of these documents as filed. Such a correction is of a strictly declaratory nature and thus does not infringe the prohibition of extension under Article 123(2) EPC." (Head note 1)*

Reading the present application, the skilled person would immediately recognise, based on his common general knowledge, that each of SEQ ID NO: 3 and SEQ ID NO: 4 contain a leader sequence as explained in our letter of 10 July 2017 and accompanying declaration of Dr Bruce Andrien.

The skilled person would also know that leader sequences are not present in an antibody ready for binding an antigen because these sequences are cleaved off during antibody maturation and production in cells. Therefore, it would be immediately evident to the skilled person that the references at page 5 of the description to an antibody comprising a light chain variable region consisting of SEQ ID NO: 3 and an antibody comprising a light chain consisting of SEQ ID NO: 4 represent an error.

As previously explained in our letter of 10 July 2017 and accompanying declaration of Dr Bruce Andrien, using his common general knowledge, the skilled person would have no difficulty determining which of the amino acids shown in SEQ ID NO: 3 and SEQ ID NO: 4 correspond to a leader peptide.

Therefore, the corrections being made to page 5 of the description to refer to the start and end positions of the light chain variable region and the light chain of the antibody would immediately be recognised by the skilled person as representing the intention of the Applicant. Accordingly, the correction of the light chain variable region and light chain sequences at page 5 of the description meets with the requirements of Rule 139 EPC.

It follows that the reference to the start and end positions of the mature part of the light chain sequence in SEQ ID NO: 4 in claim 1 complies with the requirements of Article 123(2) EPC.

Novelty

Claim 1 is novel because there was no public prior use of eculizumab before the filing date of the present application and no prior art document contains an enabling disclosure of eculizumab.

Eculizumab was not available on the market at the filing date

Contrary to the assertion in the Examination Report, none of D1-D5 indicate that eculizumab was a product available on the market prior to the filing date of 16 March 2007.

D1 is a US patent application which describes therapy trials to evaluate the effects of an anti-C5 antibody on the rare disease PNH and symptoms associated therewith. At page 9, paragraph [0082] of D1 it is stated that "*the specific anti-C5 antibody used in the study was eculizumab*". However, D1 does not say where eculizumab was obtained from, and does not indicate that eculizumab was commercially marketed or otherwise available to the public.

D2 is an abstract describing a clinical trial showing that eculizumab appears to be the first effective therapy for haemolysis in PNH. D2 discloses that "*eculizumab is a human monoclonal antibody that binds C5 and inhibits terminal complement activation*" (D2, lines 4-5). Like D1, D2 neither indicates from where eculizumab was obtained, nor does it indicate that eculizumab was commercially marketed or otherwise available to the public.

D3 is a paper describing the effect of eculizumab on haemolysis and transfusion requirements in patients with PNH. D3 states that: "*eculizumab is a recombinant humanised monoclonal antibody that was designed to block the activation of terminal complement components*". Again, no indication as to where eculizumab was obtained from is given, and there is no indication that eculizumab was commercially marketed or otherwise available to the public.

D5 is a report of an interview with Anita Hill relating to the use of eculizumab for the treatment of PNH. D5 does not provide any information as to how eculizumab may be obtained, nor does it suggest that eculizumab was commercially marketed or otherwise available to the public.

D4 is a paper describing eculizumab. D4 states that "*eculizumab (5G1.1) is a recombinant, fully humanised monoclonal antibody that inhibits the activation of*

terminal complement components" (D4, Abstract, first sentence). D4 indicates that the source of eculizumab is Alexion Pharmaceuticals, Inc. (US) (D4 page 6-7, right column, third paragraph), but it does not indicate that eculizumab was commercially marketed or otherwise available to the public.

Eculizumab was, in fact, not commercially marketed or otherwise available to the public at the publication dates of any of these references. As explicitly stated in the Declaration of Laural Boone, a Senior Director and Patent Counsellor at Alexion Pharmaceuticals, Inc. filed with our response of 10 July 2017, eculizumab was first approved for commercial marketing and sale on 16 March 2007, which is the day after the filing date of the present application (see paragraph 14 of the Boone Declaration).

The Boone Declaration is further supported by the attached letter from the Department of Health and Human Services to Alexion Pharmaceuticals dated 16 March 2007, licensing Alexion Pharmaceuticals to manufacture the product eculizumab. The attached press release made by Alexion Pharmaceuticals on 16 March 2017 announcing that it had, that day, received marketing approval from the U.S. Food and Drug Administration (FDA) for eculizumab.

Therefore, it is a matter of fact that eculizumab was not a product available on the market at the filing date of the present application.

The structure of eculizumab was not publically known at the filing date

None of the cited documents disclose the full amino acid sequence of eculizumab.

Eculizumab is an antibody that does not correspond to any naturally occurring isotypes. Rather, eculizumab has a unique, non-naturally occurring, protein-engineered full heavy chain (including the CH1-hinge-CH2-CH3 regions). This unique heavy chain sequence is not disclosed in the prior art.

D7 provides sequence information for an anti-C5 designated antibody h5G1.1. Eculizumab is derived from h5G1.1. However, D7 only discloses the heavy and light chain variable region (VH and VL) sequences of the antibody (see Figure 1 of D7). Significantly, D7 does not disclose the full, uniquely-engineered heavy chain sequence of eculizumab. There is also an amino acid difference between the light chain variable region sequence shown in D7 and the light chain variable region sequence of eculizumab. A glutamine (Gln/Q) residue is present at position 60 of SEQ ID NO: 4 but an arginine residue (Arg/R) is present at this position in the light chain variable region sequence shown in D7 (position 38 of the mature sequence of "h5G1.1 VLC" in Figure 1 of D7).

Thus, D7 does not disclose an antibody 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.

D1, D2 and D5 describe eculizumab by name only; they do not disclose the structure of eculizumab.

D3 and D4 each refer to Thomas et al. (1996) Mol. Immunol. 33: 1389-401 (reference 15 in D3) which is cited as D7 in the Examination Report of 17 November 2017. As

discussed above, D7 does not disclose the full heavy and light chain sequences of eculizumab. Therefore, D3 and D4 do not enable the skilled person to make eculizumab.

The Examining Division notes that D6 indicates that eculizumab was entered in the CAS registry database on 14 February 1999. However, D6 is a copy of the CAS registry database on 6 March 2017. D6 cannot therefore be used as evidence that SEQ ID NO: 2 and SEQ ID NO: 4 were available to the public ten years earlier at the 15 March 2007 filing date.

The sequence information for eculizumab submitted to the CAS Registry on 14 February 1999 was incorrect. The correct sequence information for eculizumab was not entered on the registry database until May 2018, which is after the filing date of the present application.

The attached Declaration of Stephen Saxe, and its annexes, show that the sequences for CAS Registry Number 219685-50-4 as shown in D6 were not available in the CAS Registry database until May 2009. In particular, Annex C, the email sent from Lois Bishop at CAS Client Services to Stephen Saxe on 1 May 2009 confirms that changes were made to the sequence for CAS Registry Number 219685-50-4 that would appear on STN a couple of days after that email was sent. Annex B, an email and letter sent from Stephen Saxe to the CAS, shows that the CAS Registry Number 219685-50-4 heavy chain sequence in the database prior to May 2009 was 650 amino acids in length, whereas SEQ ID NO: 2 is a 448 amino acid sequence, and that a single error was corrected in the light chain sequence (see paragraph 3 of the letter and point 3 of the Saxe Declaration).

Thus, the heavy chain sequence in the CAS database at the filing date of the present application contained hundreds of amino acids that are not present in SEQ ID NO: 2 and there is one amino acid difference between the light chain sequence in the CAS database at the filing date of the present application and residues 23 to 214 of SEQ ID NO: 4. Therefore, D6 is not evidence that an antibody comprising the heavy chain sequence shown in SEQ ID NO: 2 and the light chain sequence shown in residues 23 to 214 of SEQ ID NO: 4 was available to the public at the filing date of the present application.

In addition, as set out in the Declaration of Laural Boone, the heavy and light chain sequences of eculizumab (SEQ ID NOs: 2 and residues 23 to 214 of SEQ ID NO: 4 in the present application) were not made available to doctors or patients participating in any of the trials using eculizumab prior to at least 16 March 2007 (see paragraphs 6 and 7 of the Boone Declaration).

Thus, the full amino acid sequences of the heavy and light chain sequences of eculizumab were not disclosed in any of the cited documents.

The skilled person would not have been able to analyse the structure of eculizumab and reproduce it

In G1/92, the Enlarged Board of Appeal concluded that the chemical composition of a product is state of the art when the product as such is available to the public and can be analysed and reproduced by the skilled person. However, none of the studies

using eculizumab as described in D1-D5 made eculizumab available to the public such that the skilled person would have been able to analyse the structure of eculizumab and reproduce it.

As set out in the Declaration of Laural Boone, all physicians and participants in the eculizumab trials were required to maintain Alexion's confidential and/or proprietary information in strict confidence (see paragraphs 6 and 7 of the Boone Declaration). The clinical trials required strict controls to be placed over the use of eculizumab as explained in paragraphs 10 and 11 of the Boone Declaration. In particular, all eculizumab not administered to patients in the trials had to be accounted for (paragraph 10 of Boone Declaration).

Therefore, the skilled person would not have been able to obtain and analyse eculizumab to determine its structure, and would not have been able to reproduce eculizumab at the filing date of the present application.

Third Party Observations

The third party observations filed on 12 December 2018 are merely circumstantial and do not provide any evidence that eculizumab was publically available at the filing date of the present invention.

Summary

Although reports of trials describing eculizumab were available at the filing date of the present application, eculizumab was not made publically available until after the filing date. Eculizumab was not on the market at the filing date; the full structure of eculizumab was not published; and the skilled person would not have been able to analyse eculizumab and reproduce it.

Accordingly, there was no public prior use, public availability or enabling disclosure of eculizumab at the filing date. Claim 1 is therefore novel.

Inventive Step

The claimed antibody that binds C5 and comprises a heavy chain consisting of SEQ ID NO: 2 and light chain consisting of residues 23 to 214 of SEQ ID NO: 4 is inventive. In particular, SEQ ID NO: 2 is the full amino acid sequence of an engineered heavy chain that has a unique, non-naturally occurring protein engineered CH1-hinge-CH2-CH3 sequence. This unique sequence confers unexpected advantageous effects on the antibody eculizumab and to patients receiving eculizumab.

D7 may be taken to represent the closest prior art. D7 describes an antibody that has the same heavy chain variable region as the antibody of claim 1 and a light chain variable region sequence that differs from the variable region sequence of the antibody of claim 1 by just one amino acid position. The antibody of D7 is a human IgG4 isotype (see D7, page 1399, right column, line 27). The antibody of claim 1, however, is not an IgG4 isotype antibody, but has a unique, non-naturally occurring protein-engineered heavy chain.

The amino acid differences between a human IgG4 constant and hinge region and the constant and hinge regions of SEQ ID NO: 2 are shown in paragraph 5 of the Bell Declaration filed with the letter of 10 July 2017. As shown in paragraph 5 of the Bell Declaration, there are 14 amino acid differences between the IgG4 constant region and the engineered constant region of SEQ ID NO: 2.

The technical effect of these differences is that eculizumab does not bind as strongly to Fc gamma receptors as the IgG4 isotype antibody disclosed in D7 as demonstrated in the attached Experimental Report, which compares binding of eculizumab with that of the IgG4 antibody of D7 to the FCγ receptor IIB using an ELISA assay. Weakened binding to Fc gamma receptors reduces the generation of undesirable immune responses and thus improves the ability of the antibody to selectively shut down the terminal complement pathway (see paragraph 6 of the Bell Declaration) and increases plasma half-life of the antibody (see paragraph 7 of the Bell Declaration).

Accordingly, the objective technical problem in view of D7 may be defined as the provision of an improved anti-C5 antibody for therapeutic use in humans.

The solution to this problem provided by claim 1 would not have been obvious to the skilled person from D7. D7 teaches that the human IgG4 isotype was chosen because it does not activate human complement as there is only one known allotype of IgG4 and would likely preclude the development of auto-antibodies in patients (see D7 page 1399, right column, lines 27 to 31). There is no teaching or suggestion in D7 that the CH1-hinge-CH2-CH3 region of the heavy chain should be modified to further improve the properties of the antibody, let alone by engineering the exact fourteen amino acid substitutions present in SEQ ID NO: 2 into the IgG4 constant region.

None of the other documents on file provide any information about the structure of eculizumab. Accordingly, it would not have been obvious to the skilled person from any of these documents to protein engineer the heavy chain CH1-hinge-CH2-CH3 region to produce SEQ ID NO: 2. Accordingly, claim 1 involves an inventive step.

Sufficiency

The application provides a sufficient description of the claimed invention. SEQ ID NO: 2 is the complete heavy chain amino acid sequence of eculizumab, and residues 23 to 214 of SEQ ID NO: 4 represent the complete light chain amino acid sequence of eculizumab. Therefore, the complete structure of eculizumab is disclosed in the application.

In contrast, as discussed above, the full heavy and light chain sequences of eculizumab were not disclosed before the filing date of the present application. Thus, the skilled person reading any of D1 to D5 did not know the structure of the antibody that was actually tested and corresponded to the name “eculizumab.” However, in view of the inclusion of the full heavy chain sequence of SEQ ID NO: 2 and the full light chain sequence in SEQ ID NO: 4 (residues 23 to 214), the present application provides the first enabling disclosure of the antibody eculizumab.

Accordingly, the requirements of Article 83 EPC are met by the present application.

Concluding Remarks

It is believed that this application is now in an allowable form and the Examiner is asked to reconsider it favourably. If, however, there are any matters which remain outstanding, the Examiner is requested to issue a further Examination Report or telephone the undersigned. Merely as a precaution, in the event that the Examiner is minded to reject the application without first issuing a further Examination Report, oral proceedings are requested.

In addition, if the Examiner is minded to issue a Rule 71(3) Communication but wishes to propose amendments to the claims or description, it is requested that the Examiner telephones the undersigned to discuss this before the Rule 71(3) Communication is issued. I look forward to hearing from the Examiner in due course.

Yours faithfully

Electronically Signed
DR PAMELA M TUXWORTH
ASSOCIATION NO. 811

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Questions about this communication ?
Contact Customer Services at www.epo.org/contact

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Date
18-12-2017

Reference	Application No./Patent No.
	16195355.9 - 1466 / 3167888

Applicant/Proprietor
Alexion Pharmaceuticals, Inc.

Acknowledgment of receipt of observations by third parties (Article 115 EPC)

Receipt of your letter dated 12.12.2017 is hereby acknowledged.

Under Article 115 EPC you will not be a party to the proceedings before the European Patent Office.

☐ In your letter the following documents are mentioned which were not enclosed, and which are not available in the EPO:

☐ The third party observations have not been filed in an official language of the EPO (R. 114(1) EPC).

You are requested to file copy(ies) and/or translation(s) in one of the official EPO languages within **two months** of notification of this communication if they are to be taken into account.

For the Examining Division

Europäisches Patentamt • European Patent Office • Office européen des brevets

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Page 3159

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Date
18-12-2017

Reference	Application No./Patent No.
	16195355.9 - 1466 / 3167888
Applicant/Proprietor	
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Communication pursuant to Rule 114(2) EPC

Please find enclosed observations by a third party concerning the patentability of the invention of the above-mentioned patent application. That person is not a party to the proceedings before the EPO (Art. 115 EPC).

Under Rule 114(2) EPC you may comment on the observations.

For the Examining Division

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Page 3160

EPO - Munich
100
10. Juli 2017

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80298 M U N I C H

EP 16 19 5355.9 – 1466
Alexion Pharmaceuticals, Inc.
Vossius Ref.: P2913 EP/3

München, July 10, 2017
WA/FLG/SOJ

I. COMMUNICATION PURSUANT TO RULE 69 EPC

Payment of the examination fee and the designation fee for all contracting states is effected with the enclosed sheet of Payment of fees and expenses (EPO Form 1010). Examination is herewith requested.

II. REPLY TO THE EESR PURSUANT TO RULE 70a(1) EPC

This is in reply to the extended European search report issued in the above-mentioned case on March 17, 2017.

1. INADMISSIBLE BROADENING (Article 76(1) EPC)

1.1. The Examiner is of the view that the subject-matter of claim 1 cannot be clearly and unambiguously derived from the parent application as filed.

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1.2. Claim 1 is directed to an antibody comprising the complete heavy chain of eculizumab (i.e. SEQ ID NO: 2) and the three light chain CDRs of eculizumab (i.e. SEQ ID NOs 8, 9 and 10).

1.3. The basis for pending claim 1 in the parent application as filed can be found on page 9, last paragraph of the parent application as filed which reads:

> "In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region comprises one or more CDR regions having an amino acid sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7, and wherein the light chain variable region comprises one or more CDR regions having an amino acid sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10. [...] In certain embodiments, the antibody that binds C5 or an active antibody 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."

On pages 44 and 45 of the apparent application as filed SEQ ID NOs NOs 1 to 10 are shown and it is described that all of them characterize the antibody eculizumab. Hence, in order arrive at claim 1 it is required to combine features of a broader embodiment and a narrower embodiment both characterizing the structure of eculizumab. As the Examining Division is aware, this is generally seen in the EPO as resulting in a so-called "intermediate generalisation".

In T 461/05 the Board of Appeal ruled that an intermediate generalisation has to be held distinct from a simple generalisation, since in the former case a definition of the invention in general terms forms part of the original disclosure. In accordance with the pertinent case law an intermediate generalisation can be accepted under Articles 123(2) and 76(1) EPC in the absence of any clearly recognisable functional or structural relationship among the features of the specific combination (see, for example, T 1067/97 and T 25/03), or if the extracted feature is not inextricably linked with those features (see T 714/00).

Moreover, the general "gold standard" (see G 2/10) for assessing compliance with Articles 123(2) and 76(1) EPC is that amendments to the claims can only be made within the limits of what a skilled person would derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of these documents as filed.

1.4. In the present case the skilled person could be an immunologist. The structure of an antibody is part of the common general knowledge of an immunologist. As the Examining Division is aware, an antibody (monomer) is a "Y"-shaped molecular structure that consists of four polypeptide chains; two identical heavy chains and two identical light chains connected by disulfide bonds. Three variable loops each on the light and heavy chains are responsible for binding to the antigen. These loops are referred to as the complementarity determining regions (CDRs).

Thus, the skilled person knows that an antibody is composed of two different sequences, the light and heavy chain. These two chains are connected by disulfide bonds. The skilled person also knows that while the complete chains exhibit binding specificity for a particular antigen, the binding specificity is determined by the unique amino acid sequences found in the three hypervariable complementarity determining regions known as the CDRs.

Hence, the skilled person on the basis of the above-quoted last paragraph on page 9 of the parent application as filed arrives at the antibody of claim 1 comprising the heavy chain of eculizumab (i.e. SEQ ID NO: 2) and the three light chain CDRs of eculizumab (i.e. SEQ ID NOs 8, 9 and 10) without the requirement of extracting a feature being inextricably linked with other features. In line with T 714/00 this constitutes an allowable intermediate generalisation. It is of further note that since the light and heavy chain are separate there is no functional or structural relationship among these sequences that distracts a skilled person from directly and unambiguously deriving the antibody of claim 1. Thus, also in line with T 1067/97 and T 25/03 claim 1 constitutes an allowable intermediate generalisation.

In summary, it is our firm position that the claims find a clear and unambiguous basis in the parent application as filed as well as the application as filed.

2. NOVELTY AND INVENTIVE STEP (Articles 54 and 56 EPC)

2.1. The Examiner objects to the novelty of claims 1 to 3 in view of documents D1 to D5. The Examiner states that each of these documents describes eculizumab.

In view of the alleged lack of novelty the Examiner also contests inventive step of claims 1 to 3.

2.2. Because the use of eculizumab for the treatment of PNH is described in D1 to D5, the Examiner apparently assumes that eculizumab must have been publically available before the filing date of the present application.

We respectfully note that this assumption is not correct because the mere naming of a drug such as eculizumab in a publication describing a confidential clinical trial does not place the biochemical structure in the prior art or in public use. As is evident from the enclosed declaration from the corresponding US proceedings by the inventor of the present application, founder and CEO of the Applicant Alexion Pharmaceuticals, Inc. Dr. Leonard Bell, prior to the March 15, 2007 effective filing date of the present application, the complete structure of eculizumab was not disclosed in the prior art. Also, eculizumab was never provided to a member of the public absent strict confidentiality and storage restrictions before March 15, 2007, as described in the enclosed declaration from Dr. Laural Boone that was submitted in the corresponding US proceedings..

Moreover, D1 to D5 fail to teach or suggest any part of the sequence of eculizumab, and in particular the unique, non-naturally occurring, protein-engineered full heavy chain of eculizumab (including the CH1-hinge-CH2-CH3 regions) of SEQ ID NO: 2. This engineered full heavy chain confers important and unexpected functional effects to eculizumab, as described in the declaration of Dr. Bell.

In more detail and as evidenced by the sequence alignment within the declaration, the heavy chain constant regions of eculizumab and a naturally-occurring IgG4 heavy chain differ in fourteen amino acid residues (i.e., four different residues in the CH1 domain, six different residues in the hinge region, and four different residues in the CH2 domain). Furthermore and as also discussed in the accompanying declaration, these structural differences confer eculizumab with advantageous functional features, including decreased effector function, reduced immunogenicity and increased half-life.

In sum, the complete structure of eculizumab as currently claimed – being an antibody characterized by the heavy of SEQ ID NOs 2 and the light chain CDRs of SEQ ID NOs 8, 9 and 10 – was not disclosed or otherwise made available to the public prior to the March 15, 2007 effective filing date of the present application. Therefore, prior to the effective filing date of the present application the complete structure of eculizumab as currently claimed in the present application was neither taught nor suggested in the cited prior art; nor was it obtainable by way of public availability.

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We therefore submit that the new claims are novel over D1 to D5. In view of the above-discussed unexpected and advantageous technical properties being associated with the complete structure of eculizumab, we submit that the claims are also inventive.

3. CORRECTION OF AN OBVIOUS ERROR (Rule 139 EPC)

Enclosed herewith is corrected page 44 of the application as filed and a corrected sequence listing, wherein an obvious error in the sense of Rule 139 EPC has been corrected in SEQ ID NOs 3 and 4. In more detail, the light chain of eculizumab as shown in SEQ ID NO: 4 as well as the corresponding shorter V_L sequence of eculizumab as shown in SEQ ID NO: 3 as originally filed erroneously comprise the N-terminal 22-amino acids long leader peptide which cannot be found in the mature light chain as comprised with the mature antibody. In the corrected SEQ ID NOs 3 and 4 the leader peptide has been removed.

We submit that the requested correction of SEQ ID NOs 3 and 4 complies with the prerequisites of Rule 139 EPC, because the skilled person in the field of antibodies immediately recognizes

(I) that SEQ ID NOs 3 and 4 are not correct, and

(II) that the correct SEQ ID NOs 3 and 4 are nothing else than SEQ ID NOs 3 and 4 without the N-terminal 22-amino acids long leader peptide; see Guidelines for Examination in the EPO, Part H – Chapter VI-2.2.

In this respect we refer the Examining Division to the enclosed declaration from the technical expert in the field of antibodies Dr. Bruce Andrien (Director, Early Assay Development, Alexion Pharmaceuticals, Inc.).

Regarding prerequisite (I), it can be taken from section 8 of the declaration that the skilled person immediately recognizes that SEQ ID NO: 4 is not correct, because (i) SEQ ID NO: 4 starts with methionine (M), (ii) M is followed by a stretch of 21 amino acids being mostly hydrophobic, and (iii) SEQ ID NO: 4 is with 236 amino acids considerable longer than typical mature antibody light chains of 211 to 217 amino acids. It was common textbook knowledge at the priority date in 2006 that the start codon of the genetic code (ATG) codes for the amino acid M and therefore most unprocessed protein sequences start with M. It

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was also commonly known that antibody light chains originally contain a leader peptide beginning with a methionine and followed by a hydrophobic core section (known as leader or signal petide) that is removed during protein processing and maturation. As regards the length of 211 to 217 amino acids of mature antibody light chains the declaration references the textbook Janeway et al., Immunology, 5th ed. (2001). The contents of textbook as well as handbooks, monographs, encyclopaedias and reference books establish the common general knowledge of the skilled person; see 8th ed. Of the Case Law book, section I.C.2.8.1, second paragraph. Hence, it is immediately recognisable for the skilled person, using common general knowledge, from the originally-filed application documents taken by themselves that SEQ ID NOs 3 and 4 are erroneous because they contain the signal peptide that is not present in the mature protein.

Prerequisite (II) is considered to be met if the correction is within the limits of what a skilled person derives directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the originally-filed application documents. As can be taken from sections 9 to 12 of the declaration of Dr. Andrien, the skilled person immediately recognizes that the correct SEQ ID NOs 3 and 4 are nothing else than SEQ ID NOs 3 and 4 without the N-terminal 22 amino acids long leader peptide, when considering the common general knowledge of the skilled person as being evidenced by the contents of the Kabat database in the last version as a textbook. The Kabat Database was first published as textbook in 1976 and the last edition in textbook form was published in 1991. Since then the Kabat database is accessible online. Since its establishment until today the Kabat database is extensively and foremost used by people skilled in the field of antibodies in order to derive structural and functional information on primary sequences of antibodies. As of its establishment, it was the most complete compilation of data in the antibody field. In the antibody field it had an equally high importance as the Maniatis and later Sambrook, "Molecular Cloning" textbook in the field of molecular cloning. It follows that the contents of the Kabat database and in particular the early contents as published in the format of a textbook are to be considered common general knowledge of the skilled person in the field of antibodies. Hence, it was common general knowledge of the skilled person at the priority date in 2006 that (i) leader peptides of kappa antibody light chains are usually 22 amino acids long

and start with the amino acids "MDM" (see section 11 of the declaration), and (ii) that the mature form of kappa antibody light chains usually starts with the amino acids "DIQM" (see section 11 of the declaration), noting that also the leader peptide of eculizumab is 22 amino acids long and starts with the amino acids "MDM" and that also the mature antibody light chain of eculizumab starts with the amino acids "DIQM".

In summary, the requested correction of the antibody light chain of SEQ ID NO: 4 and consequently also the corresponding V_L sequence of SEQ ID NO: 3 are within the limits of what a skilled person can derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of the documents as filed; see also G 3/89 and G 11/91. We therefore submit that our request for the correction of SEQ ID NOs 3 and 4 under Rule 139 EPC is fully justified.

4. REQUESTS

With the above amendments and explanations it is submitted that the applicant has met the requirements as set forth in the EESR. It is requested that the adaptation of the description of the present application be deferred until an allowable set of claims is agreed upon.

If, however, the Examining Division does not agree to the above, it is requested that either a Communication pursuant to Article 94(3) EPC or a summons to attend oral proceedings according to Article 116(1) EPC be issued. If deemed expedient an informal interview is requested. The undersigned is prepared to discuss minor amendments over the phone.

Dr. Joachim Wachenfeld
European Patent Attorney

Enclosures:
Bell Declaration
Andrien Declaration (including Exhibits)
Boone Declaration
EPO Form 1010
Corrected Page 44
Corrected sequence listing (also on CD-ROM)

Publication Number	Title	Priority Date	Publication Date	Claims reciting "eculizumab"
EP2380907B1	METHODS AND COMPOSITIONS FOR THE TREATMENT OF ANTIBODY MEDIATED NEUROPATHIES	2006-09-05 2007-09-05	2016-11-30	6. The antibody or antigen-binding fragment thereof for use according to claim 5, wherein said antibody is eculizumab.
EP1988882B1	PROLONGATION OF SURVIVAL OF AN ALLOGRAFT BY INHIBITING COMPLEMENT ACTIVITY	2006-03-02 2007-03-02	2014-12-17	17. The anti-C5 antibody for use according to claim 13, wherein said whole antibody is eculizumab. 37. The use according to claim 34, wherein said antibody fragment is pexelizumab or said whole antibody is eculizumab.
EP1755674B1	PROLONGATION OF SURVIVAL OF AN ALLOGRAFT BY INHIBITING COMPLEMENT ACTIVITY	2004-05-14 2005-05-16	2014-11-19 (WO 2005/110481 published 11/24/2005)	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.
EP2061810B1	METHODS AND COMPOSITIONS FOR THE TREATMENT OF ANTIBODY MEDIATED NEUROPATHIES	2006-09-05 2007-09-05	2014-11-12	5. The antibody for use according to claim 1 or 2, wherein said antibody is eculizumab.
EP1720571B9	METHOD OF TREATING HEMOLYTIC DISEASE	2004-02-03 2005-02-03	2012-10-24 (A2 published 11-15-2006; WO 2005/074607A2 published 8-18-2005)	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). (All eight claims recite "eculizumab")
EP3056218A1	PROLONGATION OF SURVIVAL OF AN ALLOGRAFT BY INHIBITING COMPLEMENT ACTIVITY	2004-05-14 2005-05-16 2005-05-16	2016-08-17	10. The antibody to C5 according to claim 9 wherein said whole antibody is eculizumab.
EP2910253A1	METHOD OF TREATING HEMOLYTIC DISEASE	2004-02-03 2005-02-03 2005-02-03	2015-08-26	2. The anti-C5 antibody for use according to claim 1, wherein the anti-C5 antibody is eculizumab. 9. The use according to claim 8, wherein the anti-C5 antibody is eculizumab.
EP2815767A1	PROLONGATION OF SURVIVAL OF AN ALLOGRAFT BY INHIBITING COMPLEMENT ACTIVITY	2004-05-14 2005-05-16	2014-12-24	6. The composition according to claim 3, wherein the whole antibody is eculizumab.
EP2089058A2	METHODS OF TREATING HEMOLYTIC ANEMIA	2006-11-08 2007-11-08	2009-08-19	18. The method of claim 15, wherein said antibody is eculizumab. 34. The method of claim 32, wherein said antibody is eculizumab. 40. The method of claim 37, wherein said antibody is eculizumab. 59. The method of claim 56, wherein said antibody is eculizumab. 75. The method of claim 73, wherein said antibody is eculizumab. 100. The method of claim 97, wherein said antibody is eculizumab. 116. The method of claim 114, wherein said antibody is eculizumab. 122. The method of claim 119, wherein said antibody is eculizumab. 141. The method of claim 138, wherein said antibody is eculizumab. 157. The method of claim 155, wherein said antibody is eculizumab. 163. The method of claim 160, wherein said antibody is eculizumab.
EP2013235A1	METHODS FOR THE TREATMENT OF MUSCULAR DYSTROPHY ASSOCIATED WITH DYSFERLIN-DEFICIENCY	2006-05-01	2009-01-14	6. The method of claim 1, wherein said antibody is eculizumab.
EP1881993A2	NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DI5EASES	2005-05-11 2006-05-10	2008-01-30	7. The method of claim 1 , wherein the antibody is eculizumab or pexelizumab.

J A • K E M P

BY ONLINE FILING

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15 August 2018

Dear Sirs

European Patent Application No. 16195355.9
ALEXION PHARMACEUTICALS, INC.
Our Ref: N411599EP-B PXT/cxe

Thank you for the Summons to Oral Proceedings dated 7 May 2018. Our further submissions on the points raised by the Examining Division in the Summons are set out below. We attach the following documents:

- a second Declaration of Dr Bruce Andrien dated 15 August 2018;
- an extract from Alberts et al (1994);
- a copy of the Opposition statement filed against EP1720571 (D11);
- a redacted version of the representative clinical trial protocol C04-001; and
- a redacted version of the representative clinical trial protocol C02-002.

The comments below address each and every issue identified by the Examining Division in the Summons to Oral Proceedings. Given the early filing of this response in advance of the 5 February 2019 date set for Oral Proceedings, the Applicant would appreciate it if the Examining Division would consider the response as soon as possible and provide feedback, so that the Applicant has time to address any outstanding issues before the date set for filing final written submissions.

Articles 76(1) and 123(2) EPC

The amendment complies with Rule 139 EPC because a skilled person would have immediately recognised that the applicant intended to claim only the mature antibody.

The amendment of the description and claims to refer to an antibody having a light chain variable region consisting of residues 23-109 of SEQ ID NO: 3 and to an antibody having a light chain variable region consisting of residues 23-214 of SEQ ID NO: 4 meets the requirements of Articles 76(1) and 123(2) EPC.

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The Examining Division considers that this amendment is not a correction that complies with Rule 139 EPC because

> *"it cannot be excluded by the skilled reader that the SEQ ID NO: 3 and 4 as filed in the parent application as originally filed were actually those for which protection was sought."*

However, the skilled person would have immediately recognised that the applicant could not have intended to seek protection for an antibody comprising all of SEQ ID NO: 3 or all of SEQ ID NO: 4 because he would immediately have seen that these sequences contain a signal peptide (leader sequence) and would know that it is not possible to make a mature antibody that contains a signal peptide.

As established by the Enlarged Board of Appeal in G3/89 and G11/91:

> *"The part of a European patent application or of a European patent relating to the disclosure (the description, claims and drawings) may be corrected under Rule 88, second sentence, EPC [now Rule 139, second sentence, EPC] only within the limit of what a skilled person would derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of these documents as filed. Such a correction is of a strictly declaratory nature and thus does not infringe the prohibition of extension under Article 123(2) EPC." (Head note 1; emphasis added)*

It was common knowledge that immature heavy and light chains included a signal peptide at the N-terminus, and a skilled person could have readily identified the signal peptide in SEQ ID NOs: 3 and 4.

As addressed in the Andrien Declaration filed on 10 July 2017, it was common general knowledge in the art at the filing date of the application that a signal peptide is present at the N-terminus of immature heavy and light chain polypeptides. Kabat *et al.*, Sequences of Proteins of Immunological Interest, US Dept, Health and Human Services (1991) (Exhibits B to E filed with the Andrien Declaration on 10 July 2017), which is a database of biological sequences containing sequence alignments, is evidence of the skilled person's common general knowledge and shows the signal peptides of various human light chains.

Page 1 from Kabat *et al.* shows the 22-residue signal peptides of human kappa light chains. The first three residues are predominantly Met Asp Met and the last two residues are predominantly Arg Cys. In each of SEQ ID NO: 3 and SEQ ID NO: 4 of the patent, the first three residues are Met Asp Met and residues 21 and 22 are Arg Cys. Therefore, the skilled person would recognise that SEQ ID NO: 3 and SEQ ID NO: 4 are the amino acid sequences of the immature light chain variable region and the light chain, respectively, that contain a signal peptide that is not present in the mature antibody.

Furthermore, the skilled person would know from his common general knowledge that the framework of human kappa light chains commonly starts with the residues Asp Ile Gln Met as shown in the Table at pages 103 to 108 of Kabat *et al.* Residues 23

to 26 of SEQ ID NO: 4 are Asp Ile Gln Met, which the skilled person would recognise as being the start of the mature light chain of the disclosed antibody.

It is also part of the common general knowledge of the skilled person that antibodies are produced using cell-based recombinant methods, and not by artificially synthesising the polypeptide chains. The attached second Declaration of Dr Bruce Andrien dated 15 August 2018 refers to the textbook Alberts et al (1994) Molecular Biology of the Cell and explains that antibodies are processed by the producing-cell in the same way as other secreted proteins, and that this processing always results in the N-terminal signal peptide being cleaved off such that it is not present in the mature antibody.

Thus, during antibody production, the light chain polypeptide sequence of SEQ ID NO: 4, or of SEQ ID NO: 3, is processed to remove the signal peptide. Therefore, the skilled person would know based on his common general knowledge that it would not have been the intention of the Applicant to seek protection for an antibody that contains a signal peptide.

Accordingly, the correction to refer to an antibody comprising only the portion of SEQ ID NO: 3 or SEQ ID NO: 4 that reflects the mature antibody is an obvious correction in the sense that it is immediately evident that nothing else would have been intended than what is offered as the correction within the limit of what a skilled person would derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of the application as filed.

The corrections being made to page 5 of the description to refer to the portion of the light chain variable region or the light chain that would be found in the antibody would therefore immediately be recognised by the skilled person as representing the intention of the Applicant. Accordingly, the correction of the light chain variable region and light chain sequences at page 5 of the description meets with the requirements of Rule 139 EPC, Article 76(1) EPC and Article 123(2) EPC.

It follows that claim 1, which refers only to the portion of the light chain sequence shown in SEQ ID NO: 4 that is actually found in the mature antibody, also complies with the requirements of Article 76(1) EPC and Article 123(2) EPC.

The EPO has routinely accepted corrections of this exact type.

The European Patent Office has on other cases accepted that correction of applications defining an antibody as comprising heavy and light chain sequences including signal peptides to redefine the antibody as comprising only the mature proteins of the heavy and light chain sequences meets with the requirements of Rule 139 EPC. Two such examples include EP1558648, in which the correction allowed by the Examining Division was unsuccessfully challenged by the Opponent in an Opposition, and EP2581113.

Article 54 EPC

Eculizumab was not available to the public before the filing date of the present application.

The Examining Division has, after considering the arguments provided in relation to the question of whether eculizumab was publicly available at the filing date of the present application, expressed its opinion in paragraphs 1 to 4 of the Summons. Our comments on each of these points are set out below.

1. The fact that Alexion Pharmaceuticals received marketing authorisation for eculizumab from the FDA on 16 March 2007 means that eculizumab was not available on the market at the 15 March 2007 filing date of the present application. This appears to be accepted by the Examining Division. The Examining Division considers, however, that the antibody could still have been available to the public or that its structure could have been known before the priority/filing date of the present application. As discussed below, eculizumab was not publicly available and its structure was not publicly known at the filing date.

2. It is clear from Mr Saxe's Declaration that Alexion Pharmaceuticals inadvertently submitted the incorrect sequence of eculizumab to the Chemical Abstract Services (CAS) such that the sequence entered into the CAS STN database on 14 February 1999 did not contain the heavy and light chain sequences set out in claim 1 of the present application. It is irrelevant whether or not Alexion had any intention of keeping the substance secret after the date of submission to CAS. It is similarly also irrelevant whether anybody to whom the erroneous sequence in CAS would have been obvious could have contacted Alexion Pharmaceuticals to inquire about the correct sequence. Although Mr Saxe corrected the sequence submitted to CAS as soon as he became aware of the error, the fact remains that as of the filing date of the present application, the correct sequence of eculizumab was not available via CAS.

   It is completely alien to the concept of novelty at the EPO that a mistake in a document, the correction of which is not immediately obvious, can be treated as if the document disclosed the correct information. The fact is that the correct sequence was not disclosed in the CAS registry on 14 February 1999. What was *presumably intended to be disclosed* in the CAS registry on 14 February 1999 does not matter for purposes of assessing novelty.

   Not only was an incorrect sequence disclosed on 14 February 1999, but the corrections that would need to be made to the sequences entered on the CAS STN database on 14 February 1999 to arrive at the correct sequence of eculizumab as disclosed in SEQ ID NOs: 2 and 4 of the present application would not have been immediately obvious to the skilled person.

   Firstly, with regard to the light chain sequence, there was a single error in the sequence of the light chain that was submitted in 1999 as explained in the letter of 28 April 2009 from Dr Stephen Saxe to CAS, which letter was enclosed with the Saxe Declaration filed with our response of 16 January 2018. This error was the inclusion of an arginine (R) residue at position 38 of the light chain sequence instead of a glutamine (Q) residue at this position. It would not have been obvious to the skilled person that the inclusion of an arginine residue instead of a glutamine residue was an error. Indeed, as

shown in Figure 1B at page 1392 of D7, the 5G1.1 and h5G1.1 antibodies referred to therein contain an arginine (R) at position 38.

With regard to the heavy chain amino acid sequence, the sequence originally submitted and included in the STN database on 14 February 1999 was a 650 amino acid sequence, whereas the correct sequence has a length of only 448 amino acids. As shown in the annotated version of the incorrect heavy chain sequence originally submitted to CAS, which is included with the Saxe Declaration, the initially submitted sequence contains three blocks of amino acid residues that are not present in the eculizumab sequence. The first two blocks of amino acid sequences are inserted and the third block of amino acids substitutes a shorter stretch of amino acids present in eculizumab but missing in the incorrect sequence originally submitted to CAS. In addition, there are two single amino acids in the constant region of the heavy chain sequence that differ between the originally submitted sequence and the correct sequence. All of the errors in the originally submitted sequence would not have been obvious to the skilled person.

Even if the skilled person had recognised that the heavy chain sequence in the CAS STN database was unusually long for a heavy chain sequence and had turned to Thomas *et al*, 1996 (D7 in the present proceedings), which is the literature paper describing an antibody referred to as h5G1.1, he would have seen that the human IgG4 isotype was chosen to prepare a full length humanised 5G1.1 antibody in D7 (see D7, page 1396, sentence spanning left and right columns and page 1399, right column, lines 24 to 31). The skilled person would not have known that the heavy chain constant region intended to be submitted to CAS is not the IgG4 constant region used in D7.

The amino acid sequence of the IgG4 heavy chain in D7 differs from the heavy chain shown in SEQ ID NO: 2 of the present application and as correctly submitted to CAS in April 2009. The differences are illustrated at pages 2 to 3 of the Declaration of Dr Leonard Bell filed on 10 July 2017. There are 14 amino acid residues that differ between the IgG4 heavy chain constant region sequence and the heavy chain constant region sequence of eculizumab shown in SEQ ID NO: 2 of the present application. As shown in the Bell Declaration, there are four differences in the CH1 domain, six differences in the hinge region and four differences in the CH2 domain. Therefore, even if the skilled person would have turned to D7 to try and determine the correct sequence of eculizumab, D7 would have provided him with a very different sequence than that described in SEQ ID NO: 2.

In the third party observations filed 12 December 2017, attention was drawn to a statement made by the Patentee in the Opposition proceedings against EP1720571 in response to an allegation by the Opponent that the claims of that patent were insufficiently disclosed. A copy of the Opposition statement filed by Pfizer Inc against EP1720571 is attached as D11. It can be seen from Section 7.1 at pages 12 to 13 of this Opposition statement that the Opponent alleged that the complete sequence of eculizumab is not disclosed in U.S. Patent No. 6,355,245 or in Thomas *et al*, *Molecular Immunology*, Vol.33, No.17/18, pages 1389-1401, 1996 (D7 in the present proceedings).

In response to this objection, in paragraph 5.1.2 of the response filed on 30 December 2013, the Patentee stated that:

> *"The Patentee asserts that the sequence for eculizumab was publically available prior to the priority and filing date. A sequence for eculizumab was submitted to Chemical Abstract Services (CAS) and entered into their STN database on 14 February 1999; please see the attached copy of the CAS entry No. 219685-560-4"* [emphasis added].

The copy of the CAS entry No. 219685-50-4 that was attached with the response of 30 December 2013 did not include any sequence information. Only a one page fax dated 04/08/2008 (8 April 2008) from CAS was filed with the response (a copy of which was provided with the third party observations filed on 12 December 2017). As the sequence of eculizumab on file at CAS had not been corrected as of 8 April 2008, and certainly not as of the priority date of the opposed patent, this statement by the Patentee referenced an incorrect sequence of eculizumab and was clearly made in error. It is clear that this erroneous statement was made inadvertently and in good faith. Neither the third party observer nor the Examining Division has pointed to any evidence of bad faith in conjunction with the incorrect statement, and indeed none exists. Further, despite this unfortunate mistaken statement in the Opposition response, the fact remains that the full correct sequence of eculizumab was not publicly disclosed or publicly available before 16 March 2007.

It is also noted that Alexion's statement in the aforementioned opposition response was never challenged because the Opponent had withdrawn the Opposition on 7 October 2013. Nor was Alexion called on by the Opposition Division to provide evidence of the eculizumab sequences published by CAS in 1999. Indeed, sufficiency of disclosure was not further discussed in the Opposition proceedings on EP1720571. The assertion made by Alexion was not relied on in any way in those proceedings, and the statement had no influence on the outcome of the proceedings, in which EP1720571 was revoked for lack of inventive step. Thus, Alexion's mistaken statement amounted to nothing more than a mere assertion that turned out to be erroneous but that had no bearing on the outcome of that case. Alexion obtained no benefit as a result of the inadvertent misstatement.

The third party argued in paragraph I.3 of his observations of 12 December 2017 that:

> *"in light of Alexion's past actions, Alexion's declarations in the present case have to be considered to contravene against the generally accepted legal principle of good faith. In particular, in this case, the principle of venire contra factum proprium makes these declarations inadmissible"*.

However, the legal principle of *nemo potest venire contra factum proprium* does not apply in this situation.

The principle of *nemo potest venire contra factum proprium* has been applied by the EPO's legal Boards of Appeal where the EPO has exhibited contradictory behaviour, i.e. where the actions of the EPO have given rise to legitimate expectations which precluded the EPO from later going back on its own previous conduct (see for example, J27/94 and J14/94 summarised in Case law of the Boards of Appeal of the European Patent Office, 8th Edition, page 519, paragraph 2.3).

In the present case, the assertion made in the Opposition response was not relied on or considered any further in the Opposition proceedings. It did not affect the outcome of the Opposition proceedings in which the patent was revoked for lack of inventive step. Accordingly, the assertion made in the response to the opposition of EP1720571 does not take precedence over the signed declarations made in connection with the present application, and it does not bind Alexion Pharmaceuticals in any way.

The Examining Division considers that:

> *"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 CAS rules in order to have other patents be regarded as sufficiently disclosed".*

It appears that the Examining Division is attempting to apply the legal principle of *nemo potest venire contra factum proprium* in a different way than that suggested by the third party. Whilst the third party cited this legal principle in order to suggest that all of the declarations filed in the present application should be ignored, the Examining Division appears to consider that this principle would have bound Alexion Pharmaceuticals to disclose the correct sequence of eculizumab to anybody who requested it in order to have "*other patents be regarded as sufficiently disclosed*". It is presumed that the Examining Division is using the term "*other patents*" to refer to the patents and applications listed in D8 filed by the third party.

There are a number of points to be made here. Firstly, the legal principle of *nemo potest venire contra factum proprium* does not oblige a party who has submitted incorrect data to provide the correct data to any third party who requests it. Alexion is also unaware of what "CAS rules" the Examining Division refers to.

Secondly, the Examining Division seems to suggest that even though, in fact, the correct sequence of eculizumab was not disclosed before 16 March 2007, it must be considered to have been effectively disclosed so that the antibody defined using the term "eculizumab" used in previously-filed patents and patent applications listed in D8 is sufficiently disclosed. Clearly, this reasoning is legally flawed. Lack of novelty cannot be established based on an unsupported presumption of sufficiency of an earlier patent. Rather, the issue of novelty solely rests on the factual inquiry of whether or not the

claimed invention was in the public domain prior to the effective filing date. In the present case, it was not. Therefore, the claims are novel.

Moreover, all of the patents and patent applications listed in D8, with the exception of EP1720571 which was revoked for lack of inventive step, refer to eculizumab by name only in dependent claims (see the right column in the Table in D8). The independent claims of these patents and applications refer to anti-C5 antibodies more generally.

There is no doubt that anti-C5 antibodies were sufficiently disclosed in 2004, which is the year of the earliest priority date of any of the patents and patent applications listed in D8. For example, all of the patents and patent applications refer to US Patent 6,355,245 and many refer also to Thomas *et al*, 1996 (D7 in the present proceedings). These documents both disclose heavy and light chain variable region sequences of anti-C5 antibodies, and D7 also discloses a full length humanised IgG4 anti-C5 antibody.

In any event, as stated above, it is of no import whether disclosure of the full correct sequence of eculizumab would have been necessary in order for all of the claims of the earlier patents and patent applications to meet the sufficiency requirements of Article 83 EPC - the *fact* remains that the full correct sequence of eculizumab was *not* disclosed prior to 16 March 2007.

The Examining Division considers that:

> "*The statement from the Applicant acting as Patentee on 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*".

Again, this statement from the Patentee was an inadvertent error made in good faith. The issue of sufficiency was not further discussed in the course of the opposition and the assertion made by the Patentee was not challenged, the Opponent having withdrawn his Opposition on 7 October 2013. In the statement made in connection with the Opposition against EP1720571, Alexion Pharmaceuticals made no reference to SEQ ID NO: 2 or SEQ ID NO: 4, and did not make any admission that these particular sequences were publically available.

Also, the fact noted by the Examining Division that the date of the assertion in D9 (30 December 2013) is later than the date in which the correction of the eculizumab structure was requested to CAS is irrelevant to the discussion of novelty of the present application. The assertion cannot alter what was, or was not, disclosed at an earlier date.

The fact remains that the sequences submitted by Alexion Pharmaceuticals to CAS and entered into the CAS STN database on 14 February 1999 are not the sequences of SEQ ID NOs: 2 and 4 set out in claim 1 of the present application.

3. The Examining Division considers that 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"*.

However, this position is not in line with the case law. The Boards of Appeal have established that the mention of a compound in a published document does not mean that the compound is available to the public.

In T576/91, the Board of Appeal stated that if contractual obligations between parties resulted in access to biological material being deliberately restricted to a group of persons bound either by a research contract or a licence, it could not be concluded that this material had been made "*available to the public*" within the meaning of Article 54(2) EPC.

Further, in T128/92 the Board of Appeal stated that for a complex biochemical to be made available to the public, the minimum that would seem to be required for publication was a notice to those in the field that samples of the biochemical could be obtained upon request, and clear evidence of exactly what the biochemical was. (See Case law of the Boards of Appeal of the European Patent Office, 8th Edition, page 89, paragraph 3.2.5).

At page 2 paragraph 2.1(a) of the Summons, the Examining Division expresses the opinion that

> *"since D1-D5 are all publications referring to eculizumab and since in none of these documents it was stated that the products had not be available for public use or that the working group 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"*.

This conclusion is, however, clearly contrary to the case law discussed above. It cannot be concluded from a publication that an antibody was publicly available based merely on the absence of a statement that the antibody was *not* available. To the contrary, a positive statement to this effect would be required. No such statement is included in any of the cited documents.

The Examining Division has noted that different published patent applications belonging to the Applicant that mention "eculizumab" were available at the priority date of the present application. Such patents and applications are listed in D8 filed by the third party on 12 December 2017. None of these earlier applications, or the references they cite, disclose the full sequences of SEQ ID NO: 2 and SEQ ID NO: 4.

The mere existence of earlier patents and applications that mention "eculizumab" without precisely defining it does not destroy the novelty of

claim 1 of the present application. The present Examining Division is tasked with assessing the novelty of the claims of the present application in light of all of the *factual* information that was available in the prior art, and the fact is that none of the patents and applications listed in D8 disclose an antibody comprising the full heavy and light chain sequences of SEQ ID NO: 2 and SEQ ID NO: 4.

4. The Examining Division has requested proof of the confidentiality agreement between the company and each participant/physician involved in the studies described in D1 to D5. Dr Laural Boone, a Senior Director and Patent Counsel at Alexion, stated that she has reviewed the protocols of all of the clinical trials that were carried out with eculizumab prior to 16 March 2007 and found strict confidentiality and drug accountability provisions in each of the protocols. (See the Declaration of Dr Boone filed on 10 July 2017).

The Table at page 3 of Dr Boone's declaration lists all of the trials for which she reviewed the protocols, and paragraphs 7 and 8 of her Declaration provide examples of the confidentiality provisions she found in the protocols. The representative protocols from the studies Dr Boone refers to, C04-001 and C02-002, are now attached in redacted form. The confidentiality provision referred to in paragraph 7 of the Boone Declaration is found in paragraph 11.1 of protocol number C04-001, and the provision referred to in paragraph 8 of the Boone Declaration is found in the final paragraph at page 46 of study number C02-002.

In addition, as paragraph 10 of Dr Boone's declaration makes clear, the protocols also contained strict drug accountability provisions. The provisions referred to in paragraph 10 of Dr Boone's declaration are set out in paragraph 4.6 spanning pages 19 and 20 of protocol number C04-001, and in paragraph 6.6 at page 24 of study number C02-002. These provisions ensured that no study drug (eculizumab) was released to the public, and, as stated in the Boone Declaration, were included in all clinical trial protocols identified in the Declaration. This evidence demonstrates that all physicians and participants in the clinical trials were bound by strict confidentiality and drug accountability provisions so that there was no public disclosure or public availability of eculizumab via the trials.

Moreover, it should be noted that even if the participants and physicians involved in the studies had not had these strict conditions imposed on them by their agreements with Alexion Pharmaceuticals, the structure of eculizumab still would not have been publicly available, because, in accordance with G1/92, the skilled person would not have been able to analyse the structure of eculizumab and reproduce it without an undue burden.

In T2045/09 the Board considered whether the public sale of an antibody made that antibody publicly available in the sense that the skilled person would be able to determine its internal structure and reproduce it without unburden in accordance with G1/92.

In T2045/09, the Board noted that in a document published in 2006, the Authors emphasised that the sequencing of the variable regions of antibodies comprising the CDRs constituted a major scientific challenge and that:

> *"In particular, the unique character of the CDRs renders an alignment and the comparison of the data to already existing antibody sequences in databases impossible. A complete and sufficiently correct amino acid sequence could only be achieved by complementing the data of the Edman sequencing with sequence obtained independently from mass spectrometry".*

In view of the challenge the skilled person would have faced in trying to sequence an antibody in 2006, the Board was not persuaded that the skilled person could have correctly determined the amino acid sequence of the relevant antibody with the knowledge and the technologies available (see Reasons 35 and 36 of the Decision).

In the Summons at page 2, last paragraph, the Examining Division points to the official Communication dated 17 November 2017 in which it

> *"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".*

However, at least the uniquely-engineered, non-naturally-occurring portions of eculizumab were not disclosed in D7 and, as explained above as found in T0245/09, obtaining the full sequence of eculizumab from the eculizumab molecule alone would not have been routine in the art at the 15 March 2006 priority date of the present application.

2.2 The Examining Division maintains that the disclosure of D1-D5 teaching eculizumab and its use in the treatment of PNH are considered novelty destroying for the subject matter of the claimed invention. The claims of the present application are, however, novel in view of the disclosure of D1-D5.

Each of D1-D5 refers to eculizumab by name only. None of these documents disclose the heavy and light chain sequences of eculizumab. As discussed in paragraphs 2.1(3) above, the mere mention of a complex biochemical by name in a publication does not enable that complex biochemical to be made or make that complex biochemical available to the public. Therefore, the claims of the patent application are novel over D1-D5.

2.3 The present application is novel over the disclosure of D10. D10 is an earlier patent application of Alexion Pharmaceuticals (WO 2005/074607). This document does not provide an enabling disclosure for an antibody having the heavy and light chain sequences of SEQ ID NOs: 2 and 4.

WO 2005/074607 relates to methods of treating a hemolytic disease by administrating an anti-C5 antibody to a subject having a hemolytic disease (see claim 1 of D10). D10 discloses that:

> *"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 Humanised 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"* (page 18, lines 12 to 20).

Thus, D10 defines eculizumab by reference to U.S. Patent No. 6,355,245 and Thomas *et al*, 1996 (D7 in the present proceedings). Neither of these documents discloses the sequences of SEQ ID NOs: 2 and 4. Therefore an antibody having the heavy and light chain sequences of SEQ ID NO: 2 and SEQ ID NO: 4 is novel over D10.

The assertion made by the Applicant as Patentee in the response to the Opposition against EP1720571 derived from D10 cannot change what is disclosed in D10, and, as discussed above, is not an admission that SEQ ID NO:2 and SEQ ID NO: 4 were publically known at the filing date of EP1720571. The fact is that D10 does not disclose an antibody having the mature heavy and light chain sequences of SEQ ID NOs: 2 and 4, and nothing said later can change this. Therefore, the claims of the present application are novel over D10.

In conclusion, none of the prior art documents disclose an antibody having the sequences recited in claim 1, and such an antibody was not publically available in the sense of Article 54(2) EPC. Therefore, claim 1 is novel.

Article 56 EPC

The claims of the present application are novel for the reasons set out above. The claims also involve an inventive step in accordance with Article 56 EPC.

The claimed antibody that binds C5 and comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO: 4 is inventive.

SEQ ID NO: 2 is the full amino acid sequence of an engineered heavy chain that has a unique, non-naturally occurring protein engineered CH1-hinge-CH2-CH3 sequence. This unique sequence confers unexpected advantageous effects on the antibody eculizumab and to patients receiving eculizumab.

D7 may be taken to represent the closest prior art. D7 describes an antibody that has the same heavy chain variable region as the antibody of claim 1 and a light chain variable region sequence that differs from the variable region sequence of the antibody of claim 1 by just one amino acid position. The antibody of D7 is a human

IgG4 isotype (see D7, page 1399, right column, line 27). The antibody of claim 1, however, is not an IgG4 isotype antibody, but has a unique, non-naturally occurring protein-engineered heavy chain.

The amino acid differences between a human IgG4 constant and hinge region and the constant and hinge regions of SEQ ID NO: 2 are shown in paragraph 5 of the Bell Declaration filed with the letter of 10 July 2017. As shown in paragraph 5 of the Bell Declaration, there are 14 amino acid differences between the IgG4 constant region and the engineered constant region of SEQ ID NO: 2.

The technical effect of these differences is that eculizumab does not bind as strongly to Fc gamma receptors as the IgG4 isotype antibody disclosed in D7 as demonstrated in the attached Experimental Report, which compares binding of eculizumab with that of the IgG4 antibody of D7 to the FCγ receptor IIB using an ELISA assay. Weakened binding to Fc gamma receptors reduces the generation of undesirable immune responses and thus improves the ability of the antibody to selectively shut down the terminal complement pathway (see paragraph 6 of the Bell Declaration) and increases plasma half-life of the antibody (see paragraph 7 of the Bell Declaration).

Accordingly, the objective technical problem in view of D7 may be defined as the provision of an improved anti-C5 antibody for therapeutic use in humans.

The solution to this problem provided by claim 1 would not have been obvious to the skilled person from D7. D7 teaches that the human IgG4 isotype was chosen because it does not activate human complement as there is only one known allotype of IgG4 and it would likely preclude the development of auto-antibodies in patients (see D7 page 1399, right column, lines 27 to 31). There is no teaching or suggestion in D7 that the CH1-hinge-CH2-CH3 region of the heavy chain should be modified to further improve the properties of the antibody, let alone by engineering the exact fourteen amino acid substitutions present in SEQ ID NO: 2 into the IgG4 constant region.

None of the other documents on file provide any further information about the structure of eculizumab. It would therefore not have been obvious to the skilled person from any of these documents to engineer the heavy chain CH1-hinge-CH2-CH3 region to produce SEQ ID NO: 2.

Accordingly, claim 1 involves an inventive step.

Article 83 EPC

Regardless of the conclusions reached with respect to Articles 76(1) EPC, Article 123(2) EPC and Article 54 EPC, the claimed subject matter is sufficiently disclosed in the present application.

Claim 1 is directed to:

> *"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."*

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

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).

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:

(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

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:

(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

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.

- 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."*

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.

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VIA EPOLINE- Form 2300E

Paris, 12 March, 2013

OUR REF.: SR-01675
THP/IC

Re: European Patent No 1 720 571 B1 (ALEXION PHARMACEUTICALS, INC.)
Application No. 05712607.0
Opponent: PFIZER INC.

Dear Sirs,

In the name of Pfizer Inc., a Notice of Opposition is herewith submitted against the European Patent EP1720571 B1 (ALEXION PHARMACEUTICALS, INC.) entitled "Method of treating hemolytic disease".

It is requested that EP1720571 B1 be revoked in its entirety.

Statement under Rule 76 (c) EPC is attached hereto (Annex 1).

The fee for filing the opposition is paid by debit from deposit account with the EPO No. 28050037.

Yours faithfully,

Thomas PRINGOT
European Patent Attorney
Association Pfizer – No. 462
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Encls: - opposition statement (13 pages)
- cited documents D1-D12
- Decision T1979/09
- EPO Form 2300E

Europäisches Patentamt
European Patent Office
Office européen des brevets

Notice of opposition to a European patent

I. Patent opposed

Patent No.	EP1720571
Application No.	EP05712607.0
Date of mention of the grant in the European Patent Bulletin (Art. 97(3), Art. 99(1) EPC)	13 June 2012
Title of the invention	METHOD OF TREATING HEMOLYTIC DISEASE

II. Proprietor of the patent

first named in the patent specification	ALEXION PHARMACEUTICALS, INC.
Opponent's or representative's reference	SR-01675

III. Opponent

Name	Pfizer Inc.
Address:	235 East 42nd Street NEW YORK NY New York 10017 United States of America
State of residence or of principal place of business	United States of America
Multiple opponents (see additional sheet)	☐

IV. Authorisation

1. Representative

	Pfizer	
Department:	European Patent Department	
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Multiple representatives (see additional sheet)	☐	

Authorisation(s)

is/are enclosed ☐

has/have been registered under No. ☒ 35

V. Opposition is filed against

the patent as a whole ☒

claim(s) No(s).

VI. Grounds for opposition:

Opposition is based on the following grounds:

(a) the subject-matter of the European patent opposed is not patentable (Art. 100(a) EPC) because:

- it is not new (Art. 52(1); Art. 54 EPC) ☒
- it does not involve an inventive step (Art. 52(1); Art. 56 EPC) ☒
- patentability is excluded on other grounds, namely articles ☐

(b) the patent opposed does not disclose the invention in a manner sufficiently clear and complete for it to be carried out by a person skilled in the art (Art. 100(b) EPC; see Art. 83 EPC). ☒

(c) the subject-matter of the patent opposed extends beyond the content of the application/of the earlier application as filed (Art. 100(c) EPC, see Art. 123(2) EPC). ☒

VII. Facts (Rule 76(2)(c) EPC)

presented in support of the opposition are submitted herewith on an attached document ☒

VIII. Other 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.

IX. Evidence presented

-	Other evidence	T1979/09 original file name: T-1979-09.pdf attached as: Other-evidence-1.pdf
D1	Non-patent literature - article	Hillmen et al., "Effect of Eculizumab on Hemolysis and Transfusion Requirements in Patients with Paroxysmal Nocturnal Hemoglobinuria" The New England Journal of Medicine, Vol. 350(6), No. 6, 05 February 2004 particular relevance: pages 552-559 original file name: D1 - Hillmen New England.pdf attached as: Published-Evidence-1.pdf
D10	Non-patent literature - article	Thomas et al., "Inhibition of complement activity by humanized anti-C5 antibody and single-chain Fv." Molecular Immunology, Vol. 33, 1996 particular relevance: Pages 1389-1401 original file name: D10 Thomas et al 1996.pdf attached as: Published-Evidence-10.pdf
D11	Non-patent literature - article	Harding, "Eculizumab, Humanized Anti-C5 Monoclonal Antibody" Drugs of the Future, Vol. 29(7), 2004 particular relevance: Pages 673-676 original file name: D11 - Harding 2004.pdf attached as: Published-Evidence-11.pdf
D12	Non-patent literature - article	Hillmen et al., "Eculizumab, a C5 complement-blocking antibody, controls haemolysis in paroxysmal nocturnal haemoglobinuria (PNH) with responses maintained over a prolonged period of therapy" British Journal of Haematology, Vol. 125(1), 19 April 2004 particular relevance: Abstract 5 original file name: D12 - Hillmen 2004 BJH.pdf attached as: Published-Evidence-12.pdf
D2	Non-patent literature - article	Salodof MacNeil, Jane, "Eculizumab could be Paroxysmal Nocturnal Hemoglobinuria Breakthrough" Medscape Medical News, 13 December 2002 original file name: D2 - McNeil.pdf attached as: Published-Evidence-2.pdf
D3	Non-patent literature - article	Hillmen et al., "Eculizumab, a C5 complement blocking antibody, is the first therapy to reduce transfusion requirements in patients with paroxysmal nocturnal haemoglobinuria (PHN)" British Journal of Haematology, Vol. 121(1), 09 April 2003 particular relevance: Abstract 257 original file name: D3 - Hillmen - British Journal of Hemato - May 2003.pdf attached as: Published-Evidence-3.pdf
D4	Non-patent literature - article	Hillmen et al., "Eculizumab, a C5 Complement-Blocking Antibody, Abolishes Hemolysis and Renders Hemolytic Patients with Paroxysmal Nocturnal Hemoglobinuria (PNH) Transfusion Independent" Blood, Vol. 100;11, 16 November 2002 particular relevance: Abstract 154 original file name: D4 - Hillmen_Blood-2002.pdf attached as: Published-Evidence-4.pdf

D5	Non-patent literature - article	Hillmen et al., "Eculizumab, a C5 Complement-Blocking Antibody, Controls Hemolysis in Paroxysmal Nocturnal Hemoglobinuria (PNH) with Responses Maintained over a Prolonged period of Therapy" American Society of Hematology, Vol. 102;11, September 2003 particular relevance: Abstract 1858 original file name: D5 - Hillmen_Blood-102-2003.pdf attached as: Published-Evidence-5.pdf
D6	Non-patent literature - article	Rother et al., "Eculizumab, a C5 complement-blocking antibody, abolishes hemolysis and reduces transfusion dependency in patients with paroxysmal nocturnal hemoglobinuria (PNH)" Molecular Immunology, Vol. 40, September 2003 particular relevance: Abstract 54 original file name: D6 - Rother et al - Molecular Immunology (2003).pdf attached as: Published-Evidence-6.pdf
D7	Non-patent literature - article	Rosse et al., "Immune-mediated Hemolytic Anemia" American Society of Hematology - Education program, January 2004 particular relevance: pages 48-62 original file name: D7 - Rosse (2004).pdf attached as: Published-Evidence-7.pdf
D8	Non-patent literature - article	, "Clinics" Biotechnology News, Vol. 24(30), 17 December 2004 particular relevance: page 10 original file name: D8 - Clinics (2002).pdf attached as: Published-Evidence-8.pdf
D9	Patent document	US6355245 (B1) , 12.03.2002 original file name: D9 US6355245.pdf attached as: Published-Evidence-9.pdf

X. Payment

Mode of payment	Debit from deposit account

The European Patent Office is hereby authorised, to debit from the deposit account with the EPO any fees and costs indicated in the fees section below.

Currency:	EUR
Deposit account number:	28050037
Account holder:	Pfizer Limited

Refunds

Any refunds should be made to EPO deposit account:	28050037
Account holder:	Pfizer Limited

Fees	Factor applied	Fee schedule	Amount to be paid
010 Opposition fee	1	745.00	745.00
Total:		EUR	745.00

A	Forms	Details:	System file name:
A-1	Form for notice of opposition		ep-oppo.pdf

B	Attached document files	Original file name:	System file name:
B-1	1. Facts and arguments	2013-03 opposition statement.pdf	OPPO.pdf
B-2	1. Any annexes (other than citation) to an opposition letter - Opposition letter	SR-01675-Notice of oppo.pdf	OTHER-1.pdf

C	Attached evidence files	Original file name:	System file name:
C-1	1. Patent document	D9 US6355245.pdf	Published-Evidence-9.pdf
C-2	1. Non-patent literature - article	D1 - Hillmen New England.pdf	Published-Evidence-1.pdf
C-3	2. Non-patent literature - article	D2 - McNeil.pdf	Published-Evidence-2.pdf
C-4	3. Non-patent literature - article	D3 - Hillmen - British Journal of Hemato - May 2003.pdf	Published-Evidence-3.pdf
C-5	4. Non-patent literature - article	D4 - Hillmen_Blood-2002.pdf	Published-Evidence-4.pdf

C-6	5. Non-patent literature - article	D5 - Hillmen_Blood-102-2003.pdf	Published-Evidence-5.pdf
C-7	6. Non-patent literature - article	D6 - Rother et al - Molecular Immunology (2003).pdf	Published-Evidence-6.pdf
C-8	7. Non-patent literature - article	D7 - Rosse (2004).pdf	Published-Evidence-7.pdf
C-9	8. Non-patent literature - article	D8 - Clinics (2002).pdf	Published-Evidence-8.pdf
C-10	9. Non-patent literature - article	D10 Thomas et al 1996.pdf	Published-Evidence-10.pdf
C-11	10. Non-patent literature - article	D11 - Harding 2004.pdf	Published-Evidence-11.pdf
C-12	11. Non-patent literature - article	D12 - Hillmen 2004 BJH.pdf	Published-Evidence-12.pdf
C-13	1. Other evidence	T-1979-09.pdf	Other-evidence-1.pdf

Signature of opponent or representative

Place	**Paris**
Date:	**12 March 2013**
Signed by:	**/Thomas PRINGOT/**
Association:	**462**
Capacity:	**(Representative)**

US006355245B1

(12) **United States Patent**
Evans et al.

(10) **Patent No.: US 6,355,245 B1**
(45) **Date of Patent: Mar. 12, 2002**

(54) **C5-SPECIFIC ANTIBODIES FOR THE TREATMENT OF INFLAMMATORY DISEASES**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/487,283**

(22) Filed: **Jun. 7, 1995**

Related U.S. Application Data

(63) Continuation of application No. PCT/US95/05688, filed on May 1, 1995, which is a continuation-in-part of application No. 08/236,208, filed on May 2, 1994, now Pat. No. 6,074,642.

(51) **Int. Cl.**[7] **A61K 39/395**; C07K 16/36; C12N 5/12

(52) **U.S. Cl.** ..... **424/145.1**; 424/130.1; 424/133.1; 424/135.1; 424/141.1; 424/145.1; 424/158.1; 424/139.1; 424/130; 530/387.1; 530/387.3; 530/387.9; 530/388.1; 530/388.23; 530/388.25; 530/388.7; 435/326; 435/328; 435/331; 435/33 L; 435/337; 435/343; 435/346

(58) **Field of Search** 530/387.1, 387.3, 530/388.1, 388.7, 387.9, 388.23; 435/69.1, 172.3, 328, 343, 70.21, 325, 331, 332, 346; 424/130.1, 141.1, 139, 133.1, 145.1

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Primary Examiner—Phillip Gambel

(57) **ABSTRACT**

The use of anti-C5 antibodies, e.g., monoclonal antibodies, to treat glomerulonephritis (GN) is disclosed. The administration of such antibodies at low dosage levels has been found to significantly reduce glomerular inflammation/enlargement and other pathologic conditions associated with GN. Also disclosed are anti-C5 antibodies and anti-C5 antibody-encoding nucleic acid molecules. These antibodies are useful in the treatment of GN and other inflammatory conditions involving pathologic activation of the complement system.

23 Claims, 19 Drawing Sheets

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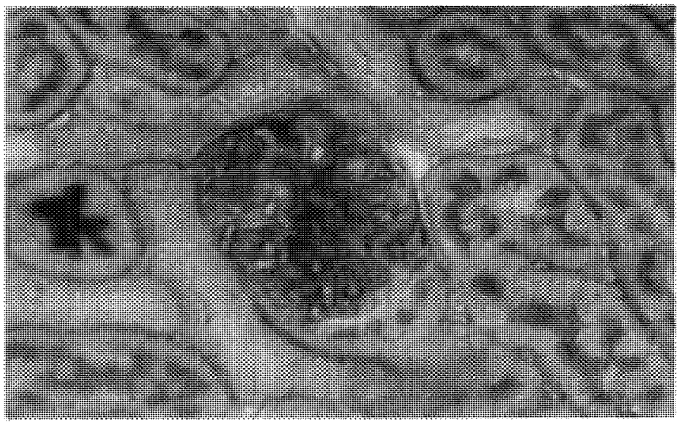


FIG. 1A

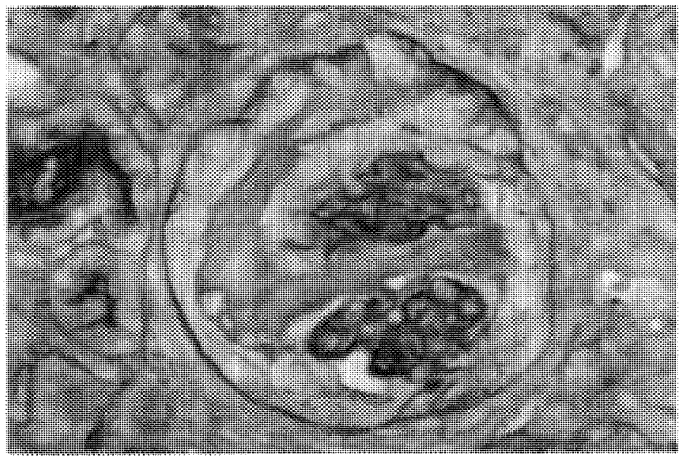


FIG. 1B

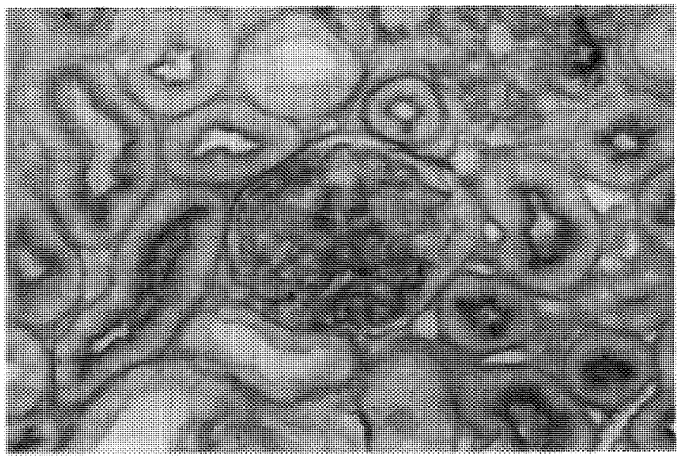


FIG. 1C

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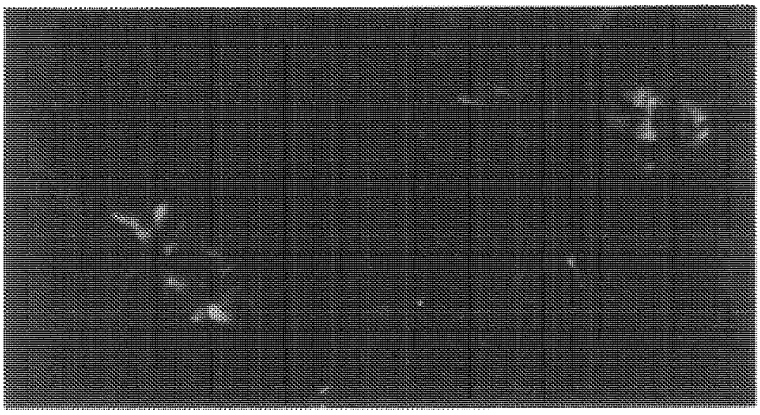


FIG. 2A

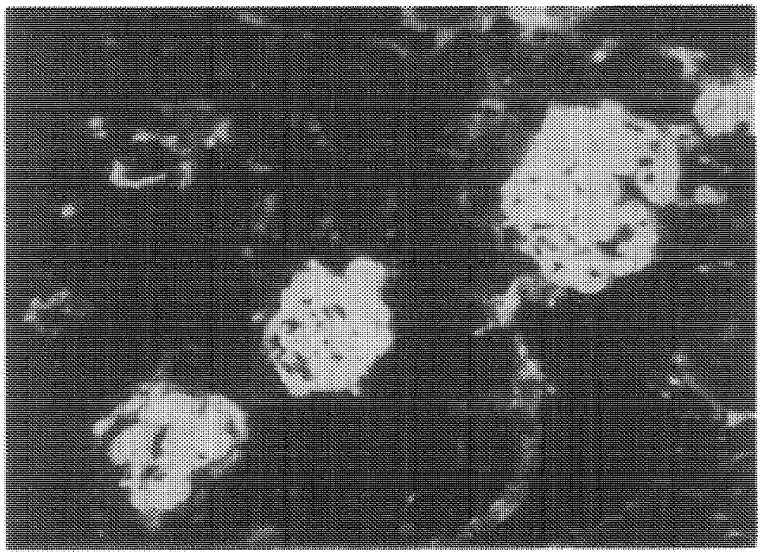


FIG. 2B

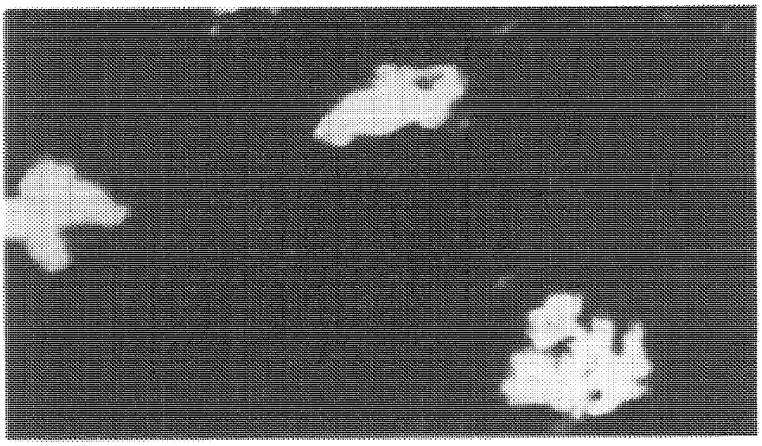


FIG. 2C

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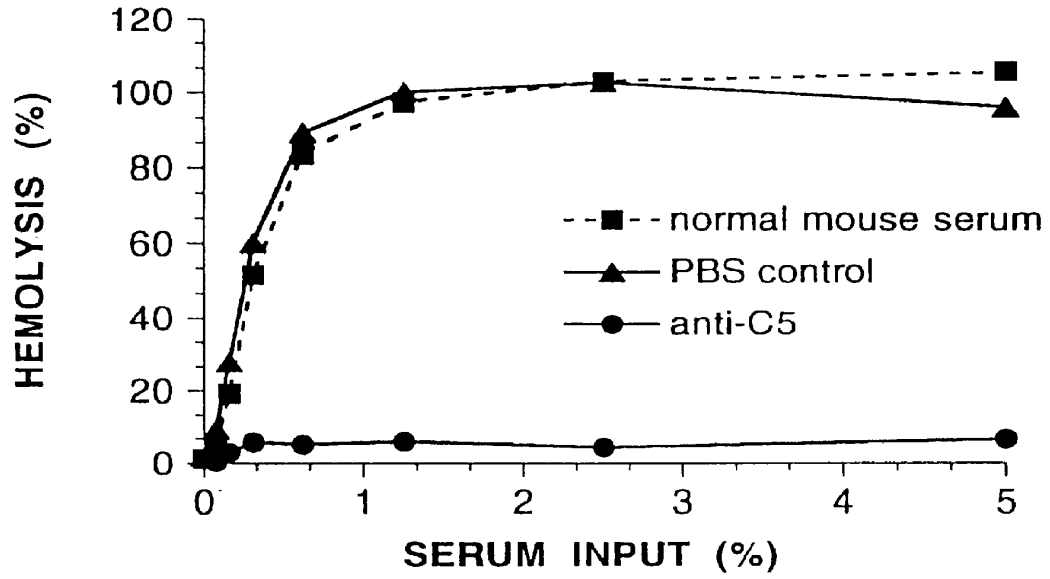


FIG.3

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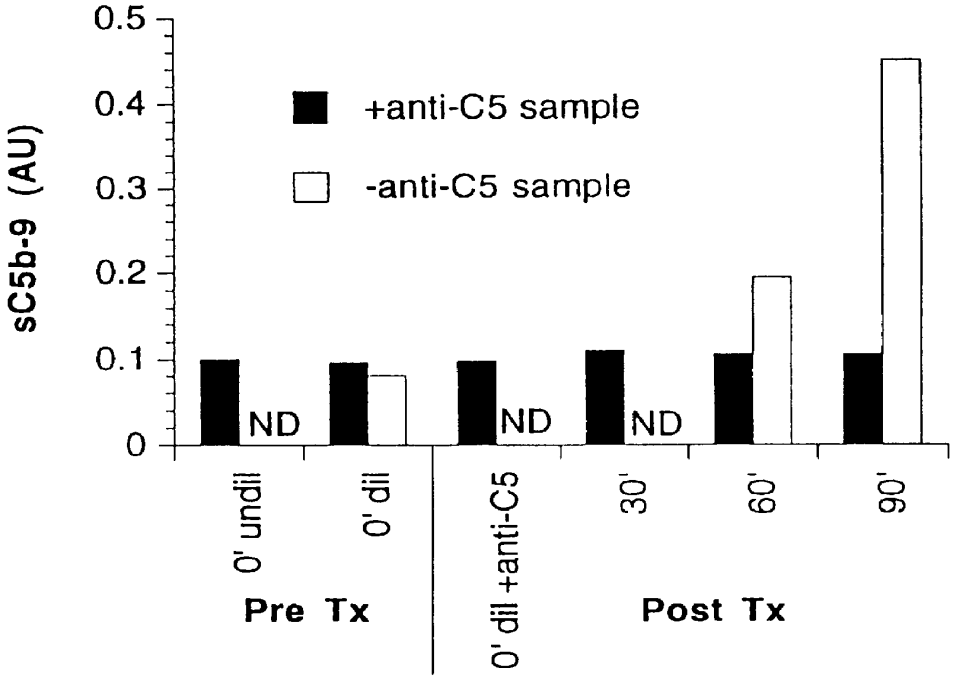


FIG.4

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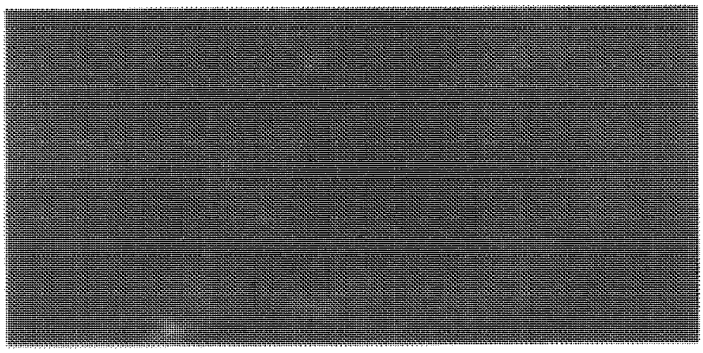


FIG.5A

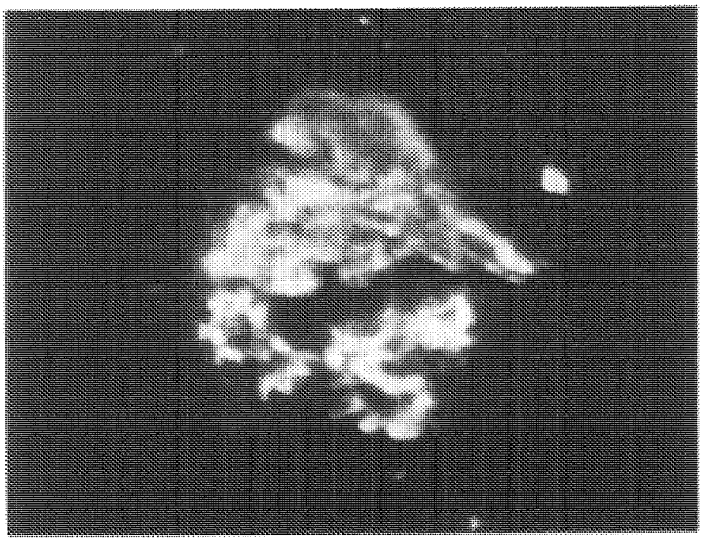


FIG.5B

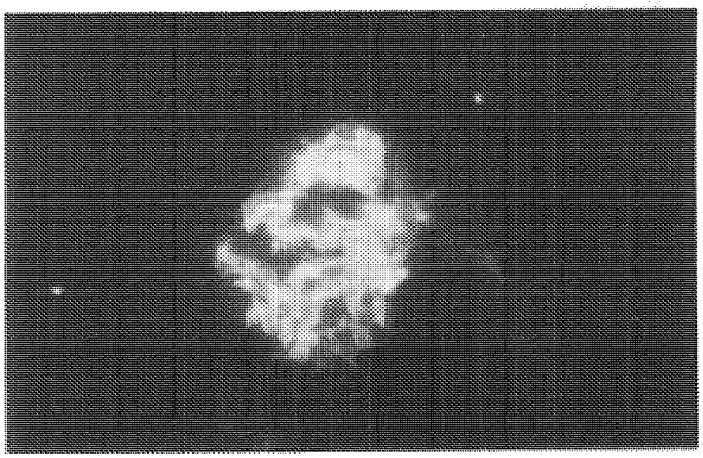


FIG.5C

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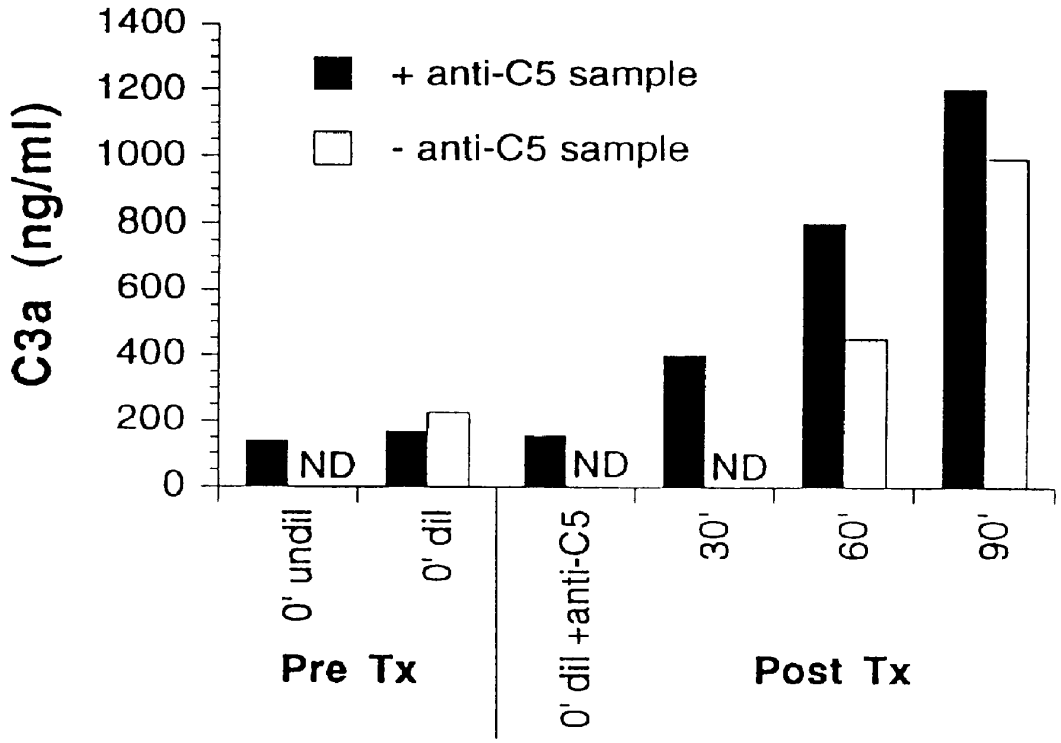


FIG.6

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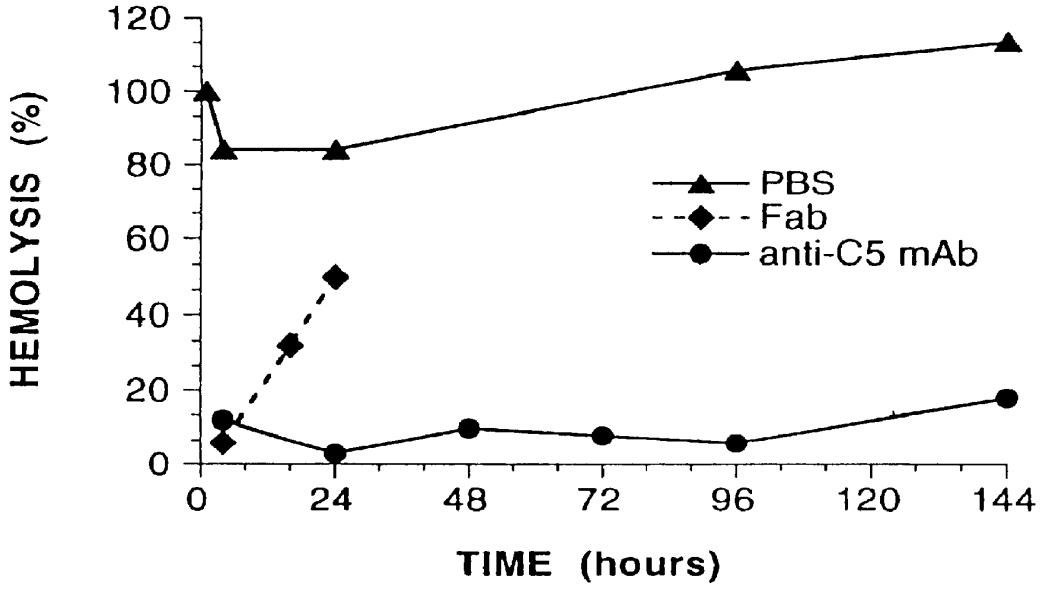


FIG.7A

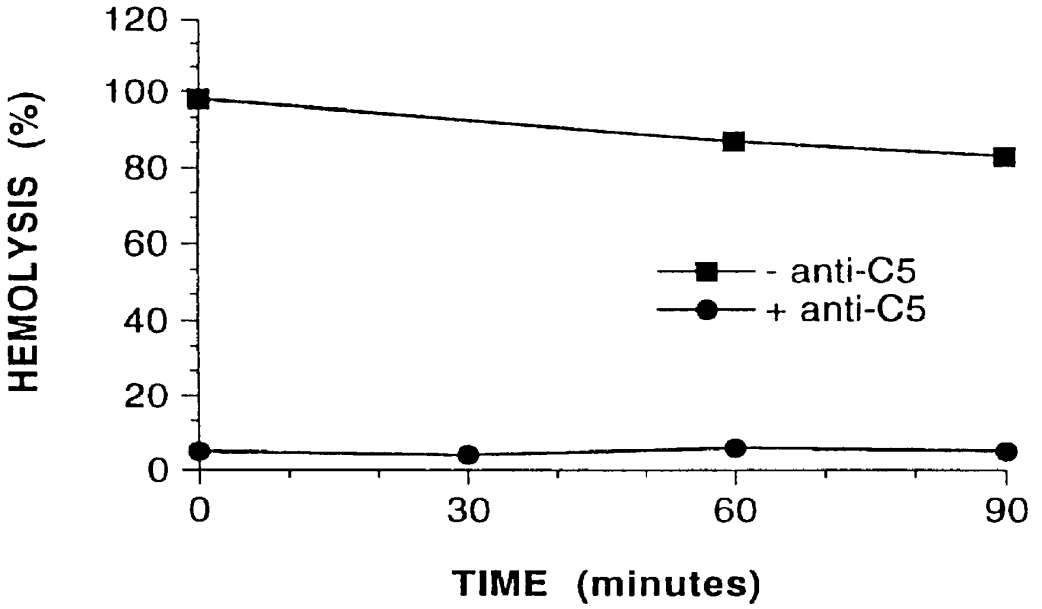


FIG.7B

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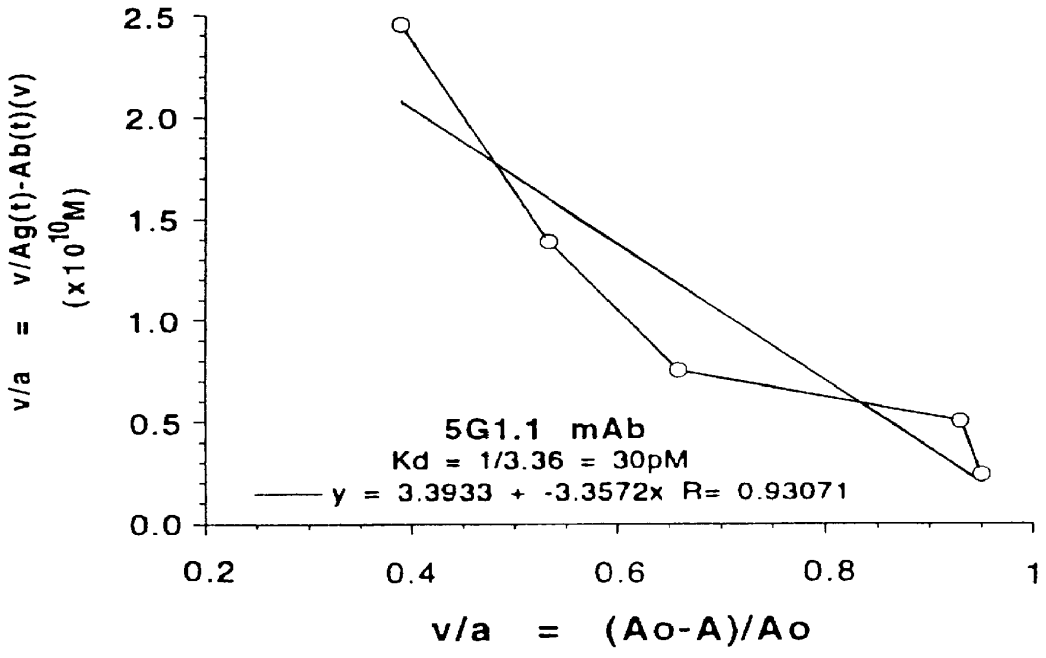


FIG.8

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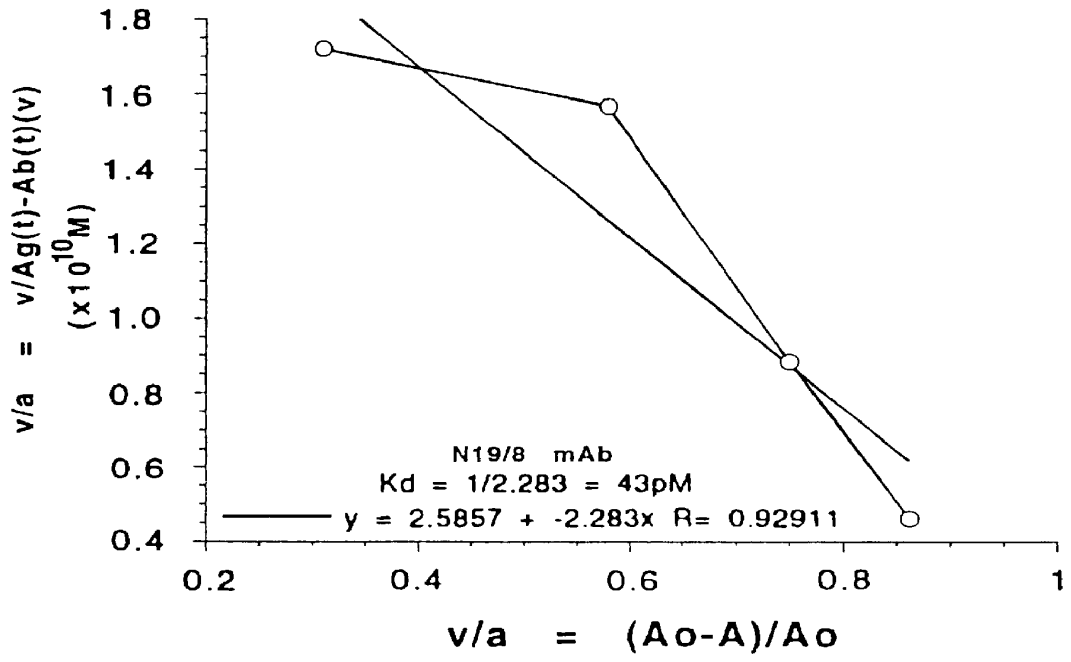


FIG.9

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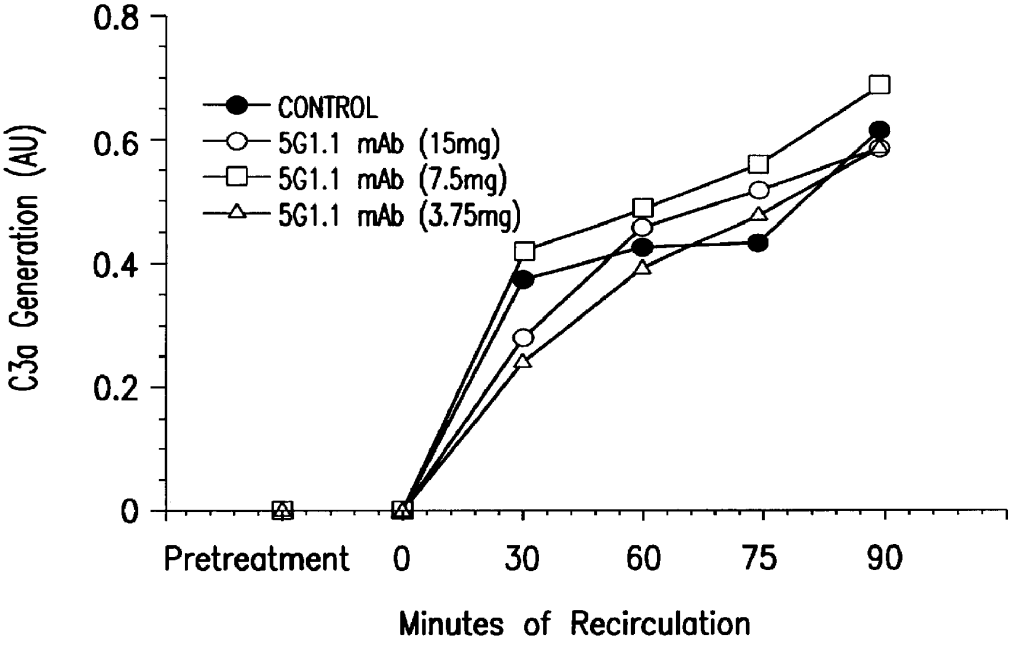


FIG.10

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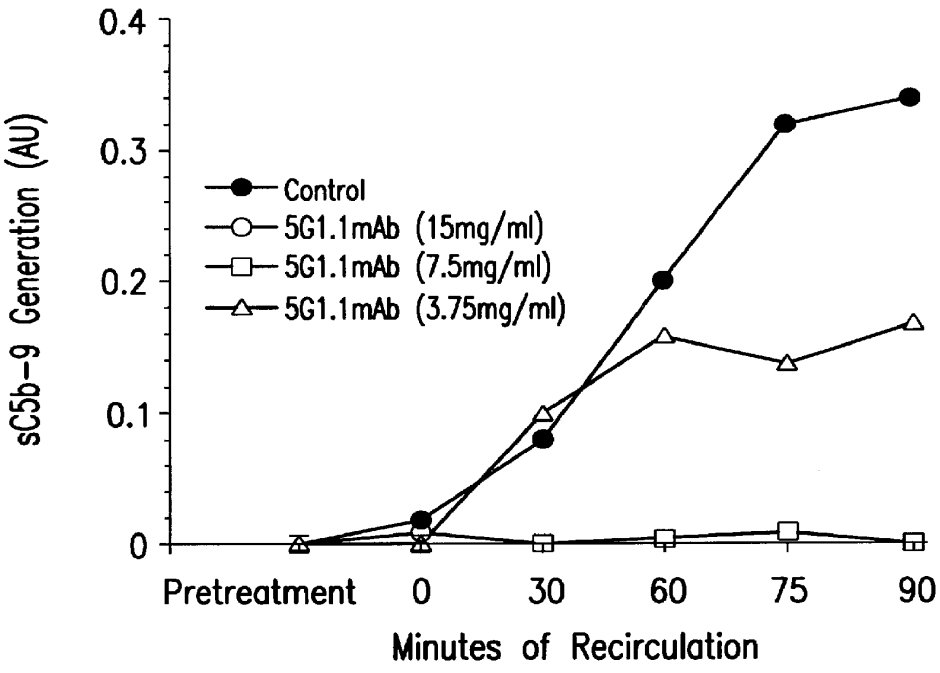


FIG. 11

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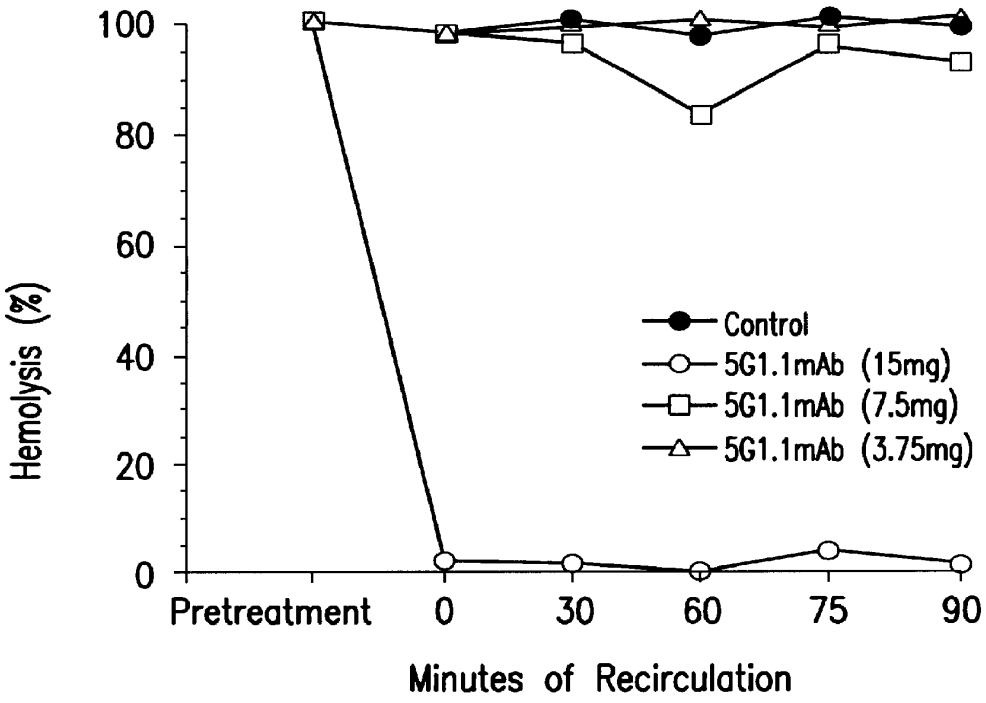


FIG. 12

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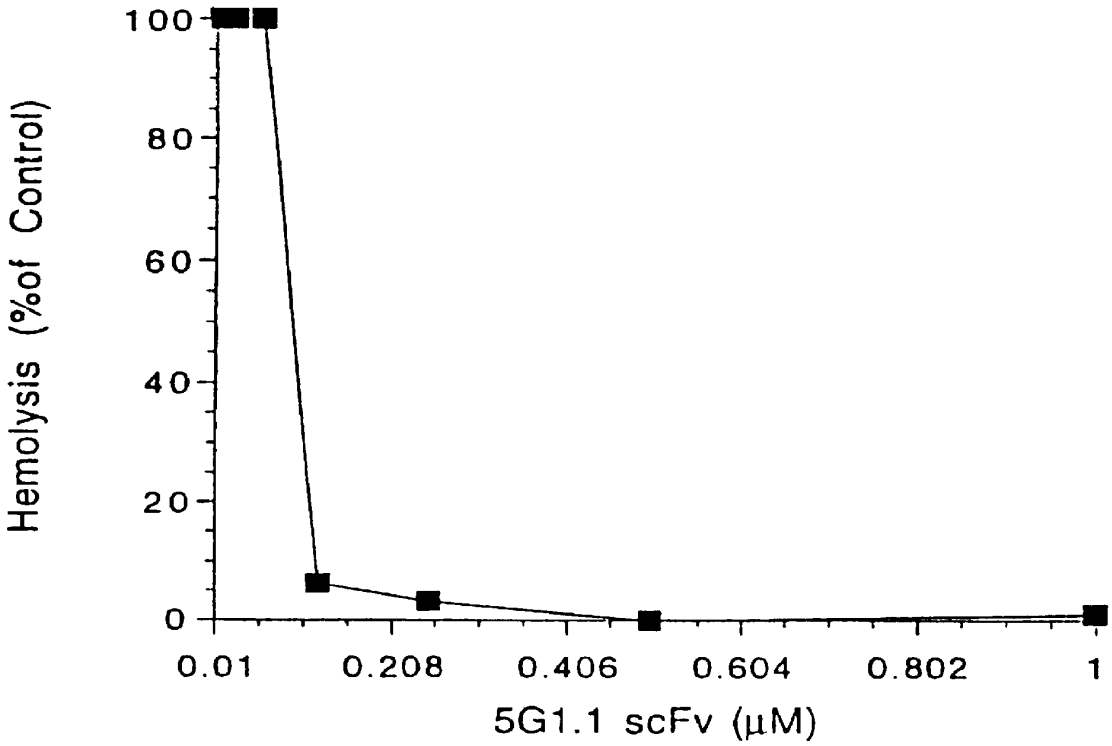


FIG.13

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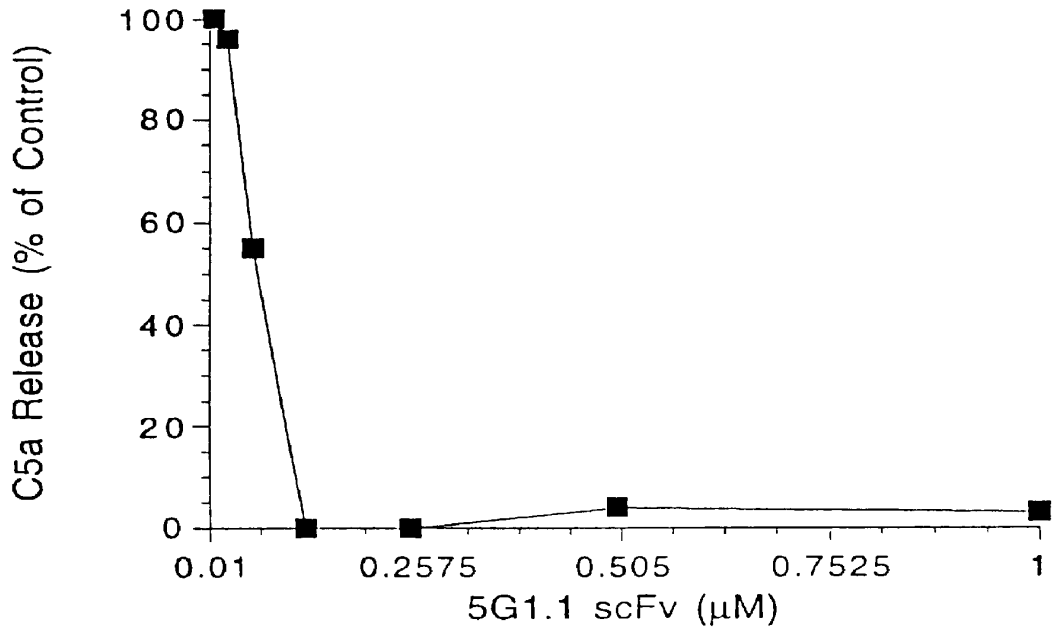


FIG.14

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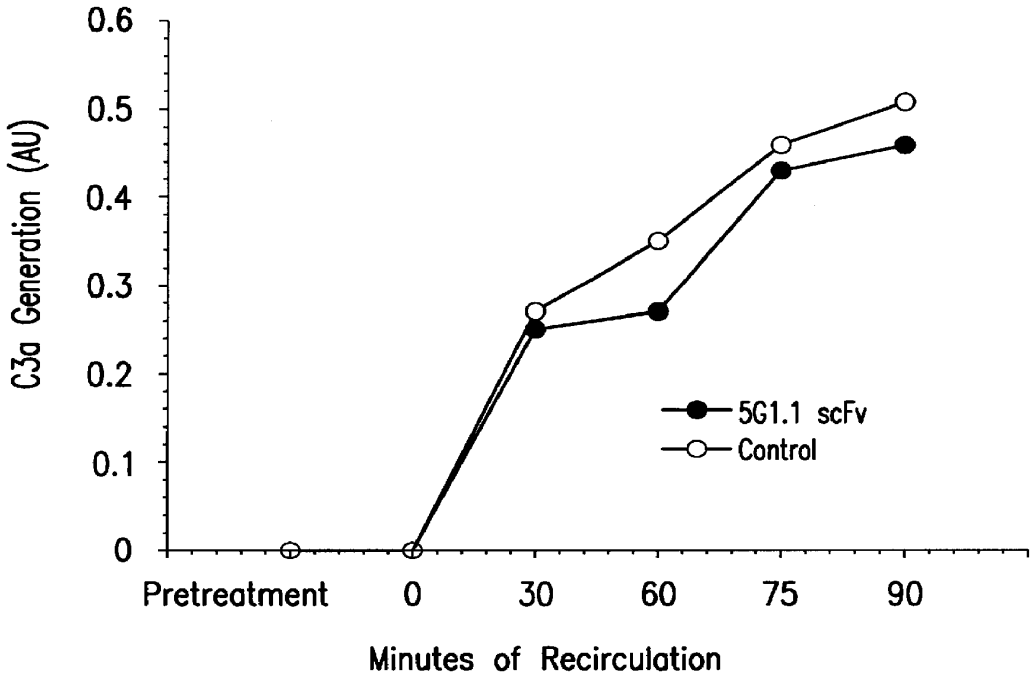


FIG. 15

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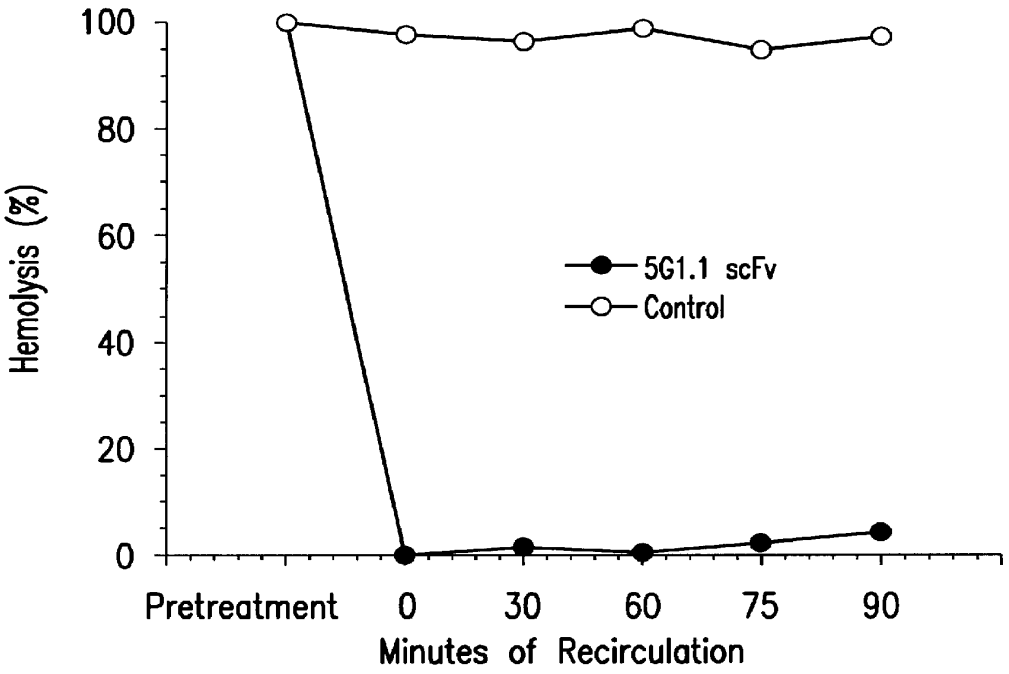


FIG. 16

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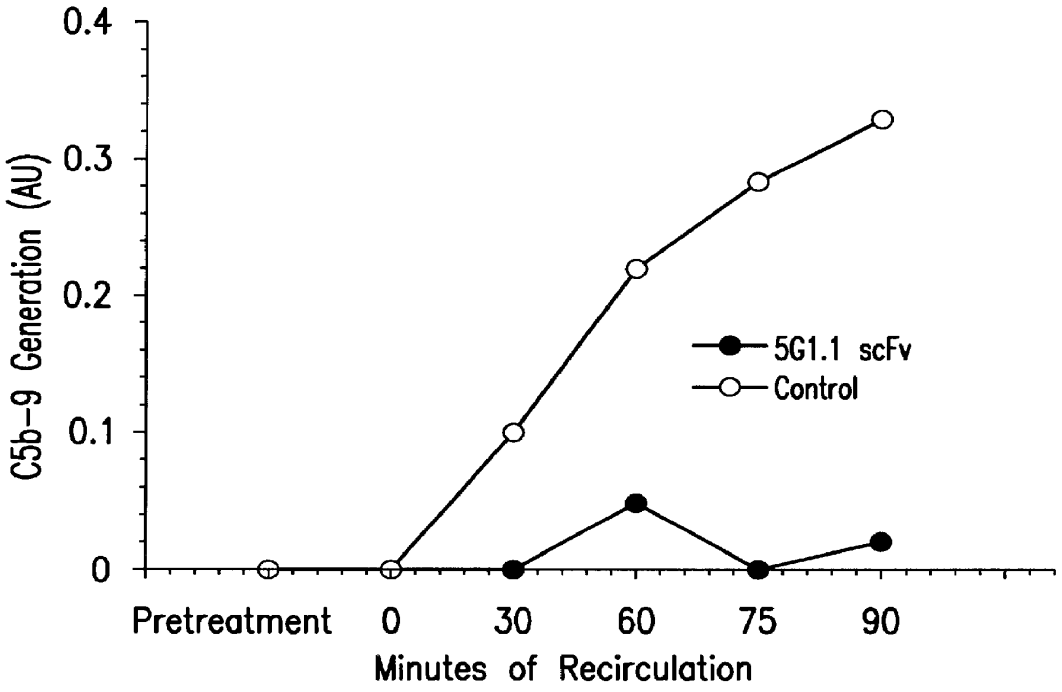


FIG. 17

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 D   I   Q   M   T   Q   S   P   A   S   L   S   A   S   V   G   E   T   V   T

                24                  30                  34                  40
ATC ACA TGT GGA GCA AGT GAG AAT ATT TAC GGT GCT TTA AAT TGG TAT CAG CGG AAA CAG
 I   T   C   G   A   S   E   N   I   Y   G   A   L   N   W   Y   Q   R   K   Q
                    CDR-L1

                50                  56                  60
GGA AAA TCT CCT CAG CTC CTG ATC TAT GGT GCA ACC AAC TTG GCA GAT GGC ATG TCA TCG
 G   K   S   P   Q   L   L   I   Y   G   A   T   N   L   A   D   G   M   S   S
                                        CDR-L2

                70                  80
AGG TTC AGT GGC AGT GGA TCT GGT AGA CAG TAT TAT CTC AAG ATC AGT AGC CTG CAT CCT
 R   F   S   G   S   G   S   G   R   Q   Y   Y   L   K   I   S   S   L   H   P

                89                  97                  100
GAC GAT GTT GCA ACG TAT TAC TGT CAA AAT GTG TTA AAT ACT CCT CTC ACG TTC GGT GCT
 D   D   V   A   T   Y   Y   C   Q   N   V   L   N   T   P   L   T   F   G   A
                                        CDR-L3

GGG ACC AAG TTG GAG CTG AAA
 G   T   K   L   E   L   K
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FIG.18

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FIG.19

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C5-SPECIFIC ANTIBODIES FOR THE TREATMENT OF INFLAMMATORY DISEASES

This is a continuation in part of International application Ser. No. PCT/US95/05688, filed May 1, 1995, which is a continuation in part of U.S. application Ser. No. 08/236,208, filed May 2, 1994, which issued as U.S. Pat. No. 6,074,642 on Jun. 13, 2000. International application Serial No. PCT/US95/05688 was published in English under PCT Article 21(2) on Nov. 9, 1995 as WO 95/29697.

FIELD OF THE INVENTION

The present invention relates to the treatment of glomerulonephritis (GN) and other inflammatory diseases, and more generally to therapeutic treatments involving the pharmacologic inhibition of a patient's complement system. In particular, the invention relates to the use of antibodies specific to human complement component C5 to accomplish such therapeutic treatment. The invention also relates to compositions comprising native monoclonal antibodies (mAbs) specific to human complement component C5 that block complement hemolytic activity and C5a generation at concentrations that substantially reach the theoretical one to two stoichiometric limit of antibody to antigen that can be achieved by a bivalent antibody. The invention further provides recombinant mabs that are derivatives (including monovalent derivatives) of these native mAbs that provide substantially the same blocking activities as the native mabs.

BACKGROUND OF THE INVENTION

I. Immune Complex Mediated Disease

The formation of immune complexes is the typical consequence of the interaction of antigens with specific antibodies. The inflammatory response that ensues when such complexes accumulate in a limited area is an important element of normal host defenses, leading to immune complex clearance and antigen destruction by phagocytic cells. In contrast, immune complex diseases are reflections of excess complex formation or retarded clearance, usually under conditions of exceptional antigen challenge or immunologic dysregulation. Under such circumstances, immune complexes are deposited or formed at specific tissue sites and resulting inflammatory responses lead to disease states due to localized or systemic tissue damage. The kidney, and more specifically the kidney structure known as the glomerulus, is a particularly important site of immune complex deposition resulting in the development of serious disease conditions.

Human studies, and studies using animal models of human diseases, have implicated the complement system in the pathologies associated with a number of immune complex associated disorders. The activation of complement that mediates the pathology associated with these disorders may be a consequence of an autoimmune mechanism, or can be non-immunologic in origin.

The hypersensitivity response that occurs when antibodies bind to antigens either in tissues or in the circulation results from the activation of complement and the release of molecules that mediate inflammation. This process is classified as either being mediated by the binding of antibody to fixed tissue or cell bound antigens (Type II hypersensitivity) or to circulating antigens, resulting in the formation of circulating immune complexes and their subsequent pathogenic deposition in tissues (Type III hypersensitivity).

Type II hypersensitivity is mediated through the activation of complement following the binding of antibodies to fixed tissue antigens. The inflammatory response that ensues results from the activation of the proinflammatory and lytic components of the complement system and the subsequent recruitment of stimulated leukocytes to the sites of immune complex formation. The increased vascular permeability that results from the anaphylatoxic activities of C3a and C5a further enhances immune complex deposition and leukocyte recruitment.

The cross-linking of antibody bound cells or tissues to effector cells such as neutrophils, platelets, NK cells, and monocytes via their Fc receptors also plays a proinflammatory role. Such cross-linking activates effector cells, stimulating the release of oxygen radicals, prostaglandins, and leukotrienes, which release is further potentiated by the actions of activated complement components.

Examples of Type II hypersensitivity-mediated conditions include hyperacute rejection of transplanted organs, autoimmune hemolytic and thrombocytopenic states, Goodpasture's syndrome (and associated glomerulonephritis and pulmonary hemorrhage), myasthenia gravis, pathologic sequellae associated with insulin-dependent diabetes melitus, and pemphigus vulgaris.

Type III hypersensitivity reactions involving circulating antigens can also result in the development of numerous pathologic conditions. These include glomerulonephritis (discussed in detail below), vasculitis (a potentially life-threatening inflammatory condition of large and/or small blood vessels), rheumatoid arthritis, dermatitis, and other disorders.

Other diseases associated with type III hypersensitivity reactions include autoimmune diseases such as systemic lupus erythematosis (SLE), many infectious diseases, neoplastic diseases, and a wide variety of other conditions (Dixon, et al. *Immune Complex Injury*, in Samter, (ed.) Immunological Diseases, 4th ed. Little Brown & Co. Boston, 1987).

II. Glomerulonephritis

The glomerulus is a key structural and functional element of the kidney. Each glomerulus is found as part of a larger structure that serves as the main functional unit of the kidney and is called a nephron. About a million nephrons are found in each kidney. Each glomerulus is a network of up to fifty parallel capillaries encased in a structure known as Bowman's capsule. The area inside Bowman's capsule that is not taken up by the glomerular capillaries is known as Bowman's space. The glomerulus functions as a filter, separating water and certain solutes from the proteins and cells of the blood into Bowman's space for further processing in the convoluted tubules, loop of Henle, and collecting duct of the nephron.

Glomerulonephritis (GN) is a disease of the kidney characterized by inflammation and resulting enlargement of the glomeruli that is typically due to immune complex formation. The accumulation of immune complexes in the glomeruli results in inflammatory responses, involving inter alia hypercellularity, that can cause total or partial blockage of the glomerulus through, among other factors, narrowing of capillary lumens. One result of this process is the inhibition of the normal filtration function of the glomerulus. Blockage may occur in large numbers of glomeruli, directly compromising kidney function and often causing the abnormal deposition of proteins in the walls of the capillaries making up the glomerulus. Such deposition can, in turn, cause damage to glomerular basement membranes. Those glomeruli that are not blocked develop increased permeability, allowing large amounts of protein to pass into the urine, a condition referred to as proteinuria.

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In many cases of severe GN, pathological structures called crescents are formed within the Bowman's space, further impeding glomerular filtration. These structures can only be seen by microscopic examination of tissue samples obtained by biopsy or necropsy, and are thus not always observed in those patients in which they occur. Crescents are a manifestation of hypercellularity and are thought to arise from the extensive abnormal proliferation of parietal epithelial cells, the cells that form the inner lining of the Bowman's capsule. Clinical research has shown that there is a rough correlation between the percentage of glomeruli with crescents and the clinical severity of the disease, and thus the patient's prognosis. When present in large numbers, crescents are a poor prognostic sign.

Symptoms of GN include: proteinuria; reduced glomerular filtration rate (GFR); serum electrolyte changes including azotemia (uremia, excessive blood urea nitrogen—BUN) and salt retention, leading to water retention resulting in hypertension and edema; hematuria and abnormal urinary sediments including red cell casts; hypoalbuminemia; hyperlipidemia; and lipiduria.

In 1990, over 210,000 patients in the United States required hemodialysis or transplantation for chronic renal failure at an annual cost in excess of 7 billion dollars, according to the United States Renal Data System (USRDS). The USRDS compiles data on kidney disease in the United States in conjunction with the National Institute of Diabetes and Digestive and Kidney Diseases, Division of Kidney, Urologic, and Hematologic Diseases, of the National Institutes of Health (NIDDKD). The USRDS estimates that the costs of treatment for renal failure are now increasing by 20 percent annually.

GN is the third leading cause of death in end-stage renal disease patients, exceeded only by diabetes and hypertension. As a result, there is a clear and long felt need in the medical community for effective treatments for this condition. Research aimed at the development of new treatments for GN is ongoing worldwide. In the United States, the NIDDKD, the National Kidney Foundation, and several other public and private organizations sponsor research in this area. The National Kidney Foundation alone supplies over two million dollars annually to fund the efforts of kidney researchers.

III. Current Treatments for GN

Corticosteroid administration, typically as high doses of "pulse" intravenous methylprednisolone or oral prednisone therapy, is currently considered the most effective pharmacologic agent available for the treatment of GN. Such steroid therapy is often administered in combination with cytotoxic general immunosuppressive agents such as azathioprine or cyclophosphamide. The overall immune suppression and resulting increased susceptibility to infection, along with other debilitating side effects associated with both steroid and cytotoxic drug administration, limit the effective use of these drugs.

Aspirin-like non-steroidal anti-inflammatory drugs (NSAIDs) have also been used to reduce the glomerular inflammation and enlargement of GN. These drugs are not routinely used for this purpose, however, probably because of their relatively weak anti-inflammatory effects and propensity to cause gastrointestinal and other side effects in many patients.

The administration of anticoagulants such as heparin or warfarin sodium, and antithrombotic agents such as cyproheptadine, dipyridamole, or sulfinpyrazone, has been used on the basis of evidence suggesting the involvement of the coagulation process in the genesis of glomerular crescents. However, objective evidence of benefit from such therapies in animals afflicted with experimentally induced crescentic GN has been inconsistent. Also, anticoagulants are dangerous drugs, as they can potentiate life-threatening bleeding episodes. They are especially hazardous in this regard in patients with advanced renal failure.

In addition to pharmacologic approaches, intensive plasma exchange (plasmapheresis) of 2 to 4 liters of plasma daily (or in some cases three times a week) can dramatically reduce high levels of circulating immune complexes when acute intervention in the inflammatory process is needed. Such treatment is expensive and requires that the patient be connected to the plasmapheresis machine for many hours each week. In addition, all procedures in which blood is removed from and returned to a patient are associated with an increased risk of infection. Nonetheless, plasma exchange is currently considered the most effective non-pharmacological treatment for removal of circulating immune complexes which can cause GN.

Circulating immune complex levels can also be decreased by eliminating or reducing the source of the antigen or antigens contained in the complexes by, for example, effective therapy of an underlying infection or change in an antibiotic. However, while such therapy is almost always a treatment of choice, great care must be taken since reduction of the antigen load alters the molar ratio of antigen to antibody involved in forming immune complexes and thus a dangerous temporary exacerbation of the inflammatory process may occur (see discussion below in Background Physiology & Pathology).

IV. Antibody Engineering

Native antibodies are multi-subunit animal protein molecules with highly specific antigen-binding properties. Animals make multiple classes of antibodies. There are five major classes (IgA, IgD, IgE, IgG and IgM) and a variety of subclasses. Native antibodies are made up of two or more heterodimeric subunits each containing one heavy (H) and one light (L) chain. The differences between antibody classes derive from their different H chains. H chains have a molecular weight of about 53 kDa, while L chains are about 23 kDa in mass.

Every individual native antibody has one type of L chain and one type of H chain, which are held together by disulfide bonds to form a heterodimeric subunit. Typically a native antibody (e.g., an IgG) has two such subunits, which are also held together by disulfide bonds. Within each chain, units of about 110 amino acid residues fold so as to form compact domains. Each domain is held together by a single intrachain disulfide bond. L chains have two domains, while H chains have four or five. Most H chains have a hinge region after the first (i.e., most amino-terminally located) two domains. The disulfide bonds linking together the heterodimeric subunits are located at the hinge regions. The hinge region is particularly sensitive to proteolytic cleavage, such proteolysis yielding two or three fragments (depending on the precise site of cleavage), a non-antigen binding fragment containing only H chain C regions (Fc) and one bivalent (Fab'2) or two monovalent (Fab) antigen binding fragments. The hinge region allows the antigen binding regions (each made up of a light chain and the first two domains of a heavy chain) to move freely relative to the rest of the native antibody, which includes the remaining heavy chain domains.

The first domain 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)

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domains. The second and subsequent (if any) domains of each chain are relatively invariant in amino acid sequence. These are known as constant heavy (CH) and constant light (CL) domains.

Each variable region contains three loops of hypervariable sequence that provide a complementary structure to that of the antigen and are critical in determining the antigen binding specificity of the antibody, as they are the contact sites for binding to the antigen. These loops are known as complementarity determining regions, or CDRs. Each variable domain is made up of three CDRs embedded in four much less variable framework segments (FRs). Together, the sets of collinear CDRs and FRs are in large part responsible for determining the three dimensional conformation of the variable regions of antibody molecules.

CDRs and FRs are features that have been deduced from structural properties of antibody variable regions. Both amino acid sequence (primary structure) and three dimensional modeling (deduced secondary and tertiary structure) of antibody variable regions have been used by various researchers to define CDRs and, by default, FRs. While the positions of the CDRs are beyond question, not all workers in the art agree upon the precise locations of the boundaries of each CDR in VH or VL regions; there is no clear cut structural marker delineating CDR/FR boundaries.

Two definitions of CDR location are currently in general use in the art. These are the "sequence variability" definition of Kabat et al. ("Sequences of Proteins of Immunological Interest," 4th ed. Washington, D.C.: Public Health Service, N.I.H.) and the "structural variability" definition of Chothia and Lesk (J. Mol. Biol. 1987, 196:901). As used herein, the terms VL CDR1, VL CDR2, VL CDR3, VH CDR1, VH CDR2, and VH CDR3 refer minimally to the region of overlap between the regions designated for each CDR by each of these two definitions, and maximally to the total region spanned by the combination of the regions designated for each CDR by each of these two definitions.

One problem that antibody engineering attempts to address is the immune activity of a human patient that occurs in response to a native murine (or other non-human animal) antibody, typically a mAb, that is being administered to the patient for therapeutic purposes. This activity against murine antibodies is characterized by a human anti-mouse antibody (HAMA) response that can have deleterious effects on treatment efficacy and patient health. It has been found that almost all such human anti-non-human antibody ("HAMA type") activity is directed at the constant domains and at the FR regions of the variable domains of native non-human antibodies.

By manipulating the nucleic acid molecules encoding antibody H and L chains it is possible to incorporate non-human variable regions into antibodies otherwise made up of human constant regions. The resulting antibodies are referred to as "chimeric antibodies," and are typically less prone to eliciting HAMA type responses than are the non-human antibodies from which the variable regions are derived.

An even more effective approach to eliminating the potential of a non-human antibody to elicit a HAMA type response is to "humanize" it, i.e., to replace its non-human framework regions with human ones. One way of achieving such humanization involves the insertion of polynucleotide fragments encoding the non-human CDRs of the antibody to be humanized into a nucleic acid molecule encoding an otherwise human antibody (with human constant regions if desired) so as to replace the human CDRs and to use the resulting nucleic acid molecule to express the encoded "humanized" antibody.

Unfortunately, however, humanization of non-human antibodies has unpredictable effects on antibody antigen interactions, e.g., antigen binding properties. Some of this unpredictability stems from the properties of the CDRs. Certain CDRs may be more amenable to the construction of humanized antibodies that retain the properties of the non-human CDR donor antibody than others. While the CDRs are key to the antigen binding properties of an antibody, CDRs and FRs must interact appropriately if the antigen specificity of an antibody is to be retained following humanization. The effects of combination with particular human FRs on uncharacterized non-human CDRs cannot be reliably predicted by any known method. However, the successful humanization of an antibody provides information that, in general, facilitates the successful humanization of the CDRs of that antibody using other human or altered human FRs. In addition, approaches are available that facilitate tailoring human FRs to enhance the likelihood of successful humanization.

Other problems addressed by antibody engineering include efficient antibody production and alteration of antibody pharmacokinetics. Recombinant protein production is generally most efficiently carried out in bacterial hosts. The large size and multimeric nature of native antibodies makes their production in bacteria difficult. One approach to dealing with production problems is to use recombinant DNA methods to construct antibodies that have their H and L chains joined by a linker peptide to form a single chain (sc) antibody. As described below, there are several types of sc antibodies that can be constructed.

As is the case for humanization, the effects on antigen binding properties of constructing a particular type of sc antibody using H and L chains that have not been characterized with regard to their ability to function as part of an sc antibody cannot be reliably predicted by any known method. However, the successful construction of any one type of sc antibody from a particular native antibody provides information that, in general, facilitates the successful construction of other types of sc antibodies from that native antibody.

Single chain antibodies may include one each of only VH and VL domains, in which case they are referred to as scFv antibodies; they may include only one each of VH, VL, CH, and CL domains, in which case they are referred to as scFab antibodies; or they may contain all of the variable and constant regions of a native antibody, in which case they are referred to as full length sc antibodies.

The differing sizes of these antibodies imparts each with differing pharmacokinetic properties. In general, smaller proteins are cleared from the circulation more rapidly than larger proteins of the same general composition. Thus, full length sc antibodies and native antibodies generally have the longest duration of action, scFab antibodies have shorter durations of action, and scFv antibodies have even shorter durations of action. Of course, depending upon the illness being treated, longer or shorter acting therapeutic agents may be desired. For example, therapeutic agents for use in the prevention of immune and hemostatic disorders associated with extracorporeal circulation procedures (which are typically of brief duration) are preferably relatively short acting, while antibodies for the treatment of long term chronic conditions (such as inflammatory joint disease or GN) are preferably relatively long acting.

Detailed discussions of antibody engineering may be found in numerous recent publications including: Borrebaek, "Antibody Engineering, A Practical Guide," 1992, W.H. Freeman and Co. NY; and Borrebaek, "Antibody Engineering," 2nd ed. 1995, Oxford University Press, NY, Oxford.

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SUMMARY OF THE INVENTION

In view of the foregoing, it is an object of the present invention to provide a new approach for reducing the glomerular inflammation and kidney dysfunction associated with GN.

The method of the invention involves the use of preparations containing antibodies to human complement component C5 as pharmaceutical agents. More particularly, the invention provides for the use of anti-C5 antibodies that bind to complement component C5 or active fragments thereof. Preferably, the antibodies block the generation and/or activity of complement components C5a and C5b. For most applications, the antibody is a monoclonal antibody.

In the preferred embodiments of the invention, the administration of the anti-C5 antibody preparation is started after the appearance of GN symptoms, e.g., after the appearance of proteinuria. Alternatively, the invention can be used prophylactically to treat patients who are at risk for an acute exacerbation of existing GN, e.g., patients experiencing a flare-up of symptoms of systemic lupus erythematosus or similar autoimmune diseases that have resulted in GN.

As shown in the examples presented below, anti-C5 antibodies administered subsequent to the onset of GN essentially eliminate glomerular inflammation/enlargement and reduce kidney dysfunction (see Examples 1 and 2).

Although not wishing to be bound by any particular theory of operation, it is believed that the anti-C5 antibodies have these and other therapeutic effects through their activity in blocking the generation or activity of the C5a and/or C5b active fragments of complement component C5. Through this blocking effect, the antibodies inhibit the proinflammatory (anaphylatoxic) effects of C5a and the generation of the C5b-9 membrane attack complex (MAC). Significantly, the blockage effected by the anti-C5 antibodies, since it occurs at the level of complement component C5, has the advantage of maintaining important opsonic, anti-infective, and immune complex clearance functions of the complement system mediated by, inter alia, complement component C3.

The invention additionally provides compositions comprising anti-C5 antibodies that block complement hemolytic activity and C5a generation. These antibodies are useful for the treatment of GN as well as a number of other conditions. These include treatment of immune and hemostatic dysfunctions associated with extracorporeal circulation (see copending U.S. patent application Ser. No. 08/217,391, now U.S. Pat. No. 5,853,722 which is incorporated herein by reference), treatment of inflammatory joint diseases (see copending U.S. patent application Ser. No. 08/311,489, which is incorporated herein by reference), and other complement associated conditions, particularly inflammatory diseases.

Although other antibodies can be used to treat GN in accordance with the present invention, the novel antibodies of the invention are preferred. Preferably, these novel antibodies bind to the alpha chain of C5, but do not exhibit substantial binding to the alpha chain cleavage product C5a (referred to hereinafter and in the claims as "free C5a"). Other preferred targets for antibody binding include fragments of the alpha chain of human C5 that are immunoreactive with the most preferred antibody of the invention, the 5G1.1 antibody discussed below. Such preferred targets include the 46 kDa acid hydrolysis fragment of C5 (the "5G46k" fragment), the 27 kDa tryptic digestion fragment of C5 (the "5G27k" fragment), the 325aa peptide spanning amino acid residues 725–1049 of SEQ ID NO:2 (the "5G325aa" peptide), the 200 amino acid peptide spanning amino acids residues 850 to 1049 of SEQ ID NO:2 (the "5G200aa" peptide)—as discussed below in Example 13.

The novel antibodies of the invention include antibodies that bind to an epitope within the amino acid sequence Val Ile Asp His Gln Gly Thr Lys Ser Ser Lys Cys Val Arg Gln Lys Val Glu Gly Ser Ser, (SEQ ID NO:1) hereinafter referred to as the KSSKC epitope. These novel antibodies that bind to the KSSKC epitope (SEQ ID NO:1) are hereinafter referred to as anti-KSSKC antibodies, and monoclonal antibodies binding to the KSSKC epitope are hereinafter referred to as anti-KSSKC mAbs.

The novel antibodies of the invention have many advantages over other anti-C5 antibodies, particularly with regard for their use as anti-inflammatory therapeutic agents. These include the ability to substantially block both complement hemolytic activity and the generation of the proinflammatory complement cleavage product C5a to substantially the same extent at the same concentration of antibody. Some of the preferred antibodies of the invention have the additional advantageous property of blocking the binding of C5 to C3 or C4.

Particularly preferred antibodies of the invention are monospecific native anti-KSSKC antibodies. The 5G1.1 native anti-KSSKC mAb has the distinct advantage of substantially blocking both complement hemolytic activity and the generation of C5a at a stoichiometric ratio of antibody to C5 that approaches the theoretical one to two (antibody to antigen) limit of binding that can be achieved by a bivalent antibody. This is a desirable property because it allows smaller doses of antibody to achieve therapeutic effects than would be required of otherwise similar antibodies that cannot function at such a ratio.

The invention further provides recombinant mAbs that are derivatives (including monovalent derivatives) of these native mAbs. These include anti-KSSKC recombinant mabs. Preferably the antibodies of the invention provide a level of blockade of both complement hemolytic activity and C5a generation (on a per mole of binding site basis) that is obtained when the antibody concentration is within an order of magnitude of that of the native mAbs. Particularly preferred anti-KSSKC recombinant mAbs provide a level of such blockade when the antibody concentration is no more than three fold that of the native mAbs of the invention.

The invention further provides nucleic acid sequences of polynucleotides encoding such recombinant anti-KSSKC mAbs, as well as amino acid sequences of the polypeptides encoded by these nucleic acid molecules of the invention.

The invention further provides CDR sequences that are useful in the construction of the humanized antibodies of the invention, as well as peptides and oligopeptides that are useful in the preparation and characterization of the antibodies of the invention.

Anti-C5 antibodies of the invention have activity in blocking the generation or activity of the C5a and/or C5b active fragments of complement component C5. Through this blocking effect, the antibodies inhibit the proinflammatory (anaphylatoxic) effects of C5a and the generation of the C5b-9 membrane attack complex (MAC). Significantly, the blockage effected by the anti-C5 antibodies, since it occurs at the level of complement component C5, has the advantage of maintaining important opsonic, anti-infective, and immune complex clearance functions of the complement system mediated by, inter alia, complement component C3.

The accompanying figures, which are incorporated in and constitute part of the specification, illustrate certain aspects

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of the invention, and together with the description, serve to explain the principles of the invention. It is to be understood, of course, that both the figures and the description are explanatory only and are not restrictive of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1**A, **1**B, and **1**C—Photomicrographs of PAS stained sections of mouse kidneys. FIG. **1**A—uninduced untreated mouse. FIG. **1**B—GN-induced PBS-(control)-treated mouse. FIG. **1**C—GN-induced anti-C5 treated mouse. Magnification for each is the same, approximately 400×.

FIGS. **2**A, **2**B, and **2**C—Photomicrographs of immunofluorescence stained sections of mouse kidneys. FIG. **2**A—uninduced untreated mouse. FIG. **2**B—GN-induced PBS-(control)-treated mouse. FIG. **2**C—GN-induced anti-C5 treated mouse. Magnification for each is the same, approximately 200×.

FIG. **3**—Results of hemolytic (cell lysis) assays of serum from GN-induced animals treated with either anti-C5 antibodies in PBS ("Anti-C5") or PBS alone ("PBS control"). Also shown are the results of assays performed with normal serum.

FIG. **4**—Results of soluble C5b-9 ("sC5b-9") assays. "ND" indicates not determined.

FIGS. **5**A, **5**B, and **5**C—Immunofluorescence photomicrographs of kidney sections stained for mouse C3. FIG. **5**A—uninduced untreated mouse. FIG. **5**B—GN-induced PBS-(control)-treated mouse. FIG. **5**C—GN-induced anti-C5 treated mouse. Magnification for each is the same, approximately 400×.

FIG. **6**—Results of C3a assays of samples of circulating human blood. "ND" indicates not determined.

FIGS. **7**A and **7**B—Pharmacokinetic analyses of the reduction of the cell lysis ability of mouse (FIG. **7**A) or human (FIG. **7**B) blood after treatment with anti-C5 antibodies.

The immunofluorescent staining of FIGS. **2** and **5** is confined to the glomerular capillary network (tuft) and thus the enlargement of the glomerulus seen in FIG. **1**B is not visible in FIGS. **2**B and **5**B.

FIG. **8**—Scatchard analysis of native 5G1.1 binding to C5.

FIG. **9**—Scatchard analysis of native N19/8 binding to C5.

FIG. **10**—C3a generation in samples of circulating human blood in the presence of native 5G1.1.

FIG. **11**—sC5b-9 generation in samples of circulating human blood in the presence of native 5G1.1.

FIG. **12**—Serum hemolytic activity of samples of circulating human blood in the presence of native 5G1.1.

FIG. **13**—Serum hemolytic activity in the presence of m5G1.1 scFv.

FIG. **14**—C5a generation in the presence of m5G1.1 scFv.

FIG. **15**—C3a generation in samples of circulating human blood in the presence of m5G1.1 scFv.

FIG. **16**—Serum hemolytic activity of samples of circulating human blood in the presence of 5G1.1 scFv.

FIG. **17**—sC5b-9 generation in samples of circulating human blood in the presence of m5G1.1 scFv.

FIG. **18**—The light chain variable region of the antibody 5G1.1. Sequence derived from the 5' oligonucleotide primer used for PCR amplification of the variable region is shown in lower case. Amino acids are number according to Kabat et al., supra. Boxed amino acids correspond to peptide sequences obtained from the mature 5G1.1 light chain or from an endoproteinase Lys C peptide of 5G1.1. The complementarity determining region (CDR) residues according to the sequence variability definition and the structural variability definition are underlined and overlined, respectively.

FIG. **19**—The heavy chain variable region of the antibody 5G1.1. Sequence derived from the 5' oligonucleotide primer used for PCR amplification of the variable region is shown in lower case. Amino acids are numbered using the scheme of Kabat et al. supra with +1 denoting the first amino acid of the processed mature variable region. Boxed amino acids correspond to peptide sequence obtained from the 5G1.1 heavy chain after treatment with pyroglutamate aminopeptidase. The complementarity determining region (CDR) residues according to the sequence variability definition or according to the structural variability definition are underlined and overlined, respectively.

BACKGROUND PHYSIOLOGY & PATHOLOGY

The discussion in this section is not limited to subject matter that qualifies as "prior art" against the present invention. Therefore, no admission of such prior art status shall be implied or inferred by reason of inclusion of particular subject matter in this discussion, and no declaration against the present inventors' interests shall be implied by reason of such inclusion.

I. Introduction

As described above, the present invention relates to therapeutic treatments for GN and other immune complex mediated diseases, as well as to the treatment of other complement mediated diseases and to the inhibition of complement component C5. To provide background for the description of the preferred embodiments and the examples presented below, we turn first to general discussions of the complement arm of the immune system, the pathophysiologic features of GN, and previous studies of the role of complement in GN pathogenesis.

General discussions of the complement system and GN can be found in, for example, Glassock and Brenner, 1994; Couser, 1993; Couser, 1992; Couser, et al, 1992; Rich, 1992; Glassock and Brenner, 1987; Robbins and Cotran, 1979; and Guyton, 1971.

II. 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

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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 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 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 (SEQ ID NO:2).

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 SEQ ID NO:2) and the alpha chain as a carboxyl terminal fragment (amino acid residues 660 to 1658 of SEQ ID NO:2), with four amino acids (amino acid residues 656–659 of SEQ ID NO:2) 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 (i.e., amino acid residues 660–733 of SEQ ID NO:2). 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 SEQ ID NO:2. 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.

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.

C5a is another anaphylatoxin (see discussion below). 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.

III. Pathophysiology of GN

Although GN may accompany an extraordinary range of pathologic processes, in general it is encountered most commonly in the course of infectious diseases, in autoimmunity, and as a consequence of therapy for some other disease process. The causative mechanism for GN is typically the deposit of circulating immune complexes in the kidney. Factors involved in the pathogenesis of GN include the specific antigen and antibody involved and the inflammatory processes that occur as a consequence of immune complex deposition.

Antigens Involved in the Formation of Immune Complexes that Cause GN

Antigens involved in the development of GN can be broadly classified as endogenous, infectious, and iatrogenic (those encountered as a consequence of medical practice). In many cases the specific antigen is unknown, although the general class can usually be identified.

The best known example of the formation of endogenous immune complexes is the DNA anti-DNA complexes produced in connection with systemic lupus erythematosus (lupus, SLE). Other important sources of endogenous antigens include malignancies in which immune complex formation may contribute to the development of paraneoplastic syndromes.

Infections with organisms of many types, particularly chronic infections, are also associated with the development of immune complexes that can cause GN. Bacterial and fungal infections that can produce such complexes include infection with certain strains of streptococci, Pseudomonas, disseminated gonococcal infection, lepromatous leprosy, subacute bacterial endocarditis, bronchopulmonary aspergillosis, secondary syphilis, and chronic infections in patients with cystic fibrosis.

Viral diseases in which immune complex deposition may be a prominent feature include hepatitis B infection, dengue, infectious mononucleosis, and subacute sclerosing panencephalitis. GN is also a prominent feature of many parasitic infestations such as the GN seen in children with quartan malaria, as well as toxoplasmosis, trypanosomiasis, and schistosomiasis.

Iatrogenic antigens constitute a special class of exogenous antigens. These include those responsible for the prototype immune complex disease, serum sickness, which follows

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formation of immune complexes between heterologous serum constituents and autologous antibodies. Serum sickness was regularly seen earlier in this century when infectious diseases were frequently treated with heterologous antisera.

An iatrogenic disease essentially indistinguishable from classic serum sickness can occur as a consequence of high-dose antibiotic therapy. The serum sickness-like manifestations of immune responses to these drugs include GN and reflect the fact that certain drugs, particularly the β-lactam and sulfonamide antibiotics, are effective haptens that are capable of inducing antibody responses upon spontaneous conjugation to autologous proteins.

Factors Affecting Immune Complex Formation and Deposition

Features of both antigen and antibody determine the likelihood of pathologic immune complex formation and subsequent deposition in the kidney. Chief among these are the absolute concentrations of the reactants and their relative molar ratios.

Most antigens display multiple epitopes and typically stimulate a polyclonal antibody response. All naturally occurring antibody molecules are at least bivalent. These properties allow for the formation of an extensive antigen-antibody lattice, the size of which is determined largely by the affinity of the antibodies and the molar ratio of antigen to antibody.

In general, antibody responses begin under conditions in which antigen is present in excess to antibody, and this relative ratio changes as the antibody response increases in magnitude. Complexes formed initially are usually small and exhibit little or no pathogenic activity. In contrast, very large complexes are often formed as the amount of antigen becomes limiting, late in the course of an antibody response under conditions of antibody excess. Because these very large complexes are readily cleared by the reticuloendothelial system in the liver, they are also relatively nonpathogenic.

The formation of immune complexes that can cause GN is believed to occur during conditions of slight antigen excess or near the point of antibody-antigen equivalence, where lattice formation is maximal and lattice size is large, but not very large.

Several factors influence the speed and location of immune complex precipitation. Interactions between Fc regions of antibody molecules promote rapid precipitation of immune complexes. The role of Fc-Fc interactions in immune complex precipitation is illustrated by studies of the properties of F(ab')2 antibody fragments, which do not contain Fc regions. Although the valence of F(ab')2 fragments does not differ from that of most whole immunoglobulins, F(ab')2 antibody fragments form lattices more slowly.

Antigen charge plays a role in determining the tissue localization of sites of deposition of immune complex precipitates. Complexes with a substantial positive charge are preferentially attracted to the strong negative charge of basement membranes, particularly in the renal glomerulus.

Localized presence of antigen may largely account for certain cases of organ specific immune complex deposition. Diseases such as Goodpasture's syndrome (a rare form of GN) are typically not classified as immune complex diseases because the complexes are formed in situ in the kidney rather than being preformed in the circulation and then deposited. Once the immune complexes are formed, the subsequent inflammatory process is believed to be essentially the same as that seen following deposition of preformed complexes. However, the different mode of deposition distinguishes this syndrome from typical GN caused by circulating immune complexes.

Features of blood flow and vascular structure are also important in determining the localization of immune complex deposits. Chief among these is capillary permeability. Because their capillary endothelium is fenestrated, renal glomeruli are preferential sites for the deposition of immune complexes. Hemodynamic variables enhancing immune complex localization include turbulence of flow and increased blood pressure, both of which are present in the renal glomeruli.

Complement and Complement Receptors as Reaulators of Immune Complex Deposition

In addition to their proinflammatory functions, complement components can also inhibit immune complex deposition and resolubilize immune complex precipitates from sites of deposition. In addition, it is known that erythrocyte receptors for C3b, e.g., CR1, are important for reticuloendothelial clearance of opsonized circulating immune complexes.

Analysis of the clinical pattern of immune complex disease in patients with deficiencies of particular complement components provides information regarding the normal role of these components in the prevention of complex deposition. The incidence of immune complex disease in patients with deficiencies of Clq, Clr, Cls, C4, C2, or C3 varies from 60 to 90 percent, with the majority of these patients exhibiting a lupus-like syndrome. Immune complex disease is rarely associated with deficiencies of late-acting or alternative pathway components.

The binding of complement components to immune complexes prevents the formation of large antigen-antibody lattices and inhibits immune precipitation. This process requires activation via the classical pathway; serum that is deficient for Clq, C4, or C2 does not effectively inhibit lattice formation and complex precipitation. Classical pathway dependence may reflect the initial binding of Cl components, impeding the Fc-Fc interactions between IgG molecules that contribute to immune precipitation. This is followed by covalent binding of C3b to the complexes, which further inhibits immune precipitation and leads to solubilization of previously deposited complexes.

The solubilization process also depends upon activation of components of the alternative pathway. Consequently, by promoting clearance of immune complexes and inhibiting their deposition at sites of inflammation, complement components and their receptors serve as negative regulators of immune complex diseases that may retard disease development.

It should be noted that the present invention involves blocking the activities of complement component C5. The targeting of this component does not alter the functions of the early complement components, and thus does not compromise the negative regulatory effects on immune complex deposition of those early components.

Immune Complex-Mediated Inflammation

Basophils are important in the initiation of immune complex-mediated inflammatory responses, as capillary permeability is markedly increased by the action of vasoactive amines such as histamine and platelet-activating factor, which are released by these cells. Vascular permeability is also promoted by aggregation of platelets at sites of an inflammatory lesion, with the release of platelet-activating factor and the formation of microthrombi.

Basophil degranulation may reflect the effects of IgE antibodies, as well as the elaboration of the anaphylatoxin components of complement, C3a and C5a.

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In addition to basophils and platelets, the primary cellular effectors of immune complex-mediated inflammation are polymorphonuclear leukocytes, monocytes, and macrophages.

IV. Previous Studies of the Role of Complement in GN Pathoaenesis

Extensive work has been performed in an attempt to understand the possible role of complement in the development of GN. This work has included studies of GN using a number of animal models by, among others, Unanue, et al., (1964); Cochrane, et al., (1965); Kniker, et al., (1965); Salant, et al., (1980); Groggel, et al., (1983); Falk and Jennette (1986); Jennette, et al., (1987); Passwell, et al., (1988); Schrijver, et al., (1988); Baker, et al., (1989); Schrijver, et al., (1990); Couser, et al., (1991); and Couser, et al., (1992).

These studies have shown that complement plays a role in GN pathogenesis. However, they have not established specific unequivocal roles for the various complement components. In particular, the relative roles of C3 and other anaphylatoxins compared to the roles of the terminal complement components in GN pathogenesis have not been unequivocally established. Also, some researchers have reported that complement depletion does not diminish glomerular injury. See Kniker, et al., (1965).

The foregoing work includes that of Falk and Jennette (1986), who reported results of experiments in which attempts were made to induce GN in mice having a genetic defect that resulted in a deficiency of complement component C5. The report concludes that C5 or some terminal complement component dependent on C5 plays a role in the pathogenesis of GN.

Significantly, with regard to the present invention, Falk and Jennette in no way disclose or suggest that an antibody to C5 can be used to treat GN. Indeed, it would be counterintuitive to use an antibody to treat disease which typically involves the formation and deposition of circulating antibody-antigen immune complexes. Plainly, the creation of more circulating immune complexes would seem to be the last way to go to solve a problem that can be caused by circulating immune complexes. Yet, as demonstrated by the surprising results presented below, anti-C5 antibodies have been found to effectively block GN, even though the creation of additional circulating immune complexes is inherent in their mode of action.

Baker et al. (1989), Couser et al. (1991), and Couser et al. (1992) (hereinafter referred to collectively as the "C6" work) discuss experiments in which high levels of an anti-C6 polyclonal antibody preparation were administered to rats, following which immune complexes were formed in situ in the rats' kidneys. Significantly, with regard to the present invention, the anti-C6 antibody preparation was not administered to animals with pre-existing kidney disease, i.e., it was not used as a therapeutic treatment. Moreover, the experimental protocol used in the C6 experiments did not involve circulating immune complexes, but rather involved complexes formed in situ. Accordingly, the experiments did not disclose or suggest the counterintuitive approach of the present invention wherein more circulating immune complexes are formed in the process of treating a disease state caused by circulating immune complexes.

Further, the anti-C6 antibody dosages used in the C6 work were too high for practical medical use. Specifically, these antibodies were used at a dosage of 1 gm/kg, a dosage which would correspond to 70 gm of antibody for a 70 kg (155 lb) individual. In contrast, the anti-C5 antibodies used in the practice of the present invention are used at concentrations at or below 0.1 gm/kg, i.e., a factor of at least ten times less than used in the C6 work. Indeed, as shown by the examples presented below, anti-C5 antibody dosages as low as 0.03 gm/kg, i.e., 33 times less than those used in the C6 work, have been found to achieve the therapeutic effects of the invention in treating GN. For a 70 kg individual, this antibody level corresponds to a dose of just 2.1 gms.

The novel anti-KSSKC antibodies of the invention allow the use of even lower dosage levels to treat GN and other inflammatory conditions. Based upon their level of activity in human blood, they are expected to provide complete complement inhibition at dosages below 0.005 g/kg, and to provide therapeutically effective complement inhibition at dosages below 0.003 g/kg. This 3 mg/kg dosage is one tenth the dosage discussed below in Examples 4 and 5 for the for the anti-C5 (beta chain specific) mAb N19/8. Some of the full length anti-KSSKC mabs of the invention will provide therapeutic benefits even at dosages below 0.0022 g/kg. This is the minimum dose providing complete complement inhibition as calculated from the data obtained using the anti-KSSKC 5G1.1 mAb in human blood in a CPB circuit, as discussed below in Example 9.

Accordingly, dosages of less than 0.005 g/kg are preferred, with dosages of below 0.003 g/kg being more preferred, and dosages below 0.0022 g/kg being particularly preferred. For a 70 kg individual, these antibody dosage levels correspond to a dose of less than 0.35 gms for the highest dosage of the preferred dosages, less than 0.21 gms for the more preferred dosage, and less than or equal to 0.15 gms for the most preferred dosage.

Of course, dosage levels of single chain and other recombinant mAbs of the invention must be adjusted according to their level of activity (e.g., their binding affinity, their ability to block C5 activation, and/or their ability to block complement hemolytic activity), their valency, and their molecular weight. For example, the humanized scFv anti-KSSKC mAbs of Example 11 are approximately 27 kDa, about one sixth the approximately 155 kDa mass of a native, full length IgG antibody. These antibodies completely block complement hemolytic activity and C5a generation at a ratio of 3:1, six fold greater than for native 5G1.1 (but only three fold greater when viewed in terms of numbers of antibody-antigen binding sites).

Thus, the number of molecules of each of these scFvs required to equal the effect of a single molecule of native 5G1.1 must be increased by a factor of six to adjust for the ratio at which blocking is complete. Since the mass of these molecules is approximately one sixth of the mass of native 5G1.1, dosages of the scFvs are in the same range as those for the native 5G1.1 mAb.

In addition to lowering dosage levels, the anti-C5 antibodies used in the practice of the present invention (i.e., in treating GN) achieve important therapeutic effects not achieved with the anti-C6 antibodies. Specifically, the control and test animals in the C6 work exhibited both hypercellularity and narrowing of capillary lumens. In direct contrast, no such hypercellularity or narrowing of capillary lumens was seen when diseased individuals were treated with anti-C5 antibodies (see FIG. **1**).

Moreover, the anti-C5 antibodies used in the present invention achieve a reduction in glomerular enlargement, thus providing a clear demonstration of the unexpectedly powerful anti-inflammatory effects of the anti-C5 antibodies used in the practice of the invention. Nowhere in the C6 work is there any disclosure or suggestion of such a powerful anti-inflammatory effect.

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V. Anti-C5 Monoclonal Antibodies that Block Complement Hemolytic Activity and Block the Generation of C5a

Anti-C5 mabs that have the desirable ability to block complement hemolytic activity and to block the generation of C5a (and are thus preferred for use in the treatment of GN and other inflammatory conditions in accordance with the present invention) 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). Significantly, none of these anti-C5 mAbs has the properties of the novel anti-C5 mabs of the invention, i.e., none of them binds to the C5 alpha chain but not to the C5 cleavage product C5a, none of them has the ability to substantially block both complement hemolytic activity and the generation of C5a to substantially the same extent at the same concentration of antibody. It is noteworthy that an scFv derivative of the N19/8 antibody of Wurzner et al. 1991, supra, has been prepared, and that the N19/8 scFv has 50% less inhibitory activity towards C5a generation than the native N19/8 antibody (see Example 15). This is in contrast to the 5G1.1 scFv, which retained substantially all of its inhibitory activity towards C5a generation (see Example 12).

While not wishing to be bound by any particular theory of operation, it is believed that these distinctions are due to the specific binding characteristics of the antibodies of the invention. Accordingly, antibodies that do not bind to sites within the alpha chain of C5, and antibodies that bind to the C5 cleavage product C5a (free C5a), are believed to lack the ability to substantially block both complement hemolytic activity and the generation of C5a to substantially the same extent at the same concentration of antibody.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As discussed above, the present invention relates to the use of anti-C5 antibodies in treating patients suffering from GN and other diseases, and to specific C5 antibodies and antibody preparations. Preferably, and when used to treat GN, the anti-C5 antibodies are used in an amount effective to substantially reduce (e.g., reduce by at least about 50%) the cell-lysing ability of complement present in the patient's blood (the "cell-lysing ability of complement present in the patient's blood" is also referred to herein as the "serum complement activity of the patient's blood"). Reduction of the cell-lysing ability of complement present in the patient's blood can be measured by methods well known in the art such as, for example, by the chicken erythrocyte hemolysis method described below under the heading "Cell Lysis Assays."

To achieve the desired reductions, the 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 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.

Subject to the judgement of the physician, a typical therapeutic treatment includes a series of doses, which will usually be administered concurrently with the monitoring of clinical endpoints such as BUN levels, proteinuria levels, etc., with the dosage levels adjusted as needed to achieve the desired clinical outcome. Alternatively, levels of serum complement activity available in the patient's blood are monitored using the techniques set forth below under the heading "Cell Lysis Assays" to determine if additional doses or higher or lower dosage levels of antibodies are needed, with such doses being administered as required to maintain at least about a 50% reduction, and preferably about a 95% or greater reduction of serum complement activity. Other protocols can, of course, be used if desired as determined by the physician.

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. 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 formulations of the invention can be distributed as articles of manufacture comprising packaging material and the anti-C5 antibodies. When prepared for use in the treatment of GN, the packaging material will include a label which indicates that the formulation is for use in the treatment of kidney disease and may specifically refer to nephritis or glomerulonephritis.

The anti-C5 antibody is preferably a monoclonal antibody, although polyclonal antibodies produced and screened by conventional techniques can also be used if desired. As discussed above, the anti-C5 antibodies must be effective in reducing the cell-lysing ability of complement present in human blood. As also discussed above, this property of the antibodies can be determined by methods well known in the art such as, for example, by the chicken erythrocyte hemolysis method described below under the heading "Cell Lysis Assays".

The anti-C5 antibodies used in the practice of the invention bind to C5 or fragments thereof, e.g., C5a or C5b. Preferably, the anti-C5 antibodies are immunoreactive against epitopes on the beta chain of purified human complement component C5 and are capable of blocking the conversion of C5 into C5a and C5b by C5 convertase. This capability can be measured using the techniques described in

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Wurzner, et al., *Complement Inflamm* 8:328–340, 1991. Preferably, the anti-C5 antibodies are used to treat GN in an amount effective to reduce the C5 convertase activity available in the patient's blood by at least about 50%.

In a particularly preferred embodiment of the invention, the anti-C5 antibodies are not immunoreactive against epitopes on the beta chain, but rather are immunoreactive against epitopes within the alpha chain of purified human complement component C5. In this embodiment the antibodies are also capable of blocking the conversion of C5 into C5a and C5b by C5 convertase. In an especially preferred example of this embodiment they can provide this blockade at substantially the same concentrations needed to block hemolytic activity.

Within the alpha chain, the most preferred antibodies bind to an amino-terminal region, however, they do not bind to free C5a. Particularly preferred targets for these antibodies within the alpha chain include the 5G46k fragment, the 5G27k fragment, the 5G325aa peptide, the 5G200aa peptide, or the KSSKC epitope. The scope of the invention also includes the 5G46k fragment, the 5G27k fragment, the 5G325aa peptide, the 5G200aa peptide, or the KSSKC epitope (SEQ ID NO:1) that are useful as immunogens and screening ligands for producing the antibodies of the invention.

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. As discussed therein, antibodies are prepared using purified components of the complement membrane attack complex as immunogens. In accordance with the present invention, complement component C5 or C5b is preferably used as the immunogen. In accordance with a particularly preferred aspect of the present invention, the immunogen is the alpha chain of C5. Within the alpha chain, the most preferred immunogens include the 5G46k fragment, the 5G27k fragment, the 5G325aa peptide, or the 5G200aa peptide. A less preferred immunogen is the KSSKC epitope.

In accordance with the invention, the antibodies of the invention all share certain required functional properties. These are the ability to substantially inhibit complement hemolytic activity and to substantially inhibit the conversion of C5 to produce C5a. Preferably, but not requisitely, they provide these functions when used at a molar ratio of antibody to antigen (C5) of 3:1 or less.

A particularly preferred antibody of the invention is the 5G1.1 antibody (5G1.1, produced by the 5G1.1 hybridoma, ATCC designation HB-11625). Other particularly preferred antibodies of the present invention share the required functional properties discussed in the preceding paragraph and have any of the following characteristics:

(1) they compete with 5G1.1 for binding to portions of C5—the C5 alpha chain, the 5G46k fragment, the 5G27k fragment, the 5G325aa peptide (SEQ ID NO:1), the 5G200aa" peptide, or the KSSKC peptide—that are specifically immunoreactive with 5G1.1; and

(2) they specifically bind to the C5 alpha chain, the 5G46k fragment, the 5G27k fragment, the 5G325aa peptide, the 5G200aa" peptide, and/or the KSSKC peptide (SEQ ID NO:1). 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).

(3) they block the binding of C5 to either C3 or C4 (which are components of C5 convertase).

Also in accordance with the invention, the antibodies preferably should 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. In a particularly preferred embodiment, these anti-C5 antibodies will not impair the opsonization function associated with the activation of complement component C3 by a C3 convertase. Plasma C3 convertase activity can be measured by assaying plasma for the presence of C3a as described below under the heading "Histology." Preferably, the anti-C5 antibody produces essentially no reduction in plasma C3a levels.

General methods for the immunization of animals (in this case with C5 or C5b or another preferred immunogen), isolation of polyclonal antibodies or 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 C5 or C5b or another preferred 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.

As used herein, the term "antibodies" refers to immunoglobulins produced in vivo, as well as those produced in vitro by a hybridoma, and antigen binding fragments (e.g., Fab' preparations) of such immunoglobulins, as well as to recombinantly expressed antigen binding proteins, including immunoglobulins, chimeric immunoglobulins, "humanized" immunoglobulins, antigen binding fragments of such immunoglobulins, single chain antibodies, and other recombinant proteins containing antigen binding domains derived from immunoglobulins. As used herein, "antibodies" also refers to antigen binding synthetic peptides comprising sequences derived from the sequences of immunoglobulin antigen binding domains. As used herein, the term "recombinant mAbs" refers to recombinantly expressed antigen binding proteins. As used herein, the term "antibody-antigen binding site" refers to an antigen binding site of an antibody comprising at least one CDR sequence.

Antibodies whose amino acid sequences are full length immunoglobulin sequences that have not been truncated (e.g., to produce an scFv or an Fab) or mutated (e.g., spliced to form a chimeric antibody or humanized) are referred to herein as "native" antibodies. Publications describing methods for the preparation of such antibodies, in addition to those listed immediately above, include: Reichmann, et al., *Nature,* 332:323–327, 1988; Winter and Milstein, *Nature,* 349:293–299, 1991; Clackson, et al., *Nature,* 352:624–628, 1991; Morrison, *Annu Rev Immunol,* 10:239–265, 1992; Haber, *Immunol Rev,* 130:189–212, 1992; and Rodrigues, et al., *J Immunol,* 151:6954–6961, 1993.

While treatment of GN in accordance with the process of the present invention may be carried out using polyclonal or monoclonal antibodies, monospecific antibodies are preferred. As used herein "monospecific antibodies" refer to

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antibodies that bind to a specific region of a particular antigen. All monoclonal antibodies are monospecific, but polyclonal antibodies are typically not monospecific.

As is known in the art, however, monospecific polyclonal antibodies may be prepared by various methods. For example, a peptide (e.g., an oligopeptide—as used hereinafter and in the claims, a polymer of 5 to 200 amino acids) may be used as an immunogen. Another procedure allowing the preparation of monospecific polyclonal antibodies is the use of antigen affinity purification techniques to isolate a monospecific antibody population from a polyclonal antibody mixture. In accordance with the present invention, peptides are preferred as immunogens for the production and as affinity ligands for the purification of monospecific polyclonal anti-KSSKC antibodies.

The native (i.e., non-engineered) monoclonal antibodies of the invention are preferably prepared by conventional means, with the 5G46k fragment, the 5G27k fragment, the 5G200aa peptide, the 5G325aa peptide, and/or the KSSKC peptide (SEQ ID NO:1) (e.g., immobilized on a polypropylene membrane as described below in Example 13) being used as screening ligand(s). This involves testing hybridoma supernatants for binding to each screening ligand.

In one preferred embodiment, the native mAbs of the invention are prepared using the alpha chain of human C5, or fragments thereof, as immunogen. Preferred fragments of the alpha chain of human C5 for this purpose include the 5G46k fragment, the 5G27k fragment, and/or the 5G200aa fragment. Although less preferred, the KSSKC peptide (SEQ ID NO:1) may also be used as an immunogen.

Another (albeit less preferred) immunogen and screening ligand for the preparation of antibodies within the scope of the novel antibodies of the present invention is the "cleavage site peptide," i.e., the peptide spanning amino acids 725 through 754 of SEQ ID NO:2 (the C5a cleavage site), as discussed below in Example 13.

In another preferred embodiment of the invention, the native mAbs of the invention are prepared in transgenic mice expressing human immunoglobulins (see, for example, Green et al., Nature Genet. 1994, 7:13–21). In this case, the same preferred immunogens and screening ligands are used as described for the preparation of other native mAbs.

In another preferred embodiment of the invention, the recombinant mAbs of the invention are prepared by screening phage display libraries expressing recombinant mab-encoding polynucleotides (preferably encoding human recombinant mabs). See, for example, Ames et al., 1994, supra; Smith and Scott, Meth. Enzymol. 1993, 217:228; Kay et al., Gene, 1993, 128:59–65. This screening is carried out with the screening ligands described above for the preparation of native mAbs. The recombinant mAbs of the invention are prepared by subcloning the recombinant mAb-encoding polynucleotides into a suitable expression vector, expressing them in a suitable host (as described below), and isolating the recombinant mAbs.

The present invention provides recombinant expression vectors which include the synthetic, genomic, or cDNA-derived nucleic acid fragments of the invention, i.e. polynucleotides encoding the mabs of the invention. The nucleotide sequence coding for any of the mAbs of the invention can be inserted into an appropriate expression vector, i.e., a vector that contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals can also be supplied by the native or source gene and/or its flanking regions.

A variety of host vector systems may be utilized to express the recombinant expression vectors of the invention. These include, but are not limited to, mammalian cell systems infected with recombinant virus (e.g., vaccinia virus, adenovirus, retroviruses, etc.); mammalian cell systems transfected with recombinant plasmids; insect cell systems infected with recombinant virus (e.g., baculovirus); microorganisms such as yeast containing yeast expression vectors, or bacteria transformed with recombinant bacteriophage DNA, recombinant plasmid DNA, or cosmid DNA (see, for example, Goeddel, 1990).

Useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well-known cloning vector pBR322 (American Type Culture Collection-"ATCC"-, 10801 University Boulevard, Manassas, Va. 20110-2209, United States of America; ATCC Accession No. 37017). These pBR322 "backbone sections," or functionally equivalent sequences, are combined with an appropriate promoter and the structural gene to be expressed. Promoters commonly used in recombinant microbial expression vectors include, but are not limited to, the lactose promoter system (Chang, et al., Nature 275:615), the tryptophan (trp) promoter (Goeddel, et al., 1980, Gene Expression Technology, Volume 185. Academic Press, Inc., San Diego, Calif.) and the tac promoter, or a fusion between the tac and trp promoters referred to as the trc promoter (Maniatis, 1982, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). Particularly preferred promoters include the T7 promoter, which is used in conjunction with host cell expression of a T7 RNA polymerase (see Studier et al. 1990, Meth. Enzymol. 185:60–89), and the trc promoter, which is found in several commercially available vectors, as described below.

Preferred bacterial expression vectors include, but are not limited to, the pET vectors (see Studier et al. 1990, supra) and the Trc vectors. Many of the pET vectors are commercially available from Stratagene Cloning Systems (La Jolla, Calif.). A particularly preferred vector is the pET Trc SO5/NI vector described below (SEQ ID NO:18). A Trc vector, pTrc 99A, is available from Pharmacia. Other Trc vectors include the pSE vectors (Invitrogen, San Diego, Calif.).

Preferred bacteria for expression of recombinant mAbs include *Bacillus subtilis* and, most preferably, *Escherichia coli*. A particularly preferred strain of *E. coli* is strain W3110 (ATCC designation 27325). Under certain unusual conditions it may be necessary to use standard bacterial genetics methods to prepare derivative strains of W3110, for example, when a contaminating bacteriophage ("phage") is present in the laboratory where the bacterial manipulations are being carried out. Generally, and particularly for large scale preparation of the recombinant anti-KSSKC mAbs of the invention, it is preferred to use unmodified W3110, or another fully characterized strain.

In cases where phage contamination is a problem and disinfection is not practicable or desirable, it is preferred to identify the phage contaminant and to then use a fully characterized bacterial strain having a known mutation rendering the bacterium resistant to the phage. Preferably the mutation is a null mutant for the receptor for the phage. In some instances, however, the generation use of a relatively uncharacterized phage-resistant derivative strain may be acceptable, particularly in small scale experimental work. When such derivative strains are desired, they may be prepared using the methods described below in Example 11.

For most purposes the use of unmodified W3110 or another fully characterized bacterial strain is generally preferred. This is particularly true for the preparation of phar-

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maceutical agents comprising the recombinant anti-KSSKC mAbs of the invention. This is because of the problems, well known in the art, of using bacterial strains containing uncharacterized or partially characterized mutations for the production of ingredients of pharmaceutical agents.

The recombinant mAbs of the invention may also be expressed in fungal hosts, preferably yeast of the Saccharomyces genus such as *S. cerevisiae*. Fungi of other genera such as Aspergillus, Pichia or Kluyveromyces may also be employed. Fungal vectors will generally contain an origin of replication from the 2 μm yeast plasmid or another autonomously replicating sequence (ARS), a promoter, DNA encoding a mAb of the invention, sequences directing polyadenylation and transcription termination, and a selectable marker gene. Preferably, fungal vectors will include an origin of replication and selectable markers permitting transformation of both *E. coli* and fungi.

Suitable promoter systems in fungi include the promoters for metallothionein, 3-phosphoglycerate kinase, or other glycolytic enzymes such as enolase, hexokinase, pyruvate kinase, glucokinase, the glucose-repressible alcohol dehydrogenase promoter (ADH2), the constitutive promoter from the alcohol dehydrogenase gene, ADH1, and others. See, for example, Schena, et al. 1991 Meth. Enzymol. 194:389–398. Secretion signals, such as those directing the secretion of yeast alpha-factor or yeast invertase, can be incorporated into the fungal vector to promote secretion of a soluble recombinant mAb into the fungal growth medium. See Moir, et al., 1991, Meth. Enzymol. 194:491–507.

Preferred fungal expression vectors can be assembled using DNA sequences from pBR322 for selection and replication in bacteria, and fungal DNA sequences, including the ADH1 promoter and the alcohol dehydrogenase ADH1 termination sequence, as found in vector pAAH5 (Ammerer, 1983, Meth. Enzymol. 101:192). The ADH1 promoter is effective in yeast in that ADH1 mRNA is estimated to be 1–2% of total poly(A) RNA.

Various mammalian or insect cell culture systems can be employed to express recombinant mAbs. Suitable baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow, et al., 1988. Examples of suitable mammalian host cell lines include the COS cell of monkey kidney origin, mouse L cells, murine C127 mammary epithelial cells, mouse Balb/3T3 cells, Chinese hamster ovary cells (CHO), human 293 EBNA and HeLa cells, myeloma, and baby hamster kidney (BHK) cells, with myeloma cells, CHO cells, and human 293 EBNA cells being particularly preferred.

Mammalian expression vectors may comprise nontranscribed elements such as origin of replication, a suitable promoter and enhancer linked to the recombinant mAb gene to be expressed, and other 5' or 3' flanking sequences such as ribosome binding sites, a polyadenylation sequence, splice donor and acceptor sites, and transcriptional termination sequences.

The transcriptional and translational control sequences in mammalian expression vector systems to be used in transforming vertebrate cells may be provided by viral sources. For example, commonly used promoters and enhancers are derived from Polyoma virus, Adenovirus, Simian Virus 40 (SV40), and human cytomegalovirus, including the cytomegalovirus immediate-early gene 1 promoter and enhancer (CMV).

Particularly preferred eukaryotic vectors for the expression of recombinant anti-KSSKC mAbs are pAPEX-1 (SEQ ID NO:3 and, more preferably, pAPEX-3p, SEQ ID NO:4. The vector pAPEX-1 is a derivative of the vector pcDNAI/Amp (Invitrogen) which was modified to increase protein expression levels. First, the 3'-untranslated SV40 small-t antigen intron was removed by deletion of a 601 base pair XbaI/HpaI fragment since this intron is susceptible to aberrant splicing into upstream coding regions (Evans and Scarpulla, 1989 Gene 84:135; Huang and Gorman, 1990, Molec. Cell Biol. 10:1805). Second, a chimeric adenovirus-immunoglobulin hybrid intron was introduced into the 5'-untranslated region by replacing a 484 base pair NdeI-NotI fragment with a corresponding 845 base pair NdeI-NotI fragment from the vector pRc/CMV7SB (Sato et al., 1994, J. Biol. Chem. 269:17267). Finally, to increase plasmid DNA yields from *E. coli*, the resulting CMV promoter expression cassette was shuttled into the vector pGEM-4Z (Promega Corp. Madison, Wis.).

The vector pAPEX-3 is a derivative of the vector pDR2 (Clontech Laboratories, Inc. Palo Alto, Calif.) in which the EBNA gene was first removed by deletion of a 2.4 kb ClaI/AccI fragment. The RSV promoter was then replaced with the CMV promoter and the adenovirus/immunoglobulin chimeric intron by exchanging a 450 bp MluI/BamHI fragment from pDR2 with a 1.0 kb MluI/BamHI fragment from the vector pAPEX-1. For construction of pAPEX-3P, a 1.7 kb BstBI/SwaI fragment containing the HSV tk promoter and hygromycin phosphotransferase (hyg) gene was removed from pAPEX-3 and replaced with a 1.1 kb SnaBI/NheI fragment containing the SV40 early promoter and puromycin acetyltransferase (pac) gene (Morgenstern and Land, 1990, Nucleic Acids Res. 18:3587–3596) plus a 137 bp XbaI/ClaI fragment containing an SV40 polyadenylation signal from the vector pAPEX-1.

A particularly preferred host cell for the expression of recombinant mAb-encoding inserts in the pAPEX vectors is the human 293 EBNA cell line (Invitrogen, San Diego, Calif.).

Another preferred eukaryotic vector for the expression of recombinant mAbs is pcDNAI/Amp (Invitrogen Corporation, San Diego, Calif.). The pcDNAI/Amp expression vector contains the human cytomegalovirus immediate-early gene I promoter and enhancer elements, the Simian Virus 40 (SV40) consensus intron donor and acceptor splice sequences, and the SV40 consensus polyadenylation signal. This vector also contains an SV40 origin of replication that allows for episomal amplification in cells (e.g., COS cells, MOP8 cells, etc.) transformed with SV40 large T antigen, and an ampicillin resistance gene for propagation and selection in bacterial hosts.

Purified recombinant mAbs are prepared by culturing suitable host/vector systems to express the recombinant mAb translation products of the nucleic acid molecules of the present invention, which are then purified from the culture media or cell extracts of the host system, e.g., the bacteria, insect cells, fungal, or mammalian cells. Fermentation of fungi or mammalian cells that express recombinant mAb proteins containing a histidine tag sequence (a sequence comprising a stretch of at least 5 histidine residues) as a secreted product greatly simplifies purification. Such a histidine tag sequence enables binding under specific conditions to metals such as nickel, and thereby to nickel (or other metal) columns for purification. Recombinant mAbs may also be purified by protein G affinity chromatography (Proudfoot et al., 1992, Protein Express. Purif. 3:368).

Additional preferred embodiments are numbered and set forth below as "favored embodiments."

FAVORED EMBODIMENTS

1. A method for the treatment of glomerulonephritis in a patient in need of such treatment comprising introducing an

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antibody that binds to complement component C5 into the patient's bloodstream in an amount effective to substantially reduce the cell-lysing ability of complement present in the patient's blood.

2. The method of favored embodiment 1 wherein the antibody reduces the conversion of complement component C5 into complement components C5a and C5b.

3. The method of favored embodiment 1 wherein the antibody binds to C5b.

4. The method of favored embodiment 1 wherein the antibody does not substantially inhibit formation of complement component C3b.

5. The method of favored embodiment 1 wherein the antibody is introduced into the patient's bloodstream in a dose that is not greater than 0.1 grams per kilogram.

6. An article of manufacture comprising packaging material and a pharmaceutical agent contained within said packaging material, wherein:

(a) said pharmaceutical agent comprises an antibody to complement component C5, said antibody being effective in substantially reducing the cell-lysing ability of complement present in the patient's blood; and

(b) said packaging material comprises a label which indicates that said pharmaceutical agent is for use in the treatment of kidney disease.

7. The article of manufacture of favored embodiment 6 wherein the label indicates that said pharmaceutical agent is for use in the treatment of nephritis.

8. The article of manufacture of favored embodiment 7 wherein the label indicates that said pharmaceutical agent is for use in the treatment of glomerulonephritis.

9. The article of manufacture of favored embodiment 6 wherein the pharmaceutical agent is to be used at a dosage level not greater than 0.1 grams per kilogram.

10. An antibody comprising at least one antibody-antigen binding site, said antibody exhibiting specific binding to human complement component C5, said specific binding being targeted to the alpha chain of human complement component C5, wherein the antibody inhibits complement activation in a human body fluid and does not specifically bind to the human complement activation product free C5a.

11. The antibody of favored embodiment 10 wherein the inhibition of complement activation in the human body fluid is measurable as a substantial increment of blockade of C5a generation and a substantial increment of blockade of complement hemolytic activity in the body fluid, said increment of blockade of C5a generation being substantially equal to said increment of blockade of complement hemolytic activity.

12. The antibody of favored embodiment 10 wherein, upon binding to human C5, the antibody substantially inhibits the ability of C5 to bind to human complement component C3.

13. The antibody of favored embodiment 10 wherein, upon binding to human C5, the antibody substantially inhibits the ability of C5 to bind to human complement component C4.

14. The antibody of favored embodiment 10 wherein the antibody binds specifically with a 5G46k fragment.

15. The antibody of favored embodiment 10 wherein the antibody binds specifically to a 5G27k fragment.

16. The antibody of favored embodiment 10 wherein the antibody binds specifically to a 5G325aa peptide.

17. The antibody of favored embodiment 10 wherein the antibody binds specifically to a 5G200aa peptide.

18. The antibody of favored embodiment 10 wherein the antibody binds specifically to a KSSKC peptide.

19. The antibody of favored embodiment 10 wherein the inhibition of complement activation in the human body fluid is measurable as a substantially complete blockade of C5a generation in the body fluid and a substantially complete blockade of complement hemolytic activity in the body fluid when the antibody is added to the body fluid at a concentration yielding a ratio equal to or less than 10 moles of antibody-antigen binding sites of the antibody to 1 mole of human C5 in the body fluid.

20. The antibody of favored embodiment 19 wherein the concentration yields a ratio equal to or less than 3 moles of antibody-antigen binding sites of the antibody to 1 mole of human C5 in the body fluid.

21. Hybridoma 5G1.1 having ATCC designation HB-11625.

22. An antibody produced by the hybridoma of favored embodiment 21.

23. An antibody that can compete with the antibody of favored embodiment 22 for binding to the alpha chain of human C5.

24. A nucleic acid molecule comprising a nucleotide sequence encoding an scFv polypeptide comprising an amino acid sequence corresponding to amino acid 1 through amino acid 248 of SEQ ID NO:7.

25. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:9.

26. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:10.

27. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:9.; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:10.

28. An isolated polypeptide comprising an amino acid sequence encoded by the nucleic acid molecule of favored embodiment 24, favored embodiment 25, or favored embodiment 26, wherein the polypeptide is an antibody.

29. A nucleic acid vector comprising a first nucleic acid molecule covalently and operatively linked to a second nucleic acid molecule so that a host containing the vector expresses the polypeptide coded for by the first nucleic acid molecule, wherein the first nucleic acid molecule is the nucleic acid molecule of favored embodiment 24, favored embodiment 25, or favored embodiment 26.

30. A recombinant host cell containing the nucleic acid vector of favored embodiment 29.

31. A method for producing an isolated C5 antibody polypeptide comprising growing the recombinant host cell of favored embodiment 30 such that the polypeptide encoded by the first nucleic acid molecule of the vector is expressed by the host cell, and isolating the expressed polypeptide, wherein the expressed polypeptide is an anti-C5 antibody.

32. The isolated anti-C5 antibody of favored embodiment 31.

33. A nucleic acid molecule comprising a nucleotide sequence encoding an scFv comprising an amino acid sequence corresponding to amino acid 1 through amino acid 248 of SEQ ID NO:8.

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34. A nucleic acid molecule comprising a nucleotide sequence encoding an scFv comprising an amino acid sequence corresponding to amino acid 1 through amino acid 248 of SEQ ID NO:17.

35. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable light chain region amino acid sequence corresponding to amino acid 1 through amino acid 108 of SEQ ID NO:15.

36. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:14.

37. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:16.

38. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:12.

39. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:11.

40. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 1 through amino acid 108 of SEQ ID NO:15; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:16.

41. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 1 through amino acid 1 through amino acid 108 of SEQ ID NO:15; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:12.

42. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 1 through amino acid 108 of SEQ ID NO:15; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:11.

43. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:14; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:16.

44. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:14; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:12.

45. An isolated protein comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:14; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:11.

46. An isolated protein comprising the amino acid sequence encoded by the nucleic acid molecule of favored embodiment 33, favored embodiment 34, favored embodiment 35, favored embodiment 36, favored embodiment 37, favored embodiment 38, or favored embodiment 39, wherein the isolated protein is an anti-C5 antibody.

47. A nucleic acid vector comprising a first nucleic acid molecule covalently and operatively linked to a second nucleic acid molecule so that a host containing the vector expresses the polypeptide coded for by the first nucleic acid molecule, wherein the first nucleic acid molecule is the nucleic acid molecule of favored embodiment 33, favored embodiment 34, favored embodiment 35, favored embodiment 36, favored embodiment 37, favored embodiment 38, or favored embodiment 39.

48. A recombinant host cell containing the nucleic acid vector of favored embodiment 47.

49. A method for producing an isolated anti-C5 antibody protein comprising growing the recombinant host cell of favored embodiment 48 such that a protein encoded by the nucleic acid molecule is expressed by the host cell, and isolating the expressed protein, wherein the expressed protein is an anti-C5 antibody.

50. The isolated anti-C5 antibody of favored embodiment 47.

51. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid 93 through amino acid 98 of SEQ ID NO:7;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

52. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid 91 through amino acid 99 of SEQ ID NO:8;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

53. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 156 through amino acid 159 of SEQ ID NO:7;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

54. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 152 through amino acid 161 of SEQ ID NO:8;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

55. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence cor-

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responding to amino acid 179 through amino acid 182 of SEQ ID NO:7;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

56. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 176 through amino acid 186 of SEQ ID NO:8;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

57. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 226 through amino acid 236 of SEQ ID NO:7;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

58. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 225 through amino acid 237 of SEQ ID NO:8;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

59. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid 91 through amino acid 99 of SEQ ID NO:8;

(b) a nucleotide sequence encoding a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 152 through amino acid 161 of SEQ ID NO:8;

(c) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 176 through amino acid 186 of SEQ ID NO:8; and

(d) a nucleotide sequence encoding a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 225 through amino acid 237 of SEQ ID NO:8.

60. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid 91 through amino acid 99 of SEQ ID NO:8;

(b) a nucleotide sequence encoding a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 152 through amino acid 161 of SEQ ID NO:8;

(c) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 176 through amino acid 192 of SEQ ID NO:8; and

(d) a nucleotide sequence encoding a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 225 through amino acid 237 of SEQ ID NO:8.

61. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid 91 through amino acid 99 of SEQ ID NO:8;

(b) a nucleotide sequence encoding a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 152 through amino acid 161 of SEQ ID NO:8;

(c) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 179 through amino acid 182 of SEQ ID NO:7; and

(d) a nucleotide sequence encoding a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 225 through amino acid 237 of SEQ ID NO:8.

62. An isolated protein comprising the amino acid sequence encoded by the nucleic acid molecule of favored embodiment 51, favored embodiment 52, favored embodiment 53, favored embodiment 54, favored embodiment 55, favored embodiment 56, favored embodiment 57, favored embodiment 58, favored embodiment 59, favored embodiment 60 or favored embodiment 87.

63. The isolated protein of favored embodiment 62 wherein the protein is an anti-C5 antibody.

64. A nucleic acid vector comprising a first nucleic acid molecule, said first nucleic acid molecule corresponding to the nucleic acid molecule of favored embodiment 51, favored embodiment 52, favored embodiment 53, favored embodiment 54, favored embodiment 55, favored embodiment 56, favored embodiment 57, favored embodiment 58, or favored embodiment 87 covalently and operatively linked to a second nucleic acid molecule so that a host containing the vector expresses the protein encoded by the first nucleic acid molecule.

65. A recombinant host cell containing the nucleic acid vector of favored embodiment 64.

66. A method for producing an anti-C5 antibody comprising growing the recombinant host cell of favored embodiment 65 so that the protein encoded by the nucleic acid molecule is expressed by the host cell, and isolating the expressed protein, wherein the expressed protein is an anti-C5 antibody.

67. The anti-C5 antibody of favored embodiment 66.

68. An isolated 5G46k fragment of human complement component C5.

69. An isolated 5G27k fragment of human complement component C5.

70. An isolated 5G325aa peptide.

71. An isolated 5G200aa peptide.

72. An isolated oligopeptide comprising an amino acid sequence corresponding to amino acid 8 through amino acid 12 of SEQ ID NO:1, i.e., Lys Ser Ser Lys Cys, or in single letter notation, KSSKC.

73. A method of inducing an animal to produce an anti-C5 antibody comprising repeatedly immunizing an animal with the isolated alpha chain of human C5.

74. A method of inducing an animal to produce an anti-C5 antibody comprising immunizing an animal with the isolated 5G46k fragment of favored embodiment 68.

75. A method of inducing an animal to produce an anti-C5 antibody comprising immunizing an animal with the isolated 5G27k fragment of favored embodiment 69.

76. A method of inducing an animal to produce an anti-C5 antibody comprising immunizing an animal with the isolated 5G325aa peptide of favored embodiment 70.

77. A method of inducing an animal to produce an anti-C5 antibody comprising immunizing an animal with the isolated 5G200aa peptide of favored embodiment 71.

78. A method of inducing an animal to produce an anti-C5 antibody comprising immunizing an animal with the isolated oligopeptide of favored embodiment 72.

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79. A method of identifying an anti-C5 antibody comprising screening candidate antibodies with the isolated alpha chain of human C5.

80. A method of identifying an anti-C5 antibody comprising screening candidate antibodies with the isolated 5G46k fragment of favored embodiment 68.

81. A method of identifying an anti-C5 antibody comprising screening candidate antibodies with the isolated 5G27k fragment of favored embodiment 69.

82. A method of identifying an anti-C5 antibody comprising screening candidate antibodies with the isolated 5G325aa peptide of favored embodiment 70.

83. A method of identifying an anti-C5 antibody comprising screening candidate antibodies with the isolated 5G200aa peptide of favored embodiment 71.

84. A method of identifying an anti-C5 antibody comprising screening candidate antibodies with the isolated oligopeptide of favored embodiment 72.

85. A method of treating a patient in need of complement inhibition comprising administering the antibody of favored embodiment 10, favored embodiment 22, favored embodiment 23, favored embodiment 28, favored embodiment 32, favored embodiment 46, favored embodiment 50, favored embodiment 63, or favored embodiment 67 to the patient in an amount effective to substantially reduce hemolytic activity in a body fluid of the patient.

86. The antibody of favored embodiment 10 wherein the antibody is a recombinant antibody that comprises a human constant domain.

87. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 176 through amino acid 192 of SEQ ID NO:8;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

88. An isolated nucleic acid molecule comprising:

(a) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 176 through amino acid 192 of SEQ ID NO:8;

(b) a sequence complementary to (a); or

(c) both (a) and (b).

89. An isolated antibody comprising any one of the CDR regions of CO12, CO13, CO14, Co15, DO12b, DO12C, DO12D.

90. An isolated nucleic acid molecule encoding the antibody of favored embodiment 89.

EXAMPLES

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.

Induction of GN in Mice

Four month old female B10.D2/nSnJ mice averaging approximately 25 gms each were obtained from the Jackson Laboratory, Bar Harbor, Me. Mice were injected with 0.1 mL daily (six days per week) of a 40 mg/mL solution of horse apoferritin (HAF), which was prepared by dilution of a saline solution of HAF (Sigma Chemical Company Catalog No. A-3641) with PBS.

Anti-C5 Monoclonal Antibodies

Monoclonal antibodies that bind to complement component C5 of the mouse were prepared by standard methods as an IgG fraction from supernatants of cultures of hybridoma BB5.1 (Frei, et al., 1987), which was obtained from Dr. Brigitta Stockinger of the National Institute for Medical Research, Mill Hill, London, England.

Histology

Kidneys were subjected to microscopic analysis using standard histochemical staining and immunofluorescence techniques. Periodic Acid Schiff (PAS) staining of 5 paraffin sections was by standard methods using a HARLECO PAS histochemical reaction set (EM Diagnostic Systems, Gibbstown, N.J., number 64945/93) according to the manufacturer's directions.

Immunofluorescence staining of 5μ cryostat sections was carried out by standard methods using FITC conjugated sheep anti-mouse C3 (Biodesign International, Kennebunk, Me, Catalog No. W90280F) to detect murine complement component C3, or FITC conjugated goat anti-mouse IgG, IgA, and IgM (Zymed Laboratories, South San Francisco, Calif., Catalog No. 65-6411) to detect immune complexes.

Urine Assays

Protein and glucose levels were determined by spotting urine samples on CHEMSTRIP 2GP dipsticks (Boehringer Mannheim Diagnostics, Indianapolis, Ind., Catalog No. 200743). The detection areas of these strips change color when exposed to urine containing protein or glucose; a lack of color change indicates no detectable protein or glucose is present. The level of analyte in the urine being tested is read out by matching changed colors with color charts supplied by the manufacturer. The urine protein chart shows colors corresponding to trace, 30, 100, and 500 mg/dL.

Cell Lysis Assays

The cell-lysing ability of complement in blood can be determined using hemolytic assays that are performed as follows: Chicken erythrocytes are washed well in GVBS (Rollins, et al., *J Immunol* 144:3478–3483, 1990, Sigma Chemical Co. St. Louis, Mo., catalog No. G-6514) and resuspended to 2×10^8/mL in GVBS. Anti-chicken erythrocyte antibody (IgG fraction of anti-chicken-RBC antiserum, Intercell Technologies, Hopewell, N.J.) is added to the cells at a final concentration of 25 μg/mL and the cells are incubated for 15 min. at 23° C. The cells are washed 2× with GVBS and 5×10^6 cells are resuspended to 30 μL in GVBS. A 100 μL volume of serum test solution is then added to yield a final reaction mixture volume of 130 μL. As used herein, reference to the serum percentage and/or serum input in these assays indicates the percent serum in the 100 μL volume of serum test solution.

For assays of mouse serum activity, the 100 μL volume of serum test solution contained 50 μL of diluted (in GVBS) mouse serum and 50 μL of human C5 deficient serum (Quidel Corporation, San Diego, Calif.). For assays of human serum activity, the serum test solution may contain up to 100% human plasma or serum, with hybridoma supernatants and/or GVBS being added to yield the 100 μL volume. For the assays used to screen hybridoma supernatants discussed below in Example 7, each 100 μL volume of serum test solution contained 50 μL of hybridoma supernatant and 50 μL of a 10% solution of human serum in GVBS, yielding a 5% human serum input.

After incubation for 30 min. at 37° C., percent hemolysis was calculated relative to a fully lysed control sample. Hemolysis was determined by spinning the cells down and measuring released hemoglobin in the supernatant as the optical density at 415 nm.

A 50% reduction in hemolysis after treatment with the anti-C5 antibodies used in the practice of the invention means that the percent hemolysis after treatment is one half of the percent hemolysis before treatment.

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Example 1

Anti-C5 Antibodies Inhibit Glomerular Inflammation and Enlargement

This example illustrates that anti-C5 antibodies will inhibit glomerular inflammation and enlargement.

The protocol for these experiments was as follows. GN-induced mice were treated with anti-C5 antibodies or with PBS as a control after 2 weeks of GN induction. Each mouse received 750 μg of anti-C5 monoclonal antibodies in PBS (30 mg/kg in a 25 gm mouse) or an equal volume of PBS alone. The amount injected was from 0.25 to 0.365 mL (the concentration of antibodies in PBS varied), which was administered by intraperitoneal injection once a day, six days a week. After an additional 2 weeks of induction and treatment, the animals were sacrificed and kidneys were harvested and prepared for histological examination as described above. Kidneys were also obtained from age-matched uninduced and untreated control mice.

FIG. **1** shows sections of mouse kidneys with a single glomerulus located centrally amidst surrounding interstitium and cross sections of convoluted tubules in each section. As can be seen therein, the kidneys of the GN-induced, PBS-treated mice (FIG. **1**B) developed severe crescentic glomerular pathology, including inflammatory glomerular hypercellularity, apparent basement membrane thickening, and glomerular enlargement, while the glomeruli of the GN-induced, anti-C5-treated animals (FIG. **1**C) were essentially indistinguishable from the glomeruli of the normal healthy kidneys of the uninduced untreated mice (FIG. **1**A).

Note that in the glomeruli with severe crescentic pathology, the size of the glomerular capillary network (glomerular tuft) is not enlarged, but shows signs of compression by a crescentic-shaped proliferation of epithelial cells and PAS-positive material, and the Bowman's capsule is dramatically enlarged. Also note that in the section of diseased glomerulus shown in FIG. **1**B, the capillary network is split in half by a projection of the hypercellular crescentic mass.

The non-inflamed glomerulus of the uninduced untreated mouse shown in FIG. **1**A is approximately 100μ in diameter; the inflamed glomerulus of the GN-induced, PBS treated mouse shown in FIG. **1**B is approximately 175μ in diameter; the non-inflamed glomerulus of the GN-induced, anti-C5-treated mouse shown in FIG. **1**C is approximately 90μ in diameter.

Example 2

Anti-C5 Antibodies Prevent/Reduce Proteinuria Associated with GN

This example demonstrates that treatment with anti-C5 antibodies results in the prevention/reduction of kidney damage as evidenced by the lack of significant amounts of protein in the urine (i.e. the presence of less than 100 mg/dL of protein in the urine).

The protocol for the experiments of this example was the same as that used in the experiments of Example 1. Five PBS-treated, GN-induced mice, 6 anti-C5-treated, GN-induced mice, and 4 age-matched untreated uninduced mice were used in this study. A first set of urine samples was analyzed prior to treatment after the initial 2 week induction period. A second set of urine samples was analyzed after the 2 week treatment period. None of the untreated uninduced control animals had detectable protein in their urine at either of these timepoints.

The results obtained with the GN-induced mice are set forth in Table 1. As shown therein, at the end of the 2 week PBS treatment period, 4 out of the 5 PBS treated (control) animals developed significant proteinuria, i.e., at least 100 mg/dL of protein in the urine. The fifth animal (mouse D in Table 1) did not have detectable protein in the urine at either timepoint but, unlike the other mice in the study, was found to have very high levels of glucose in the urine after the 2 week PBS treatment period, suggesting that this animal was physiologically compromised.

In the anti-C5-treated, GN-induced group, the one mouse that developed significant proteinuria at the end of the initial 2 week induction period (mouse 6 in Table 1) improved by the end of the 2 week antibody treatment period. In addition, in contrast to the development of significant proteinuria in 4 out of 5 PBS-treated, GN-induced mice, none of the anti-C5-treated, GN-induced mice exhibited significant proteinuria at the end of the 2 week antibody treatment period.

Example 3

Anti-C5 Antibodies do not Inhibit Glomerular Immune Complex Deposition

This example demonstrates that anti-C5 antibodies used in the practice of the invention achieve their therapeutic effects even though immune complexes are deposited in the glomeruli of treated animals at equivalent levels to those seen in the glomeruli of PBS-treated animals. The example further illustrates that the mechanism of operation of the anti-C5 antibodies is not through the inhibition of immune complex deposition in the glomerulus.

The protocol used in the experiments of this example was the same as that used in the experiments of Example 1. Immunofluorescence staining as described above was performed on sections from the same kidneys harvested in Example 1.

The results are shown in FIG. **2**. As can be seen in this figure, equivalent amounts of immune complexes were deposited in the glomeruli of the kidneys of both the PBS-treated, GN-induced mice (FIG. **2**B) and the anti-C5-treated, GN-induced mice (FIG. **2**C), but not in the untreated uninduced controls (FIG. **2**A). Kidneys of GN-induced mice harvested after the 2 week induction period, but before treatment, showed immune complex deposits in the glomeruli, but at lower levels (as indicated by lower fluorescence intensity) than in the kidney sections shown in FIG. **2**B and FIG. **2**C.

Example 4

Anti-C5 Antibodies Inhibit C5b-9 Generation

This example demonstrates that the anti-C5 antibodies used in the practice of the invention inhibit C5b-9 generation. C5b-9 generation was assayed in 2 ways: (1) by testing the cell-lysing (hemolytic) ability of blood samples, and (2) by measuring levels of soluble C5b-9 in blood samples.

FIG. **3** shows the results of cell lysis assays performed as described above, with mouse serum added to the percentage indicated on the X axis ("serum input %"). In these assays, serum from GN-induced animals treated with either anti-C5 antibodies in PBS or PBS alone (see above) was assayed at the end of the two week treatment period. Serum from normal, uninduced, uninjected mice ("normal mouse serum") obtained from Sigma Chemical Company (St. Louis, Mo., Catalog No. S-3269) was also assayed as an additional control. These results indicate that the anti-C5

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monoclonal antibody administered to mice at a dosage of 30 mg/Kg completely blocked the cell lysing ability of mouse blood at serum input levels 4-fold higher than the levels of normal serum that produce maximum hemolysis in the assay.

The effects of an anti-C5 monoclonal antibody raised to human C5 was evaluated in circulating human blood. Hybridoma N19/8 (Wurzner, et al., 1991) was obtained from Dr. Otto Götze, Department of Immunology, University of G öttingen, FRG. The C5 monoclonal antibody was prepared following immunization of mice with purified human C5 protein as described in Wurzner, et al., (1991). The hybridoma was propagated in mice, and the monoclonal antibody recovered and purified as an IgG fraction from mouse ascites fluid (*Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1988; *Current Protocols In Immunology*, John Wiley & Sons, New York, 1992).

To carry out these experiments, as well as others described below in Examples 5 and 6, 300 mL of whole human blood was drawn from a healthy human donor and additionally a 1 mL sample was removed as a control sample for later analysis. The blood was diluted to 600 mL by the addition of Ringer's lactate solution containing 10 U/mL heparin. The anti-C5 mAb (30 mg in sterile PBS) was added to the diluted blood to a final concentration of 50 μg/mL (results using test samples obtained in this way are labeled "+anti-C5 sample" in FIG. **4** and FIG. **6**). In a control experiment, an equal volume of sterile PBS was added to diluted blood (results using control samples obtained in this way are labeled "−anti-C5 sample" in FIG. **4** and FIG. **6**).

The blood was then used to prime the extracorporeal circuit of a COBE CML EXCEL membrane oxygenator cardiopulmonary bypass (CPB) machine (Cobe BCT, Inc., Lakewood, Colo.) and circulation through the circuit was started. The circuit was cooled to 28° C. and circulated for 60 minutes. The circuit was then warmed to 37° C. and circulated for an additional 30 minutes, after which time the experiment was terminated. Mechanical circulation of blood in this fashion activates the complement cascade. Samples were taken at several time points.

At each time point an aliquot of blood was taken, and subaliquots were centrifuged to remove all cells and the remaining plasma diluted 1:1 in QUIDEL sample preservation solution (Quidel Corporation, San Diego, Calif.) and stored at −80° C. for subsequent evaluation of soluble C5b-9 (sC5b-9) generation. Diluted subaliquots of plasma were also frozen for evaluation of C3a generation (see Example 5, below). Undiluted subaliquots of plasma were frozen at −80° C. for analysis in hemolytic assays to evaluate the pharmacokinetics of the effects of the anti-C5 antibodies on the cell lysing ability of complement present in the blood (see Example 6, below). These experiments are also discussed in copending U.S. patent application Ser. No. 08/217,391, filed Mar. 23, 1994, now U.S. Pat. No. 5,853,722.

sC5b-9 assays were performed before the addition of the antibody or the commencement of the CPB circuit (labeled "Pre Tx" in FIG. **4** and FIG. **6**) using undiluted blood (i.e. blood from the 1 mL sample taken before the blood was diluted with Ringer's lactate solution—labeled "undil" in FIG. **4** and FIG. **6**) and Ringer's lactate solution diluted blood (labeled "dil" in FIG. **4** and FIG. **6**). Samples of Ringer's lactate solution diluted blood to which the antibody had been added (labeled "Post Tx" in FIG. **4** and FIG. **6**) were assayed at the times indicated after starting the CPB circuit.

As can be seen in FIG. **4**, while sC5b-9 levels were more than 4-fold higher in untreated samples after 90 minutes of circulation than before circulation, the anti-C5 antibody completely inhibited C5b-9 generation throughout the 90 minute time course of circulation so that sC5b-9 levels during circulation were essentially equivalent to control, uncirculated samples, at all timepoints.

Example 5

Anti-C5 Antibodies do not Inhibit C3 Deposition or Activation

This example demonstrates that treatment with anti-C5 antibodies does not result in the inhibition of the activation of complement component C3 or in the deposition of C3 or its activated fragments in glomeruli.

The deposition of C3, or the fragments generated by its activation (e.g., C3a and C3b), in the glomeruli of GN-induced and GN-uninduced mice was visualized by immunofluorescence staining with a FITC-conjugated sheep anti-mouse C3 antibody preparation using standard methods, as described above. As can be seen in FIG. **5**, kidneys of the PBS-treated (FIG. **5**B) and the anti-C5 antibody-treated (FIG. **5**C) GN-induced mice had roughly equivalent levels of C3 immunoreactive material in the glomeruli, while the uninduced untreated control mice had only traces of C3 immunoreactive material in their kidneys (FIG. **5**A).

Note that the print shown in FIG. **5**A was overexposed compared to those of FIG. **5**B and FIG. **5**C to show the very slight levels of reactivity present in normal uninduced kidneys. Kidneys of GN-induced mice harvested after the 2 week induction period, but before treatment, showed C3 immunoreactive materials in the glomeruli, but at lower levels (as indicated by lower fluorescence intensity) than in the kidney sections shown in FIG. **5**B and FIG. **5**C.

Anti-human C5 antibodies were also tested for possible inhibition of C3 activation in human blood prepared and circulated as described above in Example 4. Activation of complement component C3 was indicated by the presence in the blood of the C3 activation product C3a. C3a assays were performed as follows.

The plasma samples that had previously been diluted in QUIDEL sample preservation solution and frozen (see Example 4) were assayed for the presence of C3a by using the QUIDEL C3A EIA kit (Quidel Corporation, San Diego, Calif.) according to the manufacturer's specifications. Concentrations of C3a in the samples is expressed as ng/well as determined by comparison to a standard curve generated from samples containing known amounts of human C3a.

As seen in FIG. **6**, the addition of the anti-C5 mAb had no inhibitory effect on the production of C3a during the circulation of human blood in this experiment.

Example 6

Pharmacokinetics of Anti-C5 Antibodies

The in vivo duration of action of mAb BB5.1, and a Fab' fragment of mAb BB5.1 (prepared by standard methods) was determined in normal female BALB/cByJ mice (averaging approximately 20 gms each) which were obtained from the Jackson Laboratory, Bar Harbor, Me. The mice were given a single intravenous injection (at 35 mg/kg body weight) of the mAB or the Fab' fragment of the mAb (or an equal volume of PBS as a control). Blood samples were collected from the retroorbital plexus at 1, 4, 24, 96, and 144 hours after administration of PBS; 4, 16, and 24 hours after administration of the Fab' fragment of mAb

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BB5.1; and 4, 24, 48, 72, 96, and 144 hours after administration of intact mAb BB5.1.

FIG. 7A shows the time course of inhibition of the cell-lysing ability of complement in mouse blood (determined, by testing serum obtained from the blood and diluted to 2.5%, as described above) after the in vivo administration of the mAb, the Fab' fragment, or the PBS. As shown in the figure, the mAb almost completely inhibited the hemolytic activity of the blood throughout the 6 day test period. The Fab', however, had a half-life of approximately 24 hours.

In addition to the above experiments, at the end of the 6 day testing period all of the mice were sacrificed. Kidneys, lungs, and livers were harvested and examined by gross inspection, as well as by microscopic examination of stained sections. All of the organs of the anti-C5 antibody treated animals appeared the same as those taken from PBS control treated animals. The overall appearance of the test and control mice was also indistinguishable prior to necropsy.

Anti-human C5 antibodies were also tested for pharmacokinetic properties in circulating human blood as described above in Example 4. As described therein, the hemolysis inhibiting effects of an anti-human C5 monoclonal antibody were assayed over a 90 minute period of circulation. The results of these assays are charted in FIG. 7B, and show that the N19/8 anti-C5 mAb essentially completely inhibited the cell lysing ability of the human blood during the entire 90 minute period of circulation.

The results of these experiments demonstrate that the anti-C5 antibodies will survive in the bloodstream for a substantial period of time, thus making periodic administration practical.

Example 7

Preparation of Anti-C5 Monoclonal Antibodies

A monoclonal antibody suitable for use in the practice of the present invention was prepared in accordance with the teachings of Sims, et al., U.S. Pat. No. 5,135,916, as follows.

Balb/c mice were immunized three times by intraperitoneal injection with human C5 protein (Quidel Corporation, San Diego, Calif., Cat # A403). The first injection contained 100 μg of C5 protein in a complete Freund's adjuvant emulsion, the second immunization contained 100 μg of C5 protein in an incomplete Freund's adjuvant emulsion, and the third immunization was 100 μg of protein in PBS. The mice were injected at roughly 2 month intervals.

Fusions of splenocytes to myeloma cells to generate hybridomas were performed essentially as described in Current Protocols in Immunology (John Wiley & Sons, New York, 1992, pages 2.5.1 to 2.5.17). One day prior to fusion the mice were boosted IV with 100 μg of C5 protein. On the day of fusion, the immunized mice were sacrificed and spleens was harvested. SP2/0-AG14 myeloma cells (ATCC CRL#1581) were used as the fusion partner. SP2/0-AG14 cultures were split on the day before the fusion to induce active cell division. A ratio of 1:10 (myeloma cells:splenocytes) was used in the fusions.

The cells were fused using PEG 1450 in PBS without calcium (Sigma Chemical Company, St. Louis, Mo., Catalog No. P-7181) and plated at $1–2.5\times10^5$ cells per well. Selection in EX-CELL 300 medium (JRH Biosciences, Lexena, Kans., Catalog No. 14337-78P) supplemented with 10% heat inactivated fetal bovine serum (FBS); glutamine, penicillin and streptomycin (GPS); and HAT (Sigma Chemical Company, St. Louis, Mo., Catalog No. H-0262) was started the following day. The fusions were then fed every other day with fresh FBS, GPS, and HAT supplemented medium. Cell death could be seen as early as 2 days and viable cell clusters could be seen as early as 5 days after initiating selection. After two weeks of selection in HAT, surviving hybridomas chosen for further study were transferred to EX-CELL 300 medium supplemented with FBS, GPS, and HT (Sigma Chemical Company, St. Louis, Mo., Catalog No. H-0137) for 1 week and then cultured in EX-CELL 300 medium supplemented with FBS and GPS.

Hybridomas were screened for reactivity to C5 and inhibition of complement-mediated hemolysis 10–14 days after fusion, and were carried at least until the screening results were analyzed. The screen for inhibition of hemolysis was the chicken erythrocyte lysis assay described above. The screen for C5 reactivity was an ELISA, which was carried out using the following protocol:

A 50 μL aliquot of a 2 μg/mL solution of C5 (Quidel Corporation, San Diego, Calif.) in sodium carbonate /bicarbonate buffer, pH 9.5, was incubated overnight at 4° C. in each test well of a 96 well plate (NUNC-IMMUNO F96 POLYSORP, A/S Nunc, Roskilde, Denmark). The wells were then subjected to a wash step. (Each wash step consisted of three washes with TBST.) Next, test wells were blocked with 200 μL of blocking solution, 1% BSA in TBS (BSA/TBS) for 1 hour at 37° C. After an additional wash step, a 50 μL aliquot of hybridoma supernatant was incubated in each test well for 1 hour at 37° C. with a subsequent wash step. As a secondary (detection) antibody, 50 μL of a 1:2000 dilution of horseradish peroxidase (HRP) conjugated goat anti-mouse IgG in BSA/TBS, was incubated in each test well for 1 hour at 37° C., followed by a wash step. Following the manufacturer's procedures, 10 mg of O-phenylenediamine (Sigma Chemical Company, St. Louis, Mo., Catalog No. P-8287) was dissolved in 25 mLs of phosphate-citrate buffer (Sigma Chemical Company, St. Louis, Mo., Catalog No. P-4922), and 50 μL of this substrate solution was added to each well to allow detection of peroxidase activity. Finally, to stop the peroxidase detection reaction, a 50 μL aliquot of 3N hydrochloric acid was added to each well. The presence of antibodies reactive with C5 in the hybridoma supernatants was read out by a spectrophotometric OD determination at 490 nm.

The supernatant from a hybridoma designated as 5G1.1 tested positive by ELISA and substantially reduced the cell-lysing ability of complement present in normal human blood in the chicken erythrocyte hemolysis assay. Further analyses revealed that the 5G1.1 antibody reduces the cell-lysing ability of complement present in normal human blood so efficiently that, even when present at roughly one-half the molar concentration of human C5 in the hemolytic assay, it can almost completely neutralize serum hemolytic activity.

Immunoblot analysis was undertaken to further characterize the 5G1.1 mAb. Human C5 (Quidel Corporation, San Diego, Calif., Catalog No. A403) was subjected to polyacrylamide gel electrophoresis under reducing conditions, transferred to a nitrocellulose membrane, and probed with the 5G1.1 mAb as a purified IgG preparation. Two bands were immunoreactive with the 5G1.1 mAb at apparent molecular weights corresponding to those of the alpha and beta chains of the human C5 protein. The two 5G1.1 immunoreactive bands seen on this Western blot were subsequently found to result from the binding of the 5G1.1 antibody to the 115 kDa C5 alpha chain and to a large fragment of the alpha chain that had the same apparent molecular weight (approximately 75 kDa) as the beta chain of C5 and was present in the C5 preparations used for the experiment.

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Assays were performed to determine the relative activity of the N19/8 mAb discussed in Examples 4 and 5 with the 5G1.1 mAb in functional hemolytic assays and to assess whether these mAbs blocked the cleavage of C5 to yield C5a. To this end, the N19/8 and 5G1.1 mAbs were directly compared in human complement hemolytic and C5a release assays.

Hemolytic assays performed in the presence of 20% v/v human serum revealed that the 5G1.1 mAb effectively blocked serum hemolytic activity at a final concentration of 6.25 μg/ml (0.5/1 molar ratio of 5G1.1/C5) whereas the N19/8 mAb blocked at a higher concentration of 25.0 μg/ml (2.0/1 molar ratio of N19/8/C5). When the supernatants from these assays were tested for the presence of C5a, the 5G1.1 mAb was found to have effectively inhibited C5a generation at doses identical to those required for the blockade of C5b-9 mediated hemolytic activity.

In contrast, the N19/8 mAb was 10 fold less effective in blocking the release of C5a in these assays when compared to the 5G1.1 mAb. Furthermore, the ability of the N19/8 mAb to block complement mediated hemolysis was not equivalent to its capacity to block C5a generation in that a dose of 25 μg/ml of N19/8 completely blocked hemolysis while only reducing C5a generation by 37%.

Hybridoma 5G1.1 was deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, Va. 20110-2209, United States of America, on Apr. 27, 1994, and has been assigned the designation HB-11625. This deposit were made under the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure (1977).

Example 8

Determination of the Affinity Constants (K_D) for the Anti-human C5 Monoclonal Antibodies 5G1.1 and N19/8

The procedure utilized to determine the dissociation constant (K_D) of antibody-antigen equilibria in solution was that described by Friguet et al., J. Immunol. Meth. 1985, 77:305–319. This method was used to determine the K_D for the anti-human C5 monoclonal antibodies N19/8 and 5G1.1. The monoclonal antibodies were incubated with the antigen (C5) in solution until the equilibrium was reached. The proportion of antibody that remains unbound (free) at equilibrium was measured using a conventional Enzyme Linked Immunosorbant Assay (ELISA). The experimental values of K_D obtained by this method have been shown to be equivalent to those obtained by other methods (immunoprecipitation of the radiolabeled antigen and fluorescence transfer). This method offers the advantage of dealing with unmodified antigen.

FIGS. **8** and **9** show the Scatchard plots of the binding of the anti-human C5 monoclonal antibodies 5G1.1 and N19/8 to human C5 as measured by ELISA. In each graph (v) represents the fraction of bound antibody and (a) represents the concentration of free antigen at equilibrium. The calculated K_D for the 5G1.1 mAb was 30 pM while the calculated K_D for the N19/8 mAb was 43 pM. These results indicate that the K_D for the 5G1.1 and N19/8 mab's are similar, and therefore the functional disparity between the two antibodies cannot be explained simply by the differences in affinity for the C5 antigen.

Example 9

Effect of 5G1.1 mAb on Complement Activation During CPB

Experiments involving recirculation of human blood in an CPB circuit, as described above in Examples 4 and 5, were carried out using three doses of the 5G1.1 mAb (15 mg, 7.5 mg, 3.75 mg) as well as controls in the absence of the 5G1.1 mAb. In five such control experiments performed in this series, C3a FIG. **10**) and sC5b-9 (FIG. **11**) levels increased during the first 30 min and continued to rise throughout the entire experiment. Addition of the 5G1.1 mAb to the CPB circuit had no effect on the generation of C3a in these experiments.

Conversely, addition of the two highest doses (15 mg and 7.5 mg) of the 5G1.1 mab completely blocked the generation of sC5b-9 in these experiments while the lowest dose (3.75 mg) only partially blocked sC5b-9 generation. Hemolytic assays performed on serum samples drawn throughout the time course of these experiments revealed that total serum complement activity was not affected in control experiments (**FIG. 12**). In contrast, the highest dose of the 5G1.1 mAb (15 mg) completely blocked complement hemolytic activity, while the two lower doses (7.5 mg and 3.75 mg), failed to block hemolytic activity.

These results show that the 7.5 mg dose effectively blocked C5b-9 generation in the CPB circuit but failed to block C5b-9-mediated hemolytic activity, suggesting that hemolytic assays alone may not accurately reflect the complement activation that occurs during CPB. These results further indicate that the 5G1.1 mAb can completely block complement activation in human blood, as measured by either criterion, at a dosage of 15 mg/500 ml, a dose that is approximately equivalent to a dose of 150 mg for a 70 kg patient.

Example 10

Cloning of Anti-C5 Recombinant Anti-KSSKC Variable Region Genes Amino Acid Sequencing

To determine the N-terminal amino acid sequence of the 5G1.1 mAb, a 12% acrylamide gel (37.5:1 acrylamide/N, N'-methylene-bisacrylamide) was prepared and pre-electrophoresed for 45 minutes at 10 mA using 1× pre-electrophoresis buffer (123 mM bis-Tris, pH 6.6, with the cathode buffer reservoir supplemented with 1 mM reduced glutathione). The following day, the pre-electrophoresis buffer in the cathode reservoir was replaced with cathode reservoir buffer (44 mM N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid, 113 mM bis-Tris, 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.067% (w/v) thioglycolic acid) and the pre-electrophoresis buffer in the anode reservoir was replaced with anode reservoir buffer (63 mM bis-Tris, pH 5.9).

75 μg 5G1.1 monoclonal antibody was added to Laemmli sample buffer (30 mM Tris-HCl pH 6.8, 3% (w/v) SDS, 10 mM EDTA, 0.02% (w/v) bromophenol blue, 5% (v/v) glycerol, 2.5% (v/v) beta-mercaptoethanol) and electrophoresed at 10 mA until the bromophenol blue tracking dye reached the bottom of the gel. The protein was transferred to a PROBLOTT membrane (Applied Biosystems, Foster City, Calif.) using 1× transfer buffer (10 mM cyclohexylaminopropane sulfonic acid, 0.05% (w/v) dithiothreitol, 15% (v/v) methanol) at 50 V for one hour.

Protein bands were localized by staining with 0.2% Ponceau S (in 3% trichloroacetic acid, 3% sulfosalicylic acid) followed by destaining with water. Bands were excised and subjected to amino acid sequence analysis using Edman chemistry performed on a pulsed liquid protein sequencer (ABI model 477A), with the PTH amino acids thereby obtained being analyzed with an on-line microbore HPLC system (ABI model 120A).

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To deblock the amino terminus of the 5G1.1 heavy chain, 10 mg 5G1.1 monoclonal antibody was exchanged into reducing buffer (5 M guanidine-HCl, 50 mM Tris-HCl, 10 mM dithiothreitol, pH 8.5) using a PD-10 column (Pharmacia, Piscataway, N.J.). After a one hour incubation at room temperature, 50 mM iodoacetamide was added and the incubation allowed to continue for 30 minutes. The carbamidomethylated light and heavy chains thus obtained were separated by size exclusion chromatography on a SUPEROSE 12 (Pharmacia) column equilibrated with 5 M guanidine-HCl, 50 mM Tris-HCl pH 8.5. The carbamidomethylated heavy chain was exchanged into 50 mM sodium phosphate, pH 7.0 using a PD-10 column, subjected to digestion with pyroglutamate aminopeptidase (PanVera, Madison, Wis.; 0.5 mU per nmol of heavy chain protein), and sequenced as described above.

For determination of internal amino acid sequence, the carbamidomethylated 5G1.1 light chain was exchanged into 2 M urea, 25 mM Tris-HCl, 1 mM EDTA, pH 8.0 and incubated with endoproteinase Lys-C (Promega, Madison, Wis.; protease:protein ratio of 1:40) at 37° C. overnight. The digested material was run on a C18 reversed phase HPLC column (Beckman Instruments, Fullerton, Calif.) and eluted using a linear 0–50% acetonitrile gradient in 0.1% trifluoroacetic acid. Peaks were subjected to amino acid sequence analysis as described above.

PCR Cloning

Cloning of the 5G1.1 variable heavy region was performed using a set of commercially available primers (Mouse Ig-PRIMER SET, catalogue number 69831-1, Novagen, Madison, Wis.). Total RNA was isolated from 5G1.1 hybridoma cells using the acid/guanidinium thiocyanate technique (Chomczynski and Sacchi, Anal. Biochem. 1987, 162:156–159). For first strand cDNA synthesis, ten micrograms total RNA were denatured at 65° C. for 5 min., chilled on ice, and added to a 100 μl reaction containing 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM dithiothreitol, 250 μM each dNTP, 20 units AMV reverse transcriptase (Seikagaku America, Rockville, Md.), and 10 pmole of the appropriate 3' primer (as described in the Ig-PRIMER SET kit protocol). After incubation at 37° C. for one hour, five microliters of the cDNA synthesis reaction were added to a 100 microliter PCR reaction containing: 10 mM Tris-HCl pH 9.0 at 25° C., 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% (w/v) gelatin, 1.0% (v/v) Triton X-100, 200 μM each dNTP, 2.5 U AMPLITAQ DNA polymerase (Perkin-Elmer-Cetus, Norwalk, Conn.) and 25 pmoles of the appropriate 5' and 3' primers (as described in the Ig-PRIMER SET kit protocol). The reaction conditions were 1 minute at 95° C., 1 minute at 42° C., and 1 minute at 72° C. for 30 cycles, followed by a final extension at 72° C. for 10 minutes.

PCR products having the expected size (approximately 450 bp) were cloned into the vector PCRII (Invitrogen, San Diego, Calif.) using a T/A cloning kit (Invitrogen). DNA sequence analysis of cloned DNA fragments was performed by the dideoxy chain-termination method using double-stranded plasmid DNA as a template. A unique heavy chain variable region was isolated by this procedure, with the resulting plasmid designated p5G1.1 VH 2-1-3. Several clones obtained from independent replicate PCR reactions were sequenced to detect any mutations introduced during the PCR amplification of this variable region.

To clone the 5G1.1 light chain variable region, PCR primers were designed by using the UWGCG program TFASTA (University of Wisconsin, Madison, Wis.) to search the GenBank rodent subdirectory with the 19mer query amino acid sequence Ile Gln Met Thr Gln Ser Pro Ala Ser Leu Ser Ala Ser Val Gly Glu Thr Val Thr, that was obtained by amino acid sequencing as described above. An exact match to this sequence was located in the murine germline gene encoding the v-kappa k2 variable region (Seidman et al. Proc. Natl. Acad. Sci. USA 1978 75:3881–3885). The DNA sequence of this germline gene was used to design the oligonucleotide UDEC690 (SEQ ID NO:5) for use as a variable region 5'-primer. A murine kappa gene constant region primer, UDEC395 (SEQ ID NO:6) was also synthesized and used in this reaction. Cloning of the 5G1.1 variable light region was performed using the UDEC690 variable region 5'-primer and the UDEC395 murine kappa gene constant region primer.

PolyA mRNA was isolated from hybridoma 5G1.1. The acid/guanidinium thiocyanate procedure (Chomczynski and Sacchi, supra) was used to isolate total RNA, and was followed by oligo(dT)-cellulose chromatography of 1 mg of total RNA. For first strand cDNA synthesis, one microliter of the 25 microliters of oligo(dT)-cellulose eluate (containing approximately 2 micrograms of purified 5G1.1 mRNA) was denatured at 65° C. for 5 min., chilled on ice, and incubated in extension buffer (10 mM Tris pH 8.3, 50 mM KCl, 1 mM dithithreitol, 240 μM each dNTP) containing 100 nM UDEC395 (SEQ ID NO:6) and 25 units AMV reverse transcriptase (Seikagaku America, Rockville, Md.) at 42° C. for one hour. Five microliters of the completed first strand reaction was subjected to PCR amplification using amplification buffer supplemented with 2.5 units AMPLITAQ DNA polymerase (Perkin Elmer, Foster City, Calif.) and 500 nM each of primer UDEC690 (SEQ ID NO:5) and UDEC395 (SEQ ID NO:6). Amplification was performed using 30 cycles each consisting of 1 minute at 95° C., 1 minute at 52° C., and 1 minute at 72° C., followed by a single ten minute incubation at 72° C.

The resulting PCR product was purified using GENECLEAN according to the manufacturer's directions (Bio 101, La Jolla, Calif.), digested with Sse8387 I and Hind III, gel purified, and ligated into the vector Bluescript II SK^+ (Stratagene, La Jolla, Calif.). Ligated plasmids were transformed into the bacterial strain DH10B by electroporation.

Plasmid DNA was purified from cultures of transformed bacteria by conventional methods including column chromatography using a QUIAGEN-TIP-500 column according to the manufacturer's directions (Quiagen, Chatsworth, Calif.) and sequenced by the Sanger dideoxy chain termination method using SEQUENASE enzyme (U.S. Biochemical, Cleveland, Ohio). Clones obtained from a second independent PCR reaction verified that no mutations were introduced during the amplification process. The resulting plasmid containing the cloned variable region was designated SK (+) 690/395. This light chain encoding insert in this plasmid coded for both the N-terminal and internal light chain sequences determined by amino acid sequencing of 5G1.1, as described above.

Example 11

Construction and Expression of Recombinant mAbs

Recombinant DNA constructions encoding the recombinant mAbs comprising the 5G1.1 CDRs are prepared by conventional recombinant DNA methods including restriction fragment subcloning and overlapping PCR procedures. The resulting recombinant mAb-encoding DNAs include:

(1) one encoding a non-humanized (murine) scFv designated 5G1.1M1scFv (SEQ ID NO:7), wherein CDR L1 is amino acid residues 28–34 of SEQ ID NO:7, CDR L2 is amino acid residues 52–54 of SEQ ID NO:7,

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CDR L3 is amino acid residues 93–98 of SEQ ID NO:7, CDR H1 is amino acid residues 156–159 of SEQ ID NO:7, CDR H2 is amino acid residues 179–183 of SEQ ID NO:7, and CDR H3 is amino acid residues 226–236 of SEQ ID NO:7;

(2) one encoding a humanized (CDR grafted) scFv designated 5G1.1 scFv CB (SEQ ID NO:8), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:8, CDR L2 is amino acid residues 52–58 of SEQ ID NO:8, CDR L3 is amino acid residues 91–99 of SEQ ID NO:8, CDR H1 is amino acid residues 152–161 of SEQ ID NO:8, CDR H2 is amino acid residues 176–192 of SEQ ID NO:8, H3 is amino acid residues 225–237 of SEQ ID NO:8;

(3) one encoding a chimeric light chain (which can form the light chain portion of an Fab) designated 5G1.1M1 VL HuK (SEQ ID NO:9);

(4) one encoding a chimeric Fd (the heavy chain portion of an Fab) designated 5G1.1M1 VH HuG1 (SEQ ID NO:10);

(5) one encoding a humanized (CDR grafted and framework sequence altered) Fd designated 5G1.1 VH+IGHRL (SEQ ID NO:11), wherein CDR H1 is amino acid residues 26–35 of SEQ ID NO:11, CDR H2 is amino acid residues 50–60 of SEQ ID NO:11, and CDR H3 is amino acid residues 99–111 of SEQ ID NO:11;

(6) one encoding a humanized (CDR grafted, not framework altered) Fd designated 5G1.1 VH+IGHRLC (SEQ ID NO:12), CDR H1 is amino acid residues 26–35 of SEQ ID NO:12, CDR H2 is amino acid residues 50–66 of SEQ ID NO:12, and CDR H3 is amino acid residues 99–111 of SEQ ID NO:12;

(7) one encoding a humanized (CDR grafted and framework sequence altered) light chain designated 5G1.1 VL+KLV56 (SEQ ID NO:13), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:13, CDR L2 is amino acid residues 52–58 of SEQ ID NO:13, and CDR L3 is amino acid residues 91–99 of SEQ ID NO:13;

(8) one encoding a humanized (CDR grafted, not framework altered) light chain designated 5G1.1 VL+KLV56B (SEQ ID NO:14), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:14, CDR L2 is amino acid residues 52–58 of SEQ ID NO:14, and CDR L3 is amino acid residues 91–99 of SEQ ID NO:14;

(9) one encoding a humanized (CDR grafted, not framework altered) light chain designated 5G1.1 VL+012 (SEQ ID NO:15), wherein CDR L1 is amino acid residues 24–34 of SEQ ID NO:15, CDR L2 is amino acid residues 50–56 of SEQ ID NO:15, and CDR L3 is amino acid residues 89–97 of SEQ ID NO:15; and

(10) one encoding a humanized (CDR grafted, not framework altered) Fd designated 5G1.1 VH+IGHRLD (SEQ ID NO:16), wherein CDR H1 is amino acid residues 26–35 of SEQ ID NO:16, CDR H2 is amino acid residues 50–60 of SEQ ID NO:16, and CDR H3 is amino acid residues 99–111 of SEQ ID NO:16.

(11) one encoding a humanized (CDR grafted) scFv designated 5G1.1 scFv DO12 (SEQ ID NO:17), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:17, CDR L2 is amino acid residues 52–58 of SEQ ID NO:17, CDR L3 is amino acid residues 91–99 of SEQ ID NO:17, CDR H1 is amino acid residues 152–161 of SEQ ID NO:17, CDR H2 is amino acid residues 176–186 of SEQ ID NO:17, and CDR H3 is amino acid residues 225–237 of SEQ ID NO:17;

(12) one encoding a humanized (CDR grafted and framework sequence altered) scFv designated 5G1.1 scFv CO12 (SEQ ID NO:20), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:20, CDR L2 is amino acid residues 52–58 of SEQ ID NO:20, CDR L3 is amino acid residues 91–99 of SEQ ID NO:20, CDR H1 is amino acid residues 152–161 of SEQ ID NO:20, CDR H2 is amino acid residues 176–192 of SEQ ID NO:20, H3 is amino acid residues 225–237 of SEQ ID NO:20;

(13) one encoding a humanized (CDR grafted) scFv designated 5G1.1 scFv DO12B (SEQ ID NO:21), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:21, CDR L2 is amino acid residues 52–58 of SEQ ID NO:21, CDR L3 is amino acid residues 91–99 of SEQ ID NO:21, CDR H1 is amino acid residues 152–161 of SEQ ID NO:21, CDR H2 is amino acid residues 176–192 of SEQ ID NO:21, H3 is amino acid residues 225–237 of SEQ ID NO:21;

(14) one encoding a humanized (CDR grafted) scFv designated 5G1.1 scFv DO12C (SEQ ID NO:22), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:22, CDR L2 is amino acid residues 52–58 of SEQ ID NO:22, CDR L3 is amino acid residues 91–99 of SEQ ID NO:22, CDR H1 is amino acid residues 152–161 of SEQ ID NO:22, CDR H2 is amino acid residues 176–192 of SEQ ID NO:22, H3 is amino acid residues 225–237 of SEQ ID NO:22;

(15) one encoding a humanized (CDR grafted) scFv designated 5G1.1 scFv DO12D (SEQ ID NO:23), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:23, CDR L2 is amino acid residues 52–58 of SEQ ID NO:23, CDR L3 is amino acid residues 91–99 of SEQ ID NO:23, CDR H1 is amino acid residues 152–161 of SEQ ID NO:23, CDR H2 is amino acid residues 176–192 of SEQ ID NO:23, H3 is amino acid residues 225–237 of SEQ ID NO:23;

(16) one encoding a humanized (CDR grafted and framework sequence altered) scFv designated 5G1.1 scFv CO13 (SEQ ID NO:24), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:24, CDR L2 is amino acid residues 52–58 of SEQ ID NO:24, CDR L3 is amino acid residues 91–99 of SEQ ID NO:24, CDR H1 is amino acid residues 152–161 of SEQ ID NO:24, CDR H2 is amino acid residues 176–192 of SEQ ID NO:24, H3 is amino acid residues 225–237 of SEQ ID NO:24;

(17) one encoding a humanized (CDR grafted and framework sequence altered) scFv designated 5G1.1 scFv C014 (SEQ ID NO:25), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:25, CDR L2 is amino acid residues 52–58 of SEQ ID NO:25, CDR L3 is amino acid residues 91–99 of SEQ ID NO:25, CDR H1 is amino acid residues 152–161 of SEQ ID NO:25, CDR H2 is amino acid residues 176–192 of SEQ ID NO:25, H3 is amino acid residues 225–237 of SEQ ID NO:25;

(18) one encoding a humanized (CDR grafted and framework sequence altered) scFv designated 5G1.1 scFv CO15 (SEQ ID NO:26), wherein CDR L1 is amino acid residues 26–36 of SEQ ID NO:26, CDR L2 is amino acid residues 52–58 of SEQ ID NO:26, CDR L3 is amino acid residues 91–99 of SEQ ID NO:26, CDR H1

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is amino acid residues 152–161 of SEQ ID NO:26, CDR H2 is amino acid residues 176–192 of SEQ ID NO:26, H3 is amino acid residues 225–237 of SEQ ID NO:26;

In accordance with the invention, one each of the various L1, L2 and L3 CDRs discussed in (1) to (18) above may be combined with any of the other light chain CDRs so as to make a set of 3 light chain CDRs comprising one L1, one L2, and one L3 CDR, as part of a recombinant antibody or synthetic peptide antibody (i.e., a synthetic peptide with the sequence of a recombinant peptide of the invention). Furthermore, the framework regions (i.e., the regions not included in the CDRs as described for each) of each of (1) to (18) above may be interchanged with homologous framework regions of the other recombinant antibody molecules of (1) to (18) to produce other antibodies of the invention.

In accordance with the invention, one each of the various H1, H2 and H3 CDRs discussed in (1) to (18) above may be combined with any of the other light chain CDRs so as to make a set of 3 light chain CDRs comprising one H1, one H2, and one H3 CDR, as part of a recombinant antibody or synthetic peptide antibody (i.e., a synthetic peptide with the sequence of a recombinant peptide of the invention).

In accordance with the invention, matched pairs of the variable regions (e.g., a VL and a VH region) of the various antibody molecules, Fds, and light chains described above may be combined with constant region domains by recombinant DNA or other methods known in the art to form full length antibodies of the invention. Particularly preferred constant regions for this purpose are IgG constant regions, which may be unaltered, or constructed of a mixture of constant domains from IgGs of various subtypes, e.g., IgG1 and IgG 4.

Matched pairs of the Fd and light chain encoding DNAs described immediately above—i.e. (3) and (4), (5) and (7), (6) and (8), and (6) and (9)—were subcloned together into the APEX-3P vector, essentially as described below in Example 15 for N19/8. The scFv constructs of (1) and (2) were subcloned into pET Trc SO5/NI using conventional techniques.

Plasmids so obtained were introduced by into the bacterial strain ME2 (pET plasmids) by conventional electroporation, or into human 293 EBNA cells (APEX plasmids) by lipofection using 2–3 microliters of TRANSFECTAM reagent (Promega, Madison, Wis.) per microgram of DNA according to the manufacturer's directions. Bacterial strains ME1 and ME2 are derivatives of *Escherichia coli* strain W3110 (ATCC designation 27325) prepared as follows.

Preparation of W3110 Derivatives ME1 and ME2

The non-humanized, non-chimeric murine 5G1.1-scFv "m5G1.1-scFv"—made up of light chain (3) and Fd (4)—was expressed in a derivative of *E. coli* K12 strain W3110. This derivative was prepared by inactivating an uncharacterized gene to provide protection against infections by a lytic bacteriophage. *E. coli* strain W3110 is a particularly preferred strain because it is fully characterized and is commonly used for recombinant DNA product fermentations.

A single colony of *E. coli* strain W3110 was grown overnight in L medium at 30° C. The cells were collected by centrifugation and resuspended in 10 mM $MgSO_4$. A total of 0.1 ml of the culture was added to 2.5 ml 0.7% L soft agar at 45° C. and quickly poured on an L plate. Fifty microliter aliquots of a plaque purified phage lysate, undiluted, diluted 10^{-2} and diluted 10^{-4}, were spotted onto the agar surface. Phage lysates had previously been filtered through 0.45 μm membranes and stored in sterile tubes with a drop of chloroform at 4° C. The spots were allowed to dry on the soft agar surface and incubated overnight at 37° C.

The next day L plates were spread with 10^9 phage PFU and allowed to dry. Using a sterile, flat toothpick, cells from isolated colonies growing in the zones of phage lysis on the spot plates were streaked for single colonies on the plates spread with 10^9 phage PFU and incubated overnight at 37° C. Single colonies were rechecked for phage resistance by cross-streaking after single colony purification. The cross streak test for phage sensitivity was performed as follows. Fifty μl of phage (10^8 pfu/ml) was spread in a vertical line in the left hand portion of the plate using a Pasteur pipette. Additional phage were tested parallel to the first and to the right. The plate was allowed to dry, and strains to be checked for sensitivity or resistance were spread perpendicular to and across the lines of all phages in a single swath from the left to the right. Resistant strains grow in the area of the phage streaks while sensitive strains lyse.

The phage resistant mutant strain ME1 was tested for phage production after overnight growth in L medium and treatment with the DNA damaging agent, mitomycin C. The strain failed to produce viable phage utilizing a standard plaque assay and *E. coli* W3110 as the phage sensitive indicator strain. These results suggest that strain ME1 does not harbor a resident prophage.

Strain ME2 was constructed by site specific integration of the lambdaDE3 prophage (Studier et al. 1990, Meth. Enzymol. 185:60–89) into the ME1 chromosome. Expression of the T7 RNA polymerase, directed by the prophage, allows expression of target genes cloned into pET vectors (Studier et al., supra) under the control of the T7 promoter in the lysogenized host. Lysogenization was accomplished in a three way infection with lambdaDE3, the lambda helper phage, lambdaB10 and the selection phage, lambdaB482 (Studier et al., supra).

lambdaDE3 (imm2l) was constructed by Studier and colleagues (1990, Meth. Enzymol. 185:60–89) by inserting the T7 RNA polymerase gene behind the *E. coli* lacUV5 promoter into the BamHI cloning site of lambdaD69 (imm21). Since cloning into the BamHI site of lambdaD69 interrupts the integrase gene, lambdaDE3 cannot integrate or excise from the chromosome by itself. The helper phage lambdaB10 provides the integrase function that lambdaDE3 lacks but cannot form a lysogen by itself. The selection phage, lambdaB482, lyses any lambdaDE3 host range mutants that otherwise would be among the surviving cells, but it can neither integrate into susceptible cells nor lyse lambdaDE3 lysogens since it has the same immunity region as lambdaDE3 (imm21).

Lysoaenization Protocol

Strain ME1 was grown in L medium supplemented with 0.2% maltose and 10 MM $MgSO_4$ at 37° C. to a density of approximately 10^8 cells/ml. One μl of ME1 cells were incubated with 2×10^8 plaque forming units (pfu) of lambdaDE3 and 10^8 pfu of lambdaB10 and lambdaB482. The host/phage mixture was incubated at 37° C. for 20 min to allow phage adsorption to ME1 cells. Several dilutions of the cell/phage suspension were spread on L plates to produce plates containing approximately 30–200 candidate lysogens as isolated colonies. The plates were inverted and incubated at 37° C. overnight. Several isolated colonies were screened for the acquisition of the lambdaDE3 prophage as described below.

Verification of lambdaDE3 Lysogens

lambdaDE3 lysogen candidates were tested for their ability to support the growth of the T7 phage 4107, a T7 phage deletion mutant that is completely defective unless active T7

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RNA polymerase is provided in trans. Only lambdaDE3 lysogens will support the normal growth of the phage in the presence of IPTG (isopropyl-beta-thiogalactopyranoside). The T7 phage produces very large plaques on lambdaDE3 lysogens in the presence of IPTG, while very small plaques are observed in the absence of inducer. The size of the plaque in the absence of IPTG is an indication of the basal level of T7 RNA polymerase expression in the lysogen. Putative lambdaDE3 lysogens were grown in L broth supplemented with 0.2 % maltose and 10 mM $MgSO_4$ at 37° C. to a cell density of approximately 10^8 cells/ml. A total of 0.5 ml of cells was centrifuged and the pellet was resuspended in 0.2 ml of a T7 phage lysate containing 2×10^4 pfu. The phage was allowed to adsorb for 30 min at 37° C. One-half of suspension (0.1 ml) was added to 3.0 ml of molten top agarose at 47° C. and poured onto L plates. The remaining aliquot of cell/phage suspension was poured onto an L plate supplemented with 0.4 mM IPTG to check for induction of T7 RNA polymerase. The plates were inverted and incubated at 37° C. overnight.

Strains were also tested for the presence of the lambdaDE3 lysogen by demonstrating that each strain was resistant to infection by the phage lambdaB482, which is in the same immunity group (imm21), by the cross streak method described above. A lysogen was chosen with a low basal expression level for protein production from pET vectors. The resulting strain, designated ME2, is phage resistant and overexpresses T7 RNA polymerase in the presence of IPTG.

Purification of Humanized 5G1.1-scFv from *E. coli*

The humanized 5G1.1-scFv (h5G1.1-scFv) cDNA construct was cloned into the bacterial expression plasmid pET Trc SO5/NI (SEQ ID NO:18) and transformed into *E. coli* strain ME1. The resulting strain expressing h5G1.1 scFv was grown at 37° C. in 2 liter Applikon glass vessel fermentors containing Terrific Broth (1.2 % (w/v) bacto-tryptone, 2.4% (w/v) bacto-yeast extract, 0.4% (v/v) glycerol, 90 mM KPO_4, pH 7.0) supplemented with 100 μg/ml ampicillin. The production of recombinant scFv was induced by the addition of 1 mM IPTG when the $O.D._{550}$ of the culture reached 10. After an additional 3 h incubation at 37° C., the cells were harvested by centrifugation and the cell pellets stored at −80° C.

Cells were resuspended in 1 mM EDTA, pH 5.0 at 10 ml per gram weight and lysed by a single pass through a microfluidizer (Model M110T, Microfluidics Corp., Newton, Mass.). After centrifugation at 17,500×g for 15 min, the resulting inclusion body pellet was washed by resuspension in 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.15% (w/v) deoxycholate at 10 ml per gram inclusion body using a Tekmar POLYTRON. The inclusion bodies were again pelleted by centrifugation at 17,500×g for 15 min and resuspended in 20 mM Tris-HCl pH 9.0, 8 M urea at 10 ml per g. After stirring for 1 h, the sample was centrifuged at 14,000×g for 30 min to pellet remaining insoluble material.

The extract supernatant was diluted 10-fold with 20 mM Tris-HCL pH 9.0, 7 M urea, 50 μM cupric sulfate and allowed to stir for at least 16 hours at 4° C. to refold the scFv. After addition of BIOCRYL BPA-1000 (TosoHaas, Montgomeryville, Pa.) as a flocculating agent at 3 μl per ml, the sample was centrifuged at 15,000×g for 10 minutes to remove insoluble material. The refolding mixture was exchanged into 20 mM Tris, pH 9.0, 1 mM EDTA by diafiltration and concentrated by ultrafiltration using a stirred cell fitted with a YM10 membrane (Amicon, Beverly, Mass.).

In subsequent experiments, other refolding conditions were tested. Thawed bacterial cells were resuspended with a POLYTRON homogenizer in 1 mM EDTA at 2.5 mL per gram of cells, passed through the MICROFLUIDIZER at 18,000 psi, and centrifuged at 10,000 RPM for 15 min in a Beckman JA-10 rotor, the resulting pellet was washed by resuspension in 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.15% (w/v) deoxycholate at 10 ml per gram inclusion body using a Tekmar POLYTRON. The inclusion bodies were again pelleted by centrifugation. The pellet from this centrifugation was resuspended with a POLYTRON homogenizer in 8M urea, 20 mM Tris pH9 at 10 mL per gram of pellet. After stirring for 1 hour at 4 degrees C., the resuspended pellet was diluted with 9 volumes of 7M urea, 20 mM Tris pH9. Cupric sulfate was then added to various final concentrations (0, 5, 10, 20, 25, 30, 40, 50, 100, 150, and 200 μM) before incubation overnight at 4 degrees C. with stirring. The use of 5 μM copper was found to give the highest levels of refolding of the humanized 5G1.1-scFv as assessed by analytical HPLC.

In the initial experiments, the properly refolded scFv was then separated from aggregated material and contaminating proteins by anion exchange chromatography using Q SEPHAROSE FAST FLOW (Pharmacia, Piscataway, N.J.). Bound scFv was eluted with 20 mM Tris-HCL pH 9.0, 1 mM EDTA containing a linear NaCl gradient (0 to 0.5 M). The fractions containing the scFv were combined, concentrated by ultrafiltration using a stirred cell fitted with a YM10 membrane, and applied to a SEPHACRYL S200 HR 26/100 gel filtration column (Pharmacia) equilibrated in 20 mM Tris-HCL pH 9.0, 1 mM EDTA, 150 mM NaCl. Fractions containing the scFv were combined, exchanged into phosphate-buffered saline by diafiltration, concentrated by ultrafiltration, filtered through a 0.22 μm MILLEX-GV filter (Millipore, Bedford, Mass.), and stored at 4° C.

Subsequent experiments have indicated that cation exchange chromatography (e.g., using POROS HS resin—PerSeptive Biosystems, Cambridge, Mass.) should give better yields than the Q Sepharose Fast Flow anion exchange chromatography step described in the preceding paragraph. In addition, it would be preferable to carry out the final gel filtration chromatography in a buffer that is more pharmaceutically acceptable than the Tris buffer described. A buffer such as PBS would be preferred if it does not interfere with the efficacy of the gel filtration chromatographic separation. This would reduce any trace amounts of Tris remaining in the preparation after diafiltration, and might eliminate the need for the diafiltration step.

Purification of m5G1.1-scFv from *E. coli*

Frozen bacterial cell paste was thawed and resuspended in 2.5 ml of 1 MM EDTA (pH 5) per gram of cell paste. This suspension of cells was lysed by passage through a MICROFLUIDIZER (Microfluidics) with the interaction chamber in line and a backpressure of approximately 18000 psi. The cell lysate was then centrifuged at 10,000 rpm in a JA-10 centrifuge rotor at 4° C. for 15 min. The supernatant was decanted and discarded.

The pellet was resuspended in 10 ml of 20 mM Tris, pH 8.0, 100 mM NaCl, 0.15% sodium deoxycholate per gram of pellet. This suspension was centrifuged as above for 10 min. Again the supernatant was decanted and discarded. This detergent washed pellet was then resuspended in 10 ml of 8 M urea, 20 mM Tris-HCl, pH 9 (1 mM EDTA may also be added to this buffer, but has the effect of increasing the time required to achieve a particular level of refolding). The suspension was stirred at 4° C. for 1 hr. and was then diluted 10 fold with 7 M urea, 20 mM Tris-HCl, pH 9 and stirred at 4° C. $CUSO_4$ was then added to a final concentration of 50 μM and stirring was continued overnight at 4° C.

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The majority of contaminating proteins (including incorrectly folded versions of m5G1.1 scFv) were then removed by precipitation by diluting (with stirring) the refolded sample five fold with buffer such that the final concentrations after dilution were 1.4 M urea, 25 mM NaCl, 1 mM EDTA, and 20 mM sodium acetate at 4° C. The pH of the dilution buffer when prepared at room temperature was pH 5.0. Prior to dilution the pH of the dilution buffer is determined at 4° C. After the dilution the pH of the sample was greater than pH 5.5. The pH of the sample was then adjusted with 6. N HCl to the initial pH 5.0 of the buffer at 4° C. The solution immediately became cloudy and it was left stirring at 4–8° C. for 0.5 to 24 hours.

The precipitate was removed by filtering the sample through a 300 kDa cut-off ultrafiltration membrane (Millipore Corporation, Bedford, Mass.). The permeate was collected and concentrated 5 fold using a 10 kDa cutoff ultrafiltration membrane (Millipore). This concentrated retentate was then diluted 2 fold with 20 mM sodium acetate, 1 mM EDTA, pH 5.0 in order to lower the NaCl concentration to 12.5 mM.

The diluted retentate was then loaded at 4° C. onto a SP SEPHAROSE FF column (Pharmacia) equilibrated in 0.7 M urea, 1 mM EDTA, 10 mM NaCl, 20 mM sodium acetate, pH 5.0, at a linear flowrate of 5 cm/min. Bed height was equal to or greater than 3.5 cm. Following loading the column was washed with 40 column volumes (CV) of equilibration buffer. The column was then washed with 20 CV of 20 mM sodium acetate, pH 5.0, 1 mM EDTA. The bound scFv was then eluted using 20 mM sodium citrate, pH 5.8, 1 mM EDTA. A single peak was collected in approximately 4 column volumes.

The SP SEPHAROSE eluate was then adjusted to 20 mM Tris-HCL by addition of 1 M Tris-HCL, pH 8. The pH of the sample was adjusted to 8.0 by addition of 1 N NaOH. This sample was loaded onto a Q SEPHAROSE FF column (Pharmacia) equilibrated in 20 mM Tris-HCL, pH 8.0, 1 mM EDTA at room temperature at a flowrate of 5 cm/min. The flow through fraction containing the scFv was collected.

The Q SEPHAROSE flow through fraction was then adjusted to 150 mM NaCl and concentrated to 10 mg of scFv per ml using a 10 kDa cutoff ultrafiltration membrane. This concentrated sample was then loaded onto a SEPHACRYL S200 column equilibrated in phosphate buffered saline, pH 7.4 and eluted at 0.4 cm/min. The fractions were analyzed by SDS-PAGE and silver staining. Peak fractions were combined after discarding the front and back shoulder fractions that contained the majority of contaminants.

Example 12

Functional Analysis of the m5G1.1 scFv

Titration of the m5G1.1 scFv in hemolytic assays revealed that the m5G1.1 scFv inhibited human complement-mediated lysis in a dose dependent fashion (FIG. **13**). Direct comparison of the efficacy of the m5G1.1 scFv to the 5G1.1 mAb and Fab demonstrated that the m5G1.1 scFv completely blocked C5b-9-mediated hemolysis in 20% human serum at 0.15 μM while the 5G1.1 mAb and Fab blocked at 0.06–0.08 μM. Analysis of C5a generation in these assays revealed similar results in that the 5G1.1 scfv completely blocked C5a generation at 0.15 μM while the 5G1.1 mAb and Fab blocked at 0.06–0.08 μM (FIG. **14**). Taken together these experiments indicated that unlike N19/8, which lost half of its effectiveness at blocking C5a generation upon being engineered as an scFv (SEQ ID NO:19), the 5G1.1 murine scFv retained the capacity to block the generation of both C5a and C5b-9.

Additionally, these data demonstrate that the m5G1.1 scFv retained similar activity to that of the parent molecule (the native murine 5G1.1 mAb) in that the molar concentration of 5G1.1 murine scFv required to completely block C5a and C5b-9 (0.15 μM) was within two-fold of that required for the 5G1.1 mAb and Fab (0.06–0.08 μM).

In order to determine whether the m5G1.1 scFv retained the capacity to block the activation of complement in the ex vivo model of cardiopulmonary bypass, 4.5 mg of the purified bacterially produced 5G1.1 murine scFv was added to the CPB circuit and complement activation was monitored. In control experiments, both C3a and C5b-9 levels increased throughout the time course of the experiment. In a single experiment, addition of 4.5 mg of the m5G1.1 scFv to the CPB circuit had no effect on the generation of C3a (FIG. **15**). Conversely, complement hemolytic activity as well as the generation of sC5b-9 was completely blocked in this experiment (FIG. **16** and FIG. **17**).

Example 13

Characterization of the Epitope Recognized by 5G1.1

Tryptic digestion: Twenty micrograms of purified human C5 (Advanced Technologies, San Diego, Calif.) was subjected to enzymatic digestion with 1 μg of TPCK-treated trypsin (Worthington Biochemical Corp., Freehold, N.J.). The digestion was allowed to continue for 3 minutes, after which time it was stopped by the addition of 20 μg soy bean trypsin inhibitor (Worthington). The reaction was then denatured and reduced by the addition of protein sample buffer and immediately boiled for 5 min. The digested fragments were size fractionated through a SDS-PAGE on a 12 % gel. The gel was then electroblotted in transfer buffer (20% (v/v) methanol, 25 mM Tris-base pH 8.0, and 192 mM glycine) to nitrocellulose (Bio-Rad Laboratories, Hercules, Calif.) and subjected to ECL western blot analysis using either 5G1.1 or a C5a specific monoclonal antibody (G25/2, obtained from Dr. Otto Götze, Department of Immunology, University of Göttingen, Germany).

The filters were incubated twice for 30 minutes each in blocking solution (500 mM NaCl, 5 mM Tris p-H 7.4, 10% (v/v) nonfat dry milk, and 0.2% (v/v) TWEEN-20). The filters were then changed to fresh blocking solution (20 ml) containing the primary antibody and incubated for 40 minutes on a rocking platform. The filters were rinsed briefly with washing solution (500 mM NaCl, 35 mM Tris pH7.4, 0.1% SDS, 1% NP40, and 0.5% deoxycholic acid) to remove any milk, and then fresh wash solution was added and incubated for two 20 minute intervals on an orbiting shaker. The filters were rinsed briefly with 10 to 20 mls of secondary antibody solution (500 mM NaCl, 5 mM Tris pH 7.4, 10% (v/v) Nonfat dry milk, 0.2% (v/v) TWEEN-20, and 1% NP-40) and then incubated with fresh secondary antibody solution containing a 1:2000 dilution of HRP conjugated goat anti-mouse for 20 minutes on a rocking platform. The filters were then washed as described above, incubated in ECL reagent (Amersham Corp., Arlington Heights, Ill.) for 1 minute and then exposed to ECL HYPERFILM (Amersham).

Acid Hydrolysis: Twenty micrograms of purified human C5 (Advanced Technologies) was subjected to hydrolysis in 1N acetic acid. The 20 μg of human C5 (1 μg/μl) was added to 20 μl of 2N acetic acid and incubated for 10 min at 100° C. The sample was denatured and reduced with protein sample buffer, also at 100° C., for 5 minutes. The acid was

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neutralized by dropwise addition of a saturated tris base solution until the sample turned blue. The cleavage products were then size fractionated by SDS-PAGE and western blotted as described above. For N-terminal sequencing, the gel fractionated acid hydrolysate was transferred to PVDF membrane. N-terminal sequence was obtained by excising the 46 kDa acid hydrolysis fragment band from a PVDF membrane and subjecting it to amino acid sequence analysis as discussed above in Example 10.

Deglycosylation: Reduced and denatured acid hydrolyzed or tryptic fragments of human C5 were subjected to deglycosylation with N-Glycosidase F (Peptide-N-Glycosidase F, Boehringer Mannheim Corp., Indianapolis, Ind.) according to the manufacture's directions.

Results: Acid hydrolysis of human C5 yielded a fragment with an apparent molecular weight by SDS-PAGE of 46 kDa that was immunoreactive for both the anti-C5a mAb G25/2 and the anti-C5 alpha chain mAb 5G1.1. Western blots probed with both antibodies simultaneously, as well as silver stain SDS-PAGE analysis, confirmed the presence of a single 46 kDa fragment that was immunoreactive with both antibodies. The presence of a single immunoreactive fragment containing binding sites for both 5G1.1 and G25/2 strongly suggested that the 5G1.1 epitope was contained within approximately the first 46 kDa of the N-terminus of the alpha chain of C5.

As discussed above in the description of the complement system under the heading "Background Physiology & Pathology," a compound (e.g., an antibody) that binds to a site at or immediately adjacent to the C5a cleavage site would have the potential to act as a terminal complement inhibitor. The potential inhibitory activity of antibodies binding to this site led to the expectation that the C5 alpha chain-binding 5G1.1 antibody would bind to an epitope at or near the C5a cleavage site. The finding that 5G1.1 bound to the 46 kDa acid hydrolysis fragment of C5 lent support to this expectation.

Western blot analysis of the tryptic digestion products identified one proteolytic fragment migrating at approximately 27 kDa that was immunoreactive with 5G1.1. Likewise, one immunoreactive proteolytic fragment migrating at approximately 29 kDa was observed following western blot analysis with the anti-C5a mAb G25/2. Experiments in which a blot was simultaneously probed with both 5G1.1 and G25/2 demonstrated that each band was distinct and that their apparent differential mobility was not a gel anomaly. This was surprising, because the 5G1.1 mAb was thought likely to bind to the C5 convertase cleavage site. 5G1.1 was thus expected to be immunoreactive with any fragment of C5 of over 12 kDa that exhibited immunoreactivity with G25/2. Such a fragment would contain enough of the extreme amino terminus of the C5 alpha chain to bind specifically to the anti-C5a mAb, and enough beyond that to encompass a region including and extending beyond the C5 convertase cleavage site.

The immunoreactivity of G25/2 with the 29 kDa fragment indicated that that fragment contains the N-terminal region of the alpha chain of C5 that is cleaved off to yield C5a. Furthermore, because 5G1.1 was not immunoreactive with this band, the 5G1.1 epitope was not likely to be contained within approximately the first 29 kDa of the N-terminus of the alpha chain of C5, and therefore could not be located near the C5 convertase cleavage site.

These tryptic digestion and acid hydrolysis mapping data suggested that the 5G1.1 epitope was contained within a region starting about 29 kDa (including post-translational modifications) from the N-terminus of the alpha chain of C5 and continuing 17 kDa in a C-terminal direction, i.e., ending 46 kDa from the N-terminus, a surprising finding in view of the expectation, discussed above, that the antibody would bind at or immediately adjacent to the point at which C5a is cleaved off of the C5 alpha chain, i.e., at or immediately adjacent to amino acid residue 733 of SEQ ID NO:2.

Post-translational modifications can alter the mobility of proteins during SDS-PAGE electrophoresis. One such modification is the addition of carbohydrate via N-linked glycosylation. As discussed above under the heading "Background Physiology & Pathology", C5 is glycosylated, as is C5a. C5a is glycosylated at an asparagine residue corresponding to amino acid number 723 of the full length pro-C5 precursor of human C5 (SEQ ID NO:2).

Computer analysis of the human C5 alpha chain suggests potential N-linked glycosylation sites at positions corresponding to amino acid numbers 893, 1097, and 1612 of SEQ ID NO:2. In order to determine the contribution of carbohydrate to the electrophoretic mobility of both the tryptic and acid fragments, enzymatic deglycosylation of the fragments was performed and followed by western blot analysis. It was determined that each tryptic fragment lost approximately 3 kDa in apparent molecular weight while the acid fragment lost approximately 6 kDa.

This result was interpreted as indicating that the tryptic fragments were each glycosylated at a single site and that the 46 kDa acid fragment was glycosylated at two sites (one of which was the known glycosylation site in C5a referred to above). The diminished mobility observed following deglycosylation agrees with the computed prediction of a second N-linked glycosylation site within the first 233 amino acids of the C5 alpha chain.

N-terminal sequence analysis determined that the first four amino acids of the 46 kDa fragment generated by 1N acetic acid treatment was Thr Leu Gln Lys. This sequence is found only once in the full length human pro-C5 precursor molecule—at a position corresponding to amino acids 660 through 663 of SEQ ID NO:2. This four amino acid sequence also corresponds to the sequence of the amino-terminus of the alpha chain of human C5 and, thus to the amino-terminus of human C5a.

In order to more precisely map the binding site of 5G1.1, overlapping peptide analysis was performed. The sequence predicted to be contained within the 17 kDa section of human C5 described above (SEQ ID NO:2; amino acids 893 through 1019) together with an extension of 43 amino acids towards the N-terminus and 30 amino acids towards the C-terminus (a total of 200 amino acids) was synthesized as a series of 88 overlapping peptides by solid phase synthesis on polypropylene filters (Research Genetics Inc., Huntsville, Ala.).

The 43 and 30 amino acid extensions were added to allow for possible inaccuracies in the prediction of the span of this 17 kDa region. Such inaccuracies are likely due to the uncertainty of the specific extent of glycosylation of each of the various regions of C5a, as well as to the aberrant gel mobility that is commonly seen when highly charged polypeptides (such as the 5G46k fragment and the 5G27k fragment) are analyzed by SDS-PAGE. As discussed above in the Summary of the Invention, a 200 amino acid peptide corresponding to the region covered by these overlapping peptides is referred to herein as the "5G200aa" peptide.

Because of the expectation that the 5G1.1 antibody would bind at the C5a cleavage site, an additional set of 8 overlapping peptides was synthesized that spanned a 30 amino

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acid section spanning the C5a cleavage site (amino acids 725 through 754 of SEQ ID NO:2). A peptide having the sequence of this 30 amino acid section is referred to herein as the "cleavage site peptide". A 325aa peptide spanning amino acid residues 725–1049 of SEQ ID NO:2 (this peptide spans the region covered by the cleavage site peptide and the 5G200aa peptide) is referred to herein as the "5G325aa" peptide.

These filters were probed with 5G1.1 as described above for ECL western blot analysis, and a set of 4 overlapping peptides spanning the region corresponding to amino acid residues 3–19 of the KSSKC peptide (SEQ ID NO:1) each gave a positive signal indicative of monoclonal antibody binding, while peptides corresponding to the C5a cleavage site did not bind to the 5G1.1 antibody.

Example 14

C3/C4 Binding Assay

C3 and C4 are both key components of classical C5 convertase, and C3 is also a key component of alternative C5 convertase. These C5 convertases are required for the conversion of C5 to active C5a and C5b. The ability to block C5 binding to C3 and C4 is thus a desirable property for an antibody to be used in treatment of complement mediated diseases in accordance with the present invention.

96 well microtiter plates were coated with 501 μ/well, 10 μg/ml of either purified human C3 or C4 (Quidel) for 1 hour at 37° C. The plates were then blocked with 200 μl/well of TBS containing 1% BSA for 1 hour at room temperature. After three washes in TBS 0.1% BSA, purified human C5 (Quidel, 20 μg/ml in TBS 1% BSA) was added to the plates in the presence (20 μg/ml) or absence of a 5G1.1 Fab (derived from 5G1.1 by conventional papain digestion) and allowed to incubate for 1 hour at 37° C. After three washes in TBS/0.1% BSA, a monoclonal antibody directed against the C5 beta chain (N19/8, 5 μg/ml) was added to the wells to detect C5 bound to either C3 or C4. After three final washes in TBS/0.1% BSA, the plate was developed using a horseradish peroxidase-conjugated secondary antibody and the appropriate substrate.

The results of these assays showed that the 5G1.1 mAb inhibited the binding of purified human C5 to either C3 or C4 by at least 60% to 90%. As used herein and in the claims, such a 60% to 90% reduction in C3 or C4 binding is a "substantial reduction" in C3 or C4 binding.

Example 15

Construction and Functional Analysis of N19/8 Chimeric Fab

The heavy chain and light chain variable regions from the hybridoma N19-8 were cloned by PCR using the Ig-Prime System (Novagen) as described by the manufacturer. Clones from multiple independent PCR reactions were sequenced to detect mutations introduced during the PCR amplification. An N19-8 VL/human kappa constant region chimeric cDNA was created by using a plasmid containing the N19-8 light chain variable region and the plasmid pHuCK (Hieter et al., 1980 Cell, 22:197–207) as templates in an overlapping PCR reaction.

Similarly, an N19-8 VH/human IgG1 Fd chimeric cDNA was created using a plasmid containing the N19-8 heavy chain variable region and a plasmid containing the human IgG1 gene (obtained from Ilan R. Kirsch, National Cancer Institute, Bethesda, Md.) as templates. This Fd construct contained the first nine amino acids of the IgG1 hinge region, including the cysteine residue which normally forms a disulfide bond with the terminal cysteine residue of the kappa light chain.

The resulting chimeric cDNAs were separately cloned into the APEX-1 vector using appropriate flanking restriction enzyme sites introduced during the PCR amplification procedure and sequenced. A fragment containing the promoter, intron, and cDNA insert from one of these APEX vectors was subsequently subcloned into the polylinker of the other to produce a single vector directing the expression of both the light chain and Fd. The tandem expression cassette from this APEX-1 vector was subsequently subcloned into APEX-3P, which was transfected into 293 EBNA cells for expression of the chimeric Fab.

When tested for the ability to block complement hemolytic activity and C5a generation, the chimeric N19/8 Fab retained the ability to block hemolytic activity, but lost 50% of its C5a generation blocking capacity.

Throughout this application 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.

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TABLE 1

Prevention/Reduction of Proteinuria by Treatment With Anti-C5 Antibodies

	Before Treatment Urine Protein (mg/dL)	After Treatment Urine Protein (mg/dL)
	PBS Control	
mouse A	none	100
mouse B	none	500
mouse C	none	500
mouse D*	trace	trace
mouse E	100	100
	Anti-C5 Treated	
mouse 1	none	none
mouse 2	none	30
mouse 3	30	trace
mouse 4	30	30
mouse 5	30	30
mouse 6	100	30

*Mouse D had more than 500 mg/dL urine glucose after treatment

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 26

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: Amino Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE:
(A) DESCRIPTION: KSSKC peptide

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Val Ile Asp His Gln Gly Thr Lys Ser Ser
5 10

Lys Cys Val Arg Gln Lys Val Glu Gly Ser Ser
15 20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1676 Amino Acids
(B) TYPE: Amino Acid

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(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE:
(A) DESCRIPTION: Pro-C5 Polytpeptide

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Haviland, D.L.
Haviland, J.C.
Fleischer, D.T.
Hunt, A.
Wetsel, R.A.
(B) TITLE: Complete cDNA Sequence of Human Complement Pro-C5
(C) JOURNAL: Journal of Immunology
(D) VOLUME: 146
(F) PAGES: 362-368
(G) DATE: 1991

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Gly Leu Leu Gly Ile Leu Cys Phe Leu
-15                 -10

Ile Phe Leu Gly Lys Thr Trp Gly Gln Glu Gln Thr Tyr Val
-5              -1                   5

Ile Ser Ala Pro Lys Ile Phe Arg Val Gly Ala Ser Glu Asn
10                  15                  20

Ile Val Ile Gln Val Tyr Gly Tyr Thr Glu Ala Phe Asp Ala
25                  30

Thr Ile Ser Ile Lys Ser Tyr Pro Asp Lys Lys Phe Ser Tyr
35                  40                  45

Ser Ser Gly His Val His Leu Ser Ser Glu Asn Lys Phe Gln
50                  55                  60

Asn Ser Ala Ile Leu Thr Ile Gln Pro Lys Gln Leu Pro Gly
65                  70                  75

Gly Gln Asn Pro Val Ser Tyr Val Tyr Leu Glu Val Val Ser
80                  85                  90

Lys His Phe Ser Lys Ser Lys Arg Met Pro Ile Thr Tyr Asp
95                 100

Asn Gly Phe Leu Phe Ile His Thr Asp Lys Pro Val Tyr Thr
105                 110                 115

Pro Asp Gln Ser Val Lys Val Arg Val Tyr Ser Leu Asn Asp
120                 125                 130

Asp Leu Lys Pro Ala Lys Arg Glu Thr Val Leu Thr Phe Ile
135                 140                 145

Asp Pro Glu Gly Ser Glu Val Asp Met Val Glu Glu Ile Asp
150                 155                 160

His Ile Gly Ile Ile Ser Phe Pro Asp Phe Lys Ile Pro Ser
165                 170

Asn Pro Arg Tyr Gly Met Trp Thr Ile Lys Ala Lys Tyr Lys
175                 180                 185

Glu Asp Phe Ser Thr Thr Gly Thr Ala Tyr Phe Glu Val Lys
190                 195                 200

Glu Tyr Val Leu Pro His Phe Ser Val Ser Ile Glu Pro Glu
205                 210                 215
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Tyr Asn Phe Ile Gly Tyr Lys Asn Phe Lys Asn Phe Glu Ile
220                 225                 230

Thr Ile Lys Ala Arg Tyr Phe Tyr Asn Lys Val Val Thr Glu
235                 240

Ala Asp Val Tyr Ile Thr Phe Gly Ile Arg Glu Asp Leu Lys
245                 250                 255

Asp Asp Gln Lys Glu Met Met Gln Thr Ala Met Gln Asn Thr
260                 265                 270

Met Leu Ile Asn Gly Ile Ala Gln Val Thr Phe Asp Ser Glu
275                 280                 285

Thr Ala Val Lys Glu Leu Ser Tyr Tyr Ser Leu Glu Asp Leu
290                 295                 300

Asn Asn Lys Tyr Leu Tyr Ile Ala Val Thr Val Ile Glu Ser
305                 310

Thr Gly Gly Phe Ser Glu Glu Ala Glu Ile Pro Gly Ile Lys
315                 320                 325

Tyr Val Leu Ser Pro Tyr Lys Leu Asn Leu Val Ala Thr Pro
330                 335                 340

Leu Phe Leu Lys Pro Gly Ile Pro Tyr Pro Ile Lys Val Gln
345                 350                 355

Val Lys Asp Ser Leu Asp Gln Leu Val Gly Gly Val Pro Val
360                 365                 370

Ile Leu Asn Ala Gln Thr Ile Asp Val Asn Gln Glu Thr Ser
375                 380

Asp Leu Asp Pro Ser Lys Ser Val Thr Arg Val Asp Asp Gly
385                 390                 395

Val Ala Ser Phe Val Leu Asn Leu Pro Ser Gly Val Thr Val
400                 405                 410

Leu Glu Phe Asn Val Lys Thr Asp Ala Pro Asp Leu Pro Glu
415                 420                 425

Glu Asn Gln Ala Arg Glu Gly Tyr Arg Ala Ile Ala Tyr Ser
430                 435                 440

Ser Leu Ser Gln Ser Tyr Leu Tyr Ile Asp Trp Thr Asp Asn
445                 450

His Lys Ala Leu Leu Val Gly Glu His Leu Asn Ile Ile Val
455                 460                 465

Thr Pro Lys Ser Pro Tyr Ile Asp Lys Ile Thr His Tyr Asn
470                 475                 480

Tyr Leu Ile Leu Ser Lys Gly Lys Ile Ile His Phe Gly Thr
485                 490                 495

Arg Glu Lys Phe Ser Asp Ala Ser Tyr Gln Ser Ile Asn Ile
500                 505                 510

Pro Val Thr Gln Asn Met Val Pro Ser Ser Arg Leu Leu Val
515                 520

Tyr Tyr Ile Val Thr Gly Glu Gln Thr Ala Glu Leu Val Ser
525                 530                 535

Asp Ser Val Trp Leu Asn Ile Glu Glu Lys Cys Gly Asn Gln
540                 545                 550

Leu Gln Val His Leu Ser Pro Asp Ala Asp Ala Tyr Ser Pro
555                 560                 565

Gly Gln Thr Val Ser Leu Asn Met Ala Thr Gly Met Asp Ser
570                 575                 580
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Trp Val Ala Leu Ala Ala Val Asp Ser Ala Val Tyr Gly Val
585                 590

Gln Arg Gly Ala Lys Lys Pro Leu Glu Arg Val Phe Gln Phe
595                 600                 605

Leu Glu Lys Ser Asp Leu Gly Cys Gly Ala Gly Gly Gly Leu
610                 615                 620

Asn Asn Ala Asn Val Phe His Leu Ala Gly Leu Thr Phe Leu
625                 630                 635

Thr Asn Ala Asn Ala Asp Asp Ser Gln Glu Asn Asp Glu Pro
640                 645                 650

Cys Lys Glu Ile Leu Arg Pro Arg Arg Thr Leu Gln Lys Lys
655                 660

Ile Glu Glu Ile Ala Ala Lys Tyr Lys His Ser Val Val Lys
665                 670                 675

Lys Cys Cys Tyr Asp Gly Ala Cys Val Asn Asn Asp Glu Thr
680                 685                 690

Cys Glu Gln Arg Ala Ala Arg Ile Ser Leu Gly Pro Arg Cys
695                 700                 705

Ile Lys Ala Phe Thr Glu Cys Cys Val Val Ala Ser Gln Leu
710                 715                 720

Arg Ala Asn Ile Ser His Lys Asp Met Gln Leu Gly Arg Leu
725                 730

His Met Lys Thr Leu Leu Pro Val Ser Lys Pro Glu Ile Arg
735                 740                 745

Ser Tyr Phe Pro Glu Ser Trp Leu Trp Glu Val His Leu Val
750                 755                 760

Pro Arg Arg Lys Gln Leu Gln Phe Ala Leu Pro Asp Ser Leu
765                 770                 775

Thr Thr Trp Glu Ile Gln Gly Ile Gly Ile Ser Asn Thr Gly
780                 785                 790

Ile Cys Val Ala Asp Thr Val Lys Ala Lys Val Phe Lys Asp
795                 800

Val Phe Leu Glu Met Asn Ile Pro Tyr Ser Val Val Arg Gly
805                 810                 815

Glu Gln Ile Gln Leu Lys Gly Thr Val Tyr Asn Tyr Arg Thr
820                 825                 830

Ser Gly Met Gln Phe Cys Val Lys Met Ser Ala Val Glu Gly
835                 840                 845

Ile Cys Thr Ser Glu Ser Pro Val Ile Asp His Gln Gly Thr
850                 855                 860

Lys Ser Ser Lys Cys Val Arg Gln Lys Val Glu Gly Ser Ser
865                 870

Ser His Leu Val Thr Phe Thr Val Leu Pro Leu Glu Ile Gly
875                 880                 885

Leu His Asn Ile Asn Phe Ser Leu Glu Thr Trp Phe Gly Lys
890                 895                 900

Glu Ile Leu Val Lys Thr Leu Arg Val Val Pro Glu Gly Val
905                 910                 915

Lys Arg Glu Ser Tyr Ser Gly Val Thr Leu Asp Pro Arg Gly
920                 925                 930

Ile Tyr Gly Thr Ile Ser Arg Arg Lys Glu Phe Pro Tyr Arg
935                 940
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Ile Pro Leu Asp Leu Val Pro Lys Thr Glu Ile Lys Arg Ile
945                 950                 955

Leu Ser Val Lys Gly Leu Leu Val Gly Glu Ile Leu Ser Ala
960                 965                 970

Val Leu Ser Gln Glu Gly Ile Asn Ile Leu Thr His Leu Pro
975                 980                 985

Lys Gly Ser Ala Glu Ala Glu Leu Met Ser Val Val Pro Val
990                 995                 1000

Phe Tyr Val Phe His Tyr Leu Glu Thr Gly Asn His Trp Asn
1005                1010

Ile Phe His Ser Asp Pro Leu Ile Glu Lys Gln Lys Leu Lys
1015                1020                1025

Lys Lys Leu Lys Glu Gly Met Leu Ser Ile Met Ser Tyr Arg
1030                1035                1040

Asn Ala Asp Tyr Ser Tyr Ser Val Trp Lys Gly Gly Ser Ala
1045                1050                1055

Ser Thr Trp Leu Thr Ala Phe Ala Leu Arg Val Leu Gly Gln
1060                1065                1070

Val Asn Lys Tyr Val Glu Gln Asn Gln Asn Ser Ile Cys Asn
1075                1080

Ser Leu Leu Trp Leu Val Glu Asn Tyr Gln Leu Asp Asn Gly
1085                1090                1095

Ser Phe Lys Glu Asn Ser Gln Tyr Gln Pro Ile Lys Leu Gln
1100                1105                1110

Gly Thr Leu Pro Val Glu Ala Arg Glu Asn Ser Leu Tyr Leu
1115                1120                1125

Thr Ala Phe Thr Val Ile Gly Ile Arg Lys Ala Phe Asp Ile
1130                1135                1140

Cys Pro Leu Val Lys Ile Asp Thr Ala Leu Ile Lys Ala Asp
1145                1150

Asn Phe Leu Leu Glu Asn Thr Leu Pro Ala Gln Ser Thr Phe
1155                1160                1165

Thr Leu Ala Ile Ser Ala Tyr Ala Leu Ser Leu Gly Asp Lys
1170                1175                1180

Thr His Pro Gln Phe Arg Ser Ile Val Ser Ala Leu Lys Arg
1185                1190                1195

Glu Ala Leu Val Lys Gly Asn Pro Pro Ile Tyr Arg Phe Trp
1200                1205                1210

Lys Asp Asn Leu Gln His Lys Asp Ser Ser Val Pro Asn Thr
1215                1220

Gly Thr Ala Arg Met Val Glu Thr Thr Ala Tyr Ala Leu Leu
1225                1230                1235

Thr Ser Leu Asn Leu Lys Asp Ile Asn Tyr Val Asn Pro Val
1240                1245                1250

Ile Lys Trp Leu Ser Glu Glu Gln Arg Tyr Gly Gly Gly Phe
1255                1260                1265

Tyr Ser Thr Gln Asp Thr Ile Asn Ala Ile Glu Gly Leu Thr
1270                1275                1280

Glu Tyr Ser Leu Leu Val Lys Gln Leu Arg Leu Ser Met Asp
1285                1290

Ile Asp Val Ser Tyr Lys His Lys Gly Ala Leu His Asn Tyr
1295                1300                1305
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Lys Met Thr Asp Lys Asn Phe Leu Gly Arg Pro Val Glu Val
1310                1315                1320

Leu Leu Asn Asp Asp Leu Ile Val Ser Thr Gly Phe Gly Ser
1325                1330                1335

Gly Leu Ala Thr Val His Val Thr Thr Val Val His Lys Thr
1340                1345                1350

Ser Thr Ser Glu Glu Val Cys Ser Phe Tyr Leu Lys Ile Asp
1355                1360

Thr Gln Asp Ile Glu Ala Ser His Tyr Arg Gly Tyr Gly Asn
1365                1370                1375

Ser Asp Tyr Lys Arg Ile Val Ala Cys Ala Ser Tyr Lys Pro
1380                1385                1390

Ser Arg Glu Glu Ser Ser Ser Gly Ser Ser His Ala Val Met
1395                1400                1405

Asp Ile Ser Leu Pro Thr Gly Ile Ser Ala Asn Glu Glu Asp
1410                1415                1420

Leu Lys Ala Leu Val Glu Gly Val Asp Gln Leu Phe Thr Asp
1425                1430

Tyr Gln Ile Lys Asp Gly His Val Ile Leu Gln Leu Asn Ser
1435                1440                1445

Ile Pro Ser Ser Asp Phe Leu Cys Val Arg Phe Arg Ile Phe
1450                1455                1460

Glu Leu Phe Glu Val Gly Phe Leu Ser Pro Ala Thr Phe Thr
1465                1470                1475

Val Tyr Glu Tyr His Arg Pro Asp Lys Gln Cys Thr Met Phe
1480                1485                1490

Tyr Ser Thr Ser Asn Ile Lys Ile Gln Lys Val Cys Glu Gly
1495                1500

Ala Ala Cys Lys Cys Val Glu Ala Asp Cys Gly Gln Met Gln
1505                1510                1515

Glu Glu Leu Asp Leu Thr Ile Ser Ala Glu Thr Arg Lys Gln
1520                1525                1530

Thr Ala Cys Lys Pro Glu Ile Ala Tyr Ala Tyr Lys Val Ser
1535                1540                1545

Ile Thr Ser Ile Thr Val Glu Asn Val Phe Val Lys Tyr Lys
1550                1555                1560

Ala Thr Leu Leu Asp Ile Tyr Lys Thr Gly Glu Ala Val Ala
1565                1570

Glu Lys Asp Ser Glu Ile Thr Phe Ile Lys Lys Val Thr Cys
1575                1580                1585

Thr Asn Ala Glu Leu Val Lys Gly Arg Gln Tyr Leu Ile Met
1590                1595                1600

Gly Lys Glu Ala Leu Gln Ile Lys Tyr Asn Phe Ser Phe Arg
1605                1610                1615

Tyr Ile Tyr Pro Leu Asp Ser Leu Thr Trp Ile Glu Tyr Trp
1620                1625                1630

Pro Arg Asp Thr Thr Cys Ser Ser Cys Gln Ala Phe Leu Ala
1635                1640

Asn Leu Asp Glu Phe Ala Glu Asp Ile Phe Leu Asn Gly Cys
1645                1650                1655
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4059 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Circular

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Apex-1 Eukaryotic Expression Vector

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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AATAATGACG TATGTTCCCA TAGTAACGCC AATAGGGACT TTCCATTGAC 200

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GTGTATCATA TGCCAAGTAC GCCCCCTATT GACGTCAATG ACGGTAAATG 300

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TGCCGAGAGT CCCGTAAGGG TAGACACTTC AGCTAATCCC TCGATGAGGT 1600

CTACTAGAAT AGTCAGTGCG GCTCCCATTT TGAAAATTCA CTTACTTGAT 1650

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TGCCACCTG                                                          4059
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8540 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Circular

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Apex-3P Eukaryotic Expression Vector

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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GGGTTAGGCG TTTTGCGCTG CTTCGCGATG TACGGGCCAG ATATACGCGT              300

TGACATTGAT TATTGACTAG TTATTAATAG TAATCAATTA CGGGGTCATT              350

AGTTCATAGC CCATATATGG AGTTCCGCGT TACATAACTT ACGGTAAATG              400

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TCCACAATTT CAAAAAAAAG AGTGGCCACT TGTCTTTGTT TATGGGCCCC 7400

ATTGGCGTGG AGCCCCGTTT AATTTTCGGG GGTGTTAGAG ACAACCAGTG 7450

GAGTCCGCTG CTGTCGGCGT CCACTCTCTT TCCCCTTGTT ACAAATAGAG 7500

TGTAACAACA TGGTTCACCT GTCTTGGTCC CTGCCTGGGA CACATCTTAA 7550

TAACCCCAGT ATCATATTGC ACTAGGATTA TGTGTTGCCC ATAGCCATAA 7600

ATTCGTGTGA GATGGACATC CAGTCTTTAC GGCTTGTCCC CACCCCATGG 7650

ATTTCTATTG TTAAAGATAT TCAGAATGTT TCATTCCTAC ACTAGTATTT 7700

ATTGCCCAAG GGGTTTGTGA GGGTTATATT GGTGTCATAG CACAATGCCA 7750

CCACTGAACC CCCCGTCCAA ATTTTATTCT GGGGGCGTCA CCTGAAACCT 7800

TGTTTTCGAG CACCTCACAT ACACCTTACT GTTCACAACT CAGCAGTTAT 7850

TCTATTAGCT AAACGAAGGA GAATGAAGAA GCAGGCGAAG ATTCAGGAGA 7900

GTTCACTGCC CGCTCCTTGA TCTTCAGCCA CTGCCCTTGT GACTAAAATG 7950

GTTCACTACC CTCGTGGAAT CCTGACCCCA TGTAAATAAA ACCGTGACAG 8000

CTCATGGGGT GGGAGATATC GCTGTTCCTT AGGACCCTTT TACTAACCCT 8050

AATTCGATAG CATATGCTTC CCGTTGGGTA ACATATGCTA TTGAATTAGG 8100

GTTAGTCTGG ATAGTATATA CTACTACCCG GGAAGCATAT GCTACCCGTT 8150

TAGGGTTAAC AAGGGGGCCT TATAAACACT ATTGCTAATG CCCTCTTGAG 8200

GGTCCGCTTA TCGGTAGCTA CACAGGCCCC TCTGATTGAC GTTGGTGTAG 8250

CCTCCCGTAG TCTTCCTGGG CCCCTGGGAG GTACATGTCC CCCAGCATTG 8300

GTGTAAGAGC TTCAGCCAAG AGTTACACAT AAAGGCAATG TTGTGTTGCA 8350

GTCCACAGAC TGCAAAGTCT GCTCCAGGAT GAAAGCCACT CAGTGTTGGC 8400

AAATGTGCAC ATCCATTTAT AAGGATGTCA ACTACAGTCA GAGAACCCCT 8450

TTGTGTTTGG TCCCCCCCCG TGTCACATGT GGAACAGGGC CCAGTTGGCA 8500

AGTTGTACCA ACCAACTGAA GGGATTACAT GCACTGCCCC 8540

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 bases
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer UDEC690

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGCCTGCAGG ACATCCAGAT GACTCAGTCT 30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 bases
(B) TYPE: Nucleic Acid

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(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Oligonucleotide primer UDEC395

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
CCCAAGCTTA CTGGATGGTG GGAAGATGGA                                    30
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1M1 scFv (murine)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
ATG GCC GAC ATC CAG ATG ACT CAG TCT CCA                              30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

GCT TCA CTG TCT GCA TCT GTG GGA GAA ACT                              60
Ala Ser Leu Ser Ala Ser Val Gly Glu Thr
15                  20

GTC ACC ATC ACA TGT GGA GCA AGT GAG AAT                              90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
25                  30

ATT TAC GGT GCT TTA AAT TGG TAT CAG CGG                             120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Arg
35                  40

AAA CAG GGA AAA TCT CCT CAG CTC CTG ATC                             150
Lys Gln Gly Lys Ser Pro Gln Leu Leu Ile
45                  50

TAT GGT GCA ACC AAC TTG GCA GAT GGC ATG                             180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Met
55                  60

TCA TCG AGG TTC AGT GGC AGT GGA TCT GGT                             210
Ser Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

AGA CAG TAT TAT CTC AAG ATC AGT AGC CTG                             240
Arg Gln Tyr Tyr Leu Lys Ile Ser Ser Leu
75                  80

CAT CCT GAC GAT GTT GCA ACG TAT TAC TGT                             270
His Pro Asp Asp Val Ala Thr Tyr Tyr Cys
85                  90

CAA AAT GTG TTA AAT ACT CCT CTC ACG TTC                             300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGT GCT GGG ACC AAG TTG GAG CTG AAA CGG                             330
Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg
105                 110

ACC GGA GGT GGC GGG TCG GGT GGC GGG GGA                             360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCG GGT GGC GGA GGG TCG CAG GTT CAG CTG                             390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130
```

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```
CAG CAG TCT GGA GCC GAG CTG ATG AAG CCT                                  420
Gln Gln Ser Gly Ala Glu Leu Met Lys Pro
135                 140

GGG GCC TCA GTG AAG ATG TCC TGC AAG GCT                                  450
Gly Ala Ser Val Lys Met Ser Cys Lys Ala
145                 150

ACT GGC TAC ATA TTC AGT AAC TAC TGG ATA                                  480
Thr Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAG TGG ATA AAG CAG AGG CCT GGA CAT GGC                                  510
Gln Trp Ile Lys Gln Arg Pro Gly His Gly
165                 170

CTT GAG TGG ATT GGT GAG ATT TTA CCT GGA                                  540
Leu Glu Trp Ile Gly Glu Ile Leu Pro Gly
175                 180

AGT GGT TCT ACT GAG TAC ACT GAG AAC TTC                                  570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
185                 190

AAG GAC AAG GCC GCA TTC ACT GCA GAT ACA                                  600
Lys Asp Lys Ala Ala Phe Thr Ala Asp Thr
195                 200

TCC TCC AAC ACA GCC TAC ATG CAA CTC AGC                                  630
Ser Ser Asn Thr Ala Tyr Met Gln Leu Ser
205                 210

AGC CTG ACA TCA GAG GAC TCT GCC GTC TAT                                  660
Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr
215                 220

TAC TGT GCA AGA TAT TTC TTC GGT AGT AGC                                  690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCC AAC TGG TAC TTC GAT GTC TGG GGC GCA                                  720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Ala
235                 240

GGG ACC ACG GTC ACC GTC TCC TCA TGA                                      747
Gly Thr Thr Val Thr Val Ser Ser
245
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv CB (humanized)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                   30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                   60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                                   90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CGT                                  120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Arg
35                  40
```

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```
AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                                  150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                                  180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                  210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                  240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                                  270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                                  300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                                  330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                                  360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                                  390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                                  420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                                  450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                                  480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                                  510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                                  540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                                  570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                                  600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                                  630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                                  660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                                  690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                                  720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240
```


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```
GGA ACC CTG GTC ACT GTC TCG AGC TGA                                    747
Gly Thr Leu Val Thr Val Ser Ser
245

(2) INFORMATION FOR SEQ ID NO:9:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH:  726 base pairs
          (B) TYPE:  Nucleic Acid
          (C) STRANDEDNESS:  Double
          (D) TOPOLOGY:  linear

    (ii) MOLECULE TYPE:  Other nucleic acid
          (A) DESCRIPTION:5G1.1M1 VL HuK (chimeric light chain)

    (xi) SEQUENCE DESCRIPTION:  SEQ ID NO:9:

ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC                                 30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC                                 60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10

CAT TCA GGT CAT AGC CTG CAG GAC ATC CAG                                 90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                   1                   5

ATG ACT CAG TCT CCA GCT TCA CTG TCT GCA                                 120
Met Thr Gln Ser Pro Ala Ser Leu Ser Ala
10                  15

TCT GTG GGA GAA ACT GTC ACC ATC ACA TGT                                 150
Ser Val Gly Glu Thr Val Thr Ile Thr Cys
20                  25

GGA GCA AGT GAG AAT ATT TAC GGT GCT TTA                                 180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
30                  35

AAT TGG TAT CAG CGG AAA CAG GGA AAA TCT                                 210
Asn Trp Tyr Gln Arg Lys Gln Gly Lys Ser
40                  45

CCT CAG CTC CTG ATC TAT GGT GCA ACC AAC                                 240
Pro Gln Leu Leu Ile Tyr Gly Ala Thr Asn
50                  55

TTG GCA GAT GGC ATG TCA TCG AGG TTC AGT                                 270
Leu Ala Asp Gly Met Ser Ser Arg Phe Ser
60                  65

GGC AGT GGA TCT GGT AGA CAG TAT TAT CTC                                 300
Gly Ser Gly Ser Gly Arg Gln Tyr Tyr Leu
70                  75

AAG ATC AGT AGC CTG CAT CCT GAC GAT GTT                                 330
Lys Ile Ser Ser Leu His Pro Asp Asp Val
80                  85

GCA ACG TAT TAC TGT CAA AAT GTG TTA AAT                                 360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
90                  95

ACT CCT CTC ACG TTC GGT GCT GGG ACC AAG                                 390
Thr Pro Leu Thr Phe Gly Ala Gly Thr Lys
100                 105

TTG GAG CTG AAA CGA ACT GTG GCT GCA CCA                                 420
Leu Glu Leu Lys Arg Thr Val Ala Ala Pro
110                 115

TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG                                 450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
120                 125
```


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```
CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG                                  480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG                                  510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC                                  540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
150                 155

CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC                                  570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
160                 165

ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC                                  600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
170                 175

AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA                                  630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC                                  660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG                                  690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG                                  720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
210                 215

TGT TAG                                                                  726
Cys
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 750 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1M1 VH +HuG1 (chimeric Fd)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
ATG AAA TGG AGC TGG GTT ATT CTC TTC CTC                                   30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
-15                 -10

CTG TCA GTA ACT GCA GGT GTC CAC TCC CAG                                   60
Leu Ser Val Thr Ala Gly Val His Ser Gln
-5                  1

GTT CAG CTG CAG CAG TCT GGA GCT GAG CTG                                   90
Val Gln Leu Gln Gln Ser Gly Ala Glu Leu
5                   10

ATG AAG CCT GGG GCC TCA GTG AAG ATG TCC                                  120
Met Lys Pro Gly Ala Ser Val Lys Met Ser
15                  20

TGC AAG GCT ACT GGC TAC ATA TTC AGT AAC                                  150
Cys Lys Ala Thr Gly Tyr Ile Phe Ser Asn
25                  30

TAC TGG ATA CAG TGG ATA AAG CAG AGG CCT                                  180
Tyr Trp Ile Gln Trp Ile Lys Gln Arg Pro
35                  40

GGA CAT GGC CTT GAG TGG ATT GGT GAG ATT                                  210
Gly His Gly Leu Glu Trp Ile Gly Glu Ile
45                  50
```

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```
TTA CCT GGA AGT GGT TCT ACT GAG TAC ACT                                  240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr
55                  60

GAG AAC TTC AAG GAC AAG GCC GCA TTC ACT                                  270
Glu Asn Phe Lys Asp Lys Ala Ala Phe Thr
65                  70

GCA GAT ACA TCC TCC AAC ACA GCC TAC ATG                                  300
Ala Asp Thr Ser Ser Asn Thr Ala Tyr Met
75                  80

CAA CTC AGC AGC CTG ACA TCA GAG GAC TCT                                  330
Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser
85                  90

GCC GTC TAT TAC TGT GCA AGA TAT TTC TTC                                  360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
95                 100

GGT AGT AGC CCC AAC TGG TAC TTC GAT GTC                                  390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
105                 110

TGG GGC GCA GGG ACC ACG GTC ACC GTC TCC                                  420
Trp Gly Ala Gly Thr Thr Val Thr Val Ser
115                 120

TCA GCC TCC ACC AAG GGC CCA TCG GTC TTC                                  450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                                  480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                                  510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                                  540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                                  570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                                  600
Val His Thr Phe Pro Ala Val Leu Gln Ser
175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                                  630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                                  660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                                  690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                                  720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                                  750
Pro Lys Ser Cys Asp Lys Thr His Thr
225
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 750 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 VH + IGHRL (Humanized Fd)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC                          30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
-15                 -10

CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA                          60
Leu Ser Val Thr Ala Gly Val His Ser Gln
-5                  1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC                          90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
5                   10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC                         120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
15                  20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT                         150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
25                  30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC                         180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
35                  40

GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC                         210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
45                  50

TTA CCG GGC TCT GGT AGC ACC GAA TAT GCC                         240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Ala
55                  60

CAA AAA TTC CAG GGC CGT GTT ACT ATG ACT                         270
Gln Lys Phe Gln Gly Arg Val Thr Met Thr
65                  70

GCG GAC ACT TCG ACT AGT ACA GCC TAC ATG                         300
Ala Asp Thr Ser Thr Ser Thr Ala Tyr Met
75                  80

GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG                         330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT                         360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
95                 100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT                         390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
105                 110

TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG                         420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC                         450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                         480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                         510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                         540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
155                 160
```


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```
TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                                  570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                                  600
Val His Thr Phe Pro Ala Val Leu Gln Ser
175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                                  630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                                  660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                                  690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                                  720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                                  750
Pro Lys Ser Cys Asp Lys Thr His Thr
225                 230

(2) INFORMATION FOR SEQ ID NO:12:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH:  750 base pairs
          (B) TYPE:  Nucleic Acid
          (C) STRANDEDNESS:  Double
          (D) TOPOLOGY:  linear

    (ii) MOLECULE TYPE:  Other nucleic acid
          (A) DESCRIPTION:5G1.1 VH + IGHRLC (Humanized Fd)

    (xi) SEQUENCE DESCRIPTION:  SEQ ID NO:12:

ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC                                   30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
-15                 -10

CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA                                   60
Leu Ser Val Thr Ala Gly Val His Ser Gln
-5                   1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC                                   90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
5                   10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC                                  120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
15                  20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT                                  150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
25                  30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC                                  180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
35                  40

GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC                                  210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
45                  50

TTA CCG GGC TCT GGT AGC ACC GAA TAT ACC                                  240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr
55                  60

GAA AAT TTT AAA GAC CGT GTT ACT ATG ACG                                  270
Glu Asn Phe Lys Asp Arg Val Thr Met Thr
65                  70
```


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```
CGT GAC ACT TCG ACT AGT ACA GTA TAC ATG                                  300
Arg Asp Thr Ser Thr Ser Thr Val Tyr Met
75                  80

GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG                                  330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT                                  360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
95                 100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT                                  390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
105                 110

TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG                                  420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC                                  450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                                  480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                                  510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                                  540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                                  570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                                  600
Val His Thr Phe Pro Ala Val Leu Gln Ser
175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                                  630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                                  660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                                  690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                                  720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                                  750
Pro Lys Ser Cys Asp Lys Thr His Thr
225                 230
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 726 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 VL +KLV56
(Humanized light chain)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC                          30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC                          60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10

CAT TCA GGT CAT AGC CTG CAG GAT ATC CAG                          90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                   1                   5

ATG ACC CAG TCC CCG TCC TCC CTG TCC GCC                         120
Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
10                  15

TCT GTG GGC GAT AGG GTC ACC ATC ACC TGC                         150
Ser Val Gly Asp Arg Val Thr Ile Thr Cys
20                  25

GGC GCC AGC GAA AAC ATC TAT GGC GCG CTG                         180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
30                  35

AAC TGG TAT CAA CGT AAA CCT GGG AAA GCT                         210
Asn Trp Tyr Gln Arg Lys Pro Gly Lys Ala
40                  45

CCG AAG CTT CTG ATT TAC GGT GCG ACG AAC                         240
Pro Lys Leu Leu Ile Tyr Gly Ala Thr Asn
50                  55

CTG GCA GAT GGA GTC CCT TCT CGC TTC TCT                         270
Leu Ala Asp Gly Val Pro Ser Arg Phe Ser
60                  65

GGA TCC GGC TCC GGA ACG GAT TAC ACT CTG                         300
Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu
70                  75

ACC ATC AGC AGT CTG CAA CCT GAG GAC TTC                         330
Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe
80                  85

GCT ACG TAT TAC TGT CAG AAC GTT TTA AAT                         360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
90                  95

ACT CCG TTG ACT TTC GGA CAG GGT ACC AAG                         390
Thr Pro Leu Thr Phe Gly Gln Gly Thr Lys
100                 105

GTG GAA ATA AAA CGA ACT GTG GCT GCA CCA                         420
Val Glu Ile Lys Arg Thr Val Ala Ala Pro
110                 115

TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG                         450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
120                 125

CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG                         480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG                         510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC                         540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
150                 155

CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC                         570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
160                 165
```


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```
ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC                                  600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
170                 175

AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA                                  630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC                                  660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG                                  690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG                                  720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
210                 215

TGT TAG                                                                  726
Cys
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 726 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 VL +KLV56B
(Humanized light chain)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC                                   30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC                                   60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10

CAT TCA GGT CAT AGC CTG CAG GAT ATC CAG                                   90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                   1                   5

ATG ACC CAG TCC CCG TCC TCC CTG TCC GCC                                  120
Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
10                  15

TCT GTG GGC GAT AGG GTC ACC ATC ACC TGC                                  150
Ser Val Gly Asp Arg Val Thr Ile Thr Cys
20                  25

GGC GCC AGC GAA AAC ATC TAT GGC GCG CTG                                  180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
30                  35

AAC TGG TAT CAA CGT AAA CCT GGG AAA GCT                                  210
Asn Trp Tyr Gln Arg Lys Pro Gly Lys Ala
40                  45

CCG AAG CTT CTG ATT TAC GGT GCG ACG AAC                                  240
Pro Lys Leu Leu Ile Tyr Gly Ala Thr Asn
50                  55

CTG GCA GAT GGA GTC CCT TCT CGC TTC TCT                                  270
Leu Ala Asp Gly Val Pro Ser Arg Phe Ser
60                  65

GGA TCC GGC TCC GGA ACG GAT TTC ACT CTG                                  300
Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu
70                  75
```


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```
ACC ATC AGC AGT CTG CAG CCT GAA GAC TTC                                  330
Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe
80                  85

GCT ACG TAT TAC TGT CAG AAC GTT TTA AAT                                  360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
90                  95

ACT CCG TTG ACT TTC GGA CAG GGT ACC AAG                                  390
Thr Pro Leu Thr Phe Gly Gln Gly Thr Lys
100                 105

GTG GAA ATA AAA CGA ACT GTG GCT GCA CCA                                  420
Val Glu Ile Lys Arg Thr Val Ala Ala Pro
110                 115

TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG                                  450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
120                 125

CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG                                  480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG                                  510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC                                  540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
150                 155

CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC                                  570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
160                 165

ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC                                  600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
170                 175

AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA                                  630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC                                  660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG                                  690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG                                  720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
210                 215

TGT TAG                                                                  726
Cys
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 711 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 VL + O12
(Humanized light chain)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
ATG GAC ATG AGG GTC CCC GCT CAG CTC CTG                                   30
Met Asp Met Arg Val Pro Ala Gln Leu Leu
-20                 -15
```


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```
GGG CTC CTG CTA CTC TGG CTC CGA GGT GCC                                  60
Gly Leu Leu Leu Leu Trp Leu Arg Gly Ala
-10                 -5

AGA TGT GAT ATC CAG ATG ACC CAG TCC CCG                                  90
Arg Cys Asp Ile Gln Met Thr Gln Ser Pro
1               5

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                 120
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
10                  15

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                                 150
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
20                  25

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                                 180
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
30                  35

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                                 210
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
40                  45

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                                 240
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
50                  55

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                 270
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
60                  65

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                 300
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
70                  75

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                                 330
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
80                  85

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                                 360
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
90                  95

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGA                                 390
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
100                 105

ACT GTG GCT GCA CCA TCT GTC TTC ATC TTC                                 420
Thr Val Ala Ala Pro Ser Val Phe Ile Phe
110                 115

CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA                                 450
Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
120                 125

ACT GCC TCT GTT GTG TGC CTG CTG AAT AAC                                 480
Thr Ala Ser Val Val Cys Leu Leu Asn Asn
130                 135

TTC TAT CCC AGA GAG GCC AAA GTA CAG TGG                                 510
Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp
140                 145

AAG GTG GAT AAC GCC CTC CAA TCG GGT AAC                                 540
Lys Val Asp Asn Ala Leu Gln Ser Gly Asn
150                 155

TCC CAG GAG AGT GTC ACA GAG CAG GAC AGC                                 570
Ser Gln Glu Ser Val Thr Glu Gln Asp Ser
160                 165

AAG GAC AGC ACC TAC AGC CTC AGC AGC ACC                                 600
Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr
170                 175

CTG ACG CTG AGC AAA GCA GAC TAC GAG AAA                                 630
Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys
180                 185
```


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```
CAC AAA GTC TAC GCC TGC GAA GTC ACC CAT                                  660
His Lys Val Tyr Ala Cys Glu Val Thr His
190                 195

CAG GGC CTG AGC TCG CCC GTC ACA AAG AGC                                  690
Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
200                 205

TTC AAC AGG GGA GAG TGT TAG                                              711
Phe Asn Arg Gly Glu Cys
210
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 750 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 VH + IGHRLD
(Humanized Fd)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC                                   30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
-15                 -10

CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA                                   60
Leu Ser Val Thr Ala Gly Val His Ser Gln
-5                  1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC                                   90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
5                   10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC                                   120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
15                  20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT                                   150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
25                  30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC                                   180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
35                  40

GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC                                   210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
45                  50

TTA CCG GGC TCT GGT AGC ACC GAA TAT GCC                                   240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Ala
55                  60

CAA AAA TTC CAG GGC CGT GTT ACT ATG ACT                                   270
Gln Lys Phe Gln Gly Arg Val Thr Met Thr
65                  70

CGT GAC ACT TCG ACT AGT ACA GTA TAC ATG                                   300
Arg Asp Thr Ser Thr Ser Thr Val Tyr Met
75                  80

GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG                                   330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT                                   360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
95                 100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT                                   390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
105                 110
```


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```
TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG                                  420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC                                  450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                                  480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                                  510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                                  540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                                  570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                                  600
Val His Thr Phe Pro Ala Val Leu Gln Ser
175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                                  630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                                  660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                                  690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                                  720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                                  750
Pro Lys Ser Cys Asp Lys Thr His Thr
225                 230

(2) INFORMATION FOR SEQ ID NO:17:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH:  747 base pairs
          (B) TYPE:  Nucleic Acid
          (C) STRANDEDNESS:  Double
          (D) TOPOLOGY:  linear

    (ii) MOLECULE TYPE:  Other nucleic acid
          (A) DESCRIPTION:5G1.1 scFv DO12
          (Humanized scFv)

    (xi) SEQUENCE DESCRIPTION:  SEQ ID NO:17:

ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                   30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                   60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                                   90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                                  120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40
```

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```
AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                                  150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                                  180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                  210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                  240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                                  270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                                  300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                                  330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                                  360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                                  390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                                  420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                                  450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                                  480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                                  510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                                  540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                                  570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                                  600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                                  630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                                  660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                                  690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                                  720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240
```


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GGA ACC CTG GTC ACT GTC TCG AGC TGA 747
Gly Thr Leu Val Thr Val Ser Ser
245

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5248 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Circular

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: pET Trc SO5/NI
prokaryotic expression vector

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TGGCGAATGG GACGCGCCCT GTAGCGGCGC ATTAAGCGCG GCGGGTGTGG 50

TGGTTACGCG CAGCGTGACC GCTACACTTG CCAGCGCCCT AGCGCCCGCT 100

CCTTTCGCTT TCTTCCCTTC CTTTCTCGCC ACGTTCGCCG GCTTTCCCCG 150

TCAAGCTCTA AATCGGGGGC TCCCTTTAGG GTTCCGATTT AGTGCTTTAC 200

GGCACCTCGA CCCCAAAAAA CTTGATTAGG GTGATGGTTC ACGTAGTGGG 250

CCATCGCCCT GATAGACGGT TTTTCGCCCT TTGACGTTGG AGTCCACGTT 300

CTTTAATAGT GGACTCTTGT TCCAAACTGG AACAACACTC AACCCTATCT 350

CGGTCTATTC TTTTGATTTA TAAGGGATTT TGCCGATTTC GGCCTATTGG 400

TTAAAAAATG AGCTGATTTA ACAAAAATTT AACGCGAATT TTAACAAAAT 450

ATTAACGTTT ACAATTTCAG GTGGCACTTT TCGGGGAAAT GTGCGCGGAA 500

CCCCTATTTG TTTATTTTTC TAAATACATT CAAATATGTA TCCGCTCATG 550

AGACAATAAC CCTGATAAAT GCTTCAATAA TATTGAAAAA GGAAGAGTAT 600

GAGTATTCAA CATTTCCGTG TCGCCCTTAT TCCCTTTTTT GCGGCATTTT 650

GCCTTCCTGT TTTTGCTCAC CCAGAAACGC TGGTGAAAGT AAAAGATGCT 700

GAAGATCAGT TGGGTGCACG AGTGGGTTAC ATCGAACTGG ATCTCAACAG 750

CGGTAAGATC CTTGAGAGTT TTCGCCCCGA AGAACGTTTT CCAATGATGA 800

GCACTTTTAA AGTTCTGCTA TGTGGCGCGG TATTATCCCG TATTGACGCC 850

GGGCAAGAGC AACTCGGTCG CCGCATACAC TATTCTCAGA ATGACTTGGT 900

TGAGTACTCA CCAGTCACAG AAAAGCATCT TACGGATGGC ATGACAGTAA 950

GAGAATTATG CAGTGCTGCC ATAACCATGA GTGATAACAC TGCGGCCAAC 1000

TTACTTCTGA CAACGATCGG AGGACCGAAG GAGCTAACCG CTTTTTTGCA 1050

CAACATGGGG GATCATGTAA CTCGCCTTGA TCGTTGGGAA CCGGAGCTGA 1100

ATGAAGCCAT ACCAAACGAC GAGCGTGACA CCACGATGCC TGCAGCAATG 1150

GCAACAACGT TGCGCAAACT ATTAACTGGC GAACTACTTA CTCTAGCTTC 1200

CCGGCAACAA TTAATAGACT GGATGGAGGC GGATAAAGTT GCAGGACCAC 1250

TTCTGCGCTC GGCCCTTCCG GCTGGCTGGT TTATTGCTGA TAAATCTGGA 1300

GCCGGTGAGC GTGGGTCTCG CGGTATCATT GCAGCACTGG GGCCAGATGG 1350

TAAGCCCTCC CGTATCGTAG TTATCTACAC GACGGGGAGT CAGGCAACTA 1400

TGGATGAACG AAATAGACAG ATCGCTGAGA TAGGTGCCTC ACTGATTAAG 1450

CATTGGTAAC TGTCAGACCA AGTTTACTCA TATATACTTT AGATTGATTT 1500

AAAACTTCAT TTTTAATTTA AAAGGATCTA GGTGAAGATC CTTTTTGATA 1550

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```
ATCTCATGAC CAAAATCCCT TAACGTGAGT TTTCGTTCCA CTGAGCGTCA              1600

GACCCCGTAG AAAAGATCAA AGGATCTTCT TGAGATCCTT TTTTTCTGCG              1650

CGTAATCTGC TGCTTGCAAA CAAAAAAACC ACCGCTACCA GCGGTGGTTT              1700

GTTTGCCGGA TCAAGAGCTA CCAACTCTTT TTCCGAAGGT AACTGGCTTC              1750

AGCAGAGCGC AGATACCAAA TACTGTCCTT CTAGTGTAGC CGTAGTTAGG              1800

CCACCACTTC AAGAACTCTG TAGCACCGCC TACATACCTC GCTCTGCTAA              1850

TCCTGTTACC AGTGGCTGCT GCCAGTGGCG ATAAGTCGTG TCTTACCGGG              1900

TTGGACTCAA GACGATAGTT ACCGGATAAG GCGCAGCGGT CGGGCTGAAC              1950

GGGGGGTTCG TGCACACAGC CCAGCTTGGA GCGAACGACC TACACCGAAC              2000

TGAGATACCT ACAGCGTGAG CTATGAGAAA GCGCCACGCT TCCCGAAGGG              2050

AGAAAGGCGG ACAGGTATCC GGTAAGCGGC AGGGTCGGAA CAGGAGAGCG              2100

CACGAGGGAG CTTCCAGGGG GAAACGCCTG GTATCTTTAT AGTCCTGTCG              2150

GGTTTCGCCA CCTCTGACTT GAGCGTCGAT TTTTGTGATG CTCGTCAGGG              2200

GGGCGGAGCC TATGGAAAAA CGCCAGCAAC GCGGCCTTTT TACGGTTCCT              2250

GGCCTTTTGC TGGCCTTTTG CTCACATGTT CTTTCCTGCG TTATCCCCTG              2300

ATTCTGTGGA TAACCGTATT ACCGCCTTTG AGTGAGCTGA TACCGCTCGC              2350

CGCAGCCGAA CGACCGAGCG CAGCGAGTCA GTGAGCGAGG AAGCGGAAGA              2400

GCGCCTGATG CGGTATTTTC TCCTTACGCA TCTGTGCGGT ATTTCACACC              2450

GCATATATGG TGCACTCTCA GTACAATCTG CTCTGATGCC GCATAGTTAA              2500

GCCAGTATAC ACTCCGCTAT CGCTACGTGA CTGGGTCATG GCTGCGCCCC              2550

GACACCCGCC AACACCCGCT GACGCGCCCT GACGGGCTTG TCTGCTCCCG              2600

GCATCCGCTT ACAGACAAGC TGTGACCGTC TCCGGGAGCT GCATGTGTCA              2650

GAGGTTTTCA CCGTCATCAC CGAAACGCGC GAGGCAGCTG CGGTAAAGCT              2700

CATCAGCGTG GTCGTGAAGC GATTCACAGA TGTCTGCCTG TTCATCCGCG              2750

TCCAGCTCGT TGAGTTTCTC CAGAAGCGTT AATGTCTGGC TTCTGATAAA              2800

GCGGGCCATG TTAAGGGCGG TTTTTTCCTG TTTGGTCACT GATGCCTCCG              2850

TGTAAGGGGG ATTTCTGTTC ATGGGGGTAA TGATACCGAT GAAACGAGAG              2900

AGGATGCTCA CGATACGGGT TACTGATGAT GAACATGCCC GGTTACTGGA              2950

ACGTTGTGAG GGTAAACAAC TGGCGGTATG GATGCGGCGG GACCAGAGAA              3000

AAATCACTCA GGGTCAATGC CAGCGCTTCG TTAATACAGA TGTAGGTGTT              3050

CCACAGGGTA GCCAGCAGCA TCCTGCGATG CAGATCCGGA ACATAATGGT              3100

GCAGGGCGCT GACTTCCGCG TTTCCAGACT TTACGAAACA CGGAAACCGA              3150

AGACCATTCA TGTTGTTGCT CAGGTCGCAG ACGTTTTGCA GCAGCAGTCG              3200

CTTCACGTTC GCTCGCGTAT CGGTGATTCA TTCTGCTAAC CAGTAAGGCA              3250

ACCCCGCCAG CCTAGCCGGG TCCTCAACGA CAGGAGCACG ATCATGCGCA              3300

CCCGTGGGGC CGCCATGCCG GCGATAATGG CCTGCTTCTC GCCGAAACGT              3350

TTGGTGGCGG GACCAGTGAC GAAGGCTTGA GCGAGGGCGT GCAAGATTCC              3400

GAATACCGCA AGCGACAGGC CGATCATCGT CGCGCTCCAG CGAAAGCGGT              3450

CCTCGCCGAA AATGACCCAG AGCGCTGCCG GCACCTGTCC TACGAGTTGC              3500

ATGATAAAGA AGACAGTCAT AAGTGCGGCG ACGATAGTCA TGCCCCGCGC              3550
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CCACCGGAAG GAGCTGACTG GGTTGAAGGC TCTCAAGGGC ATCGGTCGAG 3600

ATCCCGGTGC CTAATGAGTG AGCTAACTTA CATTAATTGC GTTGCGCTCA 3650

CTGCCCGCTT TCCAGTCGGG AAACCTGTCG TGCCAGCTGC ATTAATGAAT 3700

CGGCCAACGC GCGGGGAGAG GCGGTTTGCG TATTGGGCGC CAGGGTGGTT 3750

TTTCTTTTCA CCAGTGAGAC GGGCAACAGC TGATTGCCCT TCACCGCCTG 3800

GCCCTGAGAG AGTTGCAGCA AGCGGTCCAC GCTGGTTTGC CCCAGCAGGC 3850

GAAAATCCTG TTTGATGGTG GTTAACGGCG GGATATAACA TGAGCTGTCT 3900

TCGGTATCGT CGTATCCCAC TACCGAGATA TCCGCACCAA CGCGCAGCCC 3950

GGACTCGGTA ATGGCGCGCA TTGCGCCCAG CGCCATCTGA TCGTTGGCAA 4000

CCAGCATCGC AGTGGGAACG ATGCCCTCAT TCAGCATTTG CATGGTTTGT 4050

TGAAAACCGG ACATGGCACT CCAGTCGCCT TCCCGTTCCG CTATCGGCTG 4100

AATTTGATTG CGAGTGAGAT ATTTATGCCA GCCAGCCAGA CGCAGACGCG 4150

CCGAGACAGA ACTTAATGGG CCCGCTAACA GCGCGATTTG CTGGTGACCC 4200

AATGCGACCA GATGCTCCAC GCCCAGTCGC GTACCGTCTT CATGGGAGAA 4250

AATAATACTG TTGATGGGTG TCTGGTCAGA GACATCAAGA AATAACGCCG 4300

GAACATTAGT GCAGGCAGCT TCCACAGCAA TGGCATCCTG GTCATCCAGC 4350

GGATAGTTAA TGATCAGCCC ACTGACGCGT TGCGCGAGAA GATTGTGCAC 4400

CGCCGCTTTA CAGGCTTCGA CGCCGCTTCG TTCTACCATC GACACCACCA 4450

CGCTGGCACC CAGTTGATCG GCGCGAGATT TAATCGCCGC GACAATTTGC 4500

GACGGCGCGT GCAGGGCCAG ACTGGAGGTG GCAACGCCAA TCAGCAACGA 4550

CTGTTTGCCC GCCAGTTGTT GTGCCACGCG GTTGGGAATG TAATTCAGCT 4600

CCGCCATCGC CGCTTCCACT TTTTCCCGCG TTTTCGCAGA AACGTGGCTG 4650

GCCTGGTTCA CCACGCGGGA AACGGTCTGA TAAGAGACAC CGGCATACTC 4700

TGCGACATCG TATAACGTTA CTGGTTTCAC ATTCACCACC CTGAATTGAC 4750

TCTCTTCCGG GCGCTATCAT GCCATACCGC GAAAGGTTTT GCGCCATTCG 4800

ATGGTGTCCG GGATCTCGAC GCTCTCCCTT ATGCGACTCC TGCATTAGGA 4850

AGCAGCCCAG TAGTAGGTTG AGGCCGTTGA GCACCGCCGC CGCAAGGAAT 4900

GGTGCATGCG GTACCAGCTG TTGACAATTA ATCATCCGGC TCGTATAATA 4950

GTACTGTGTG GAATTGTGAG CGCTCACAAT TCCACACATC TAGAAATAAT 5000

TTTGTTTAAC TTTAAGAAGG AGATATACCA TGGAGATCTG GATCCATCGA 5050

TGAATTCGAG CTCCGTCGAC AAGCTTGCGG CCGCACTCGA GCACCACCAC 5100

CACCACCACT GAGATCCGGC TGCTAACAAA GCCCGAAAGG AAGCTGAGTT 5150

GGCTGCTGCC ACCGCTGAGC AATAACTAGC ATAACCCCTT GGGGCCTCTA 5200

AACGGGTCTT GAGGGGTTTT TTGCTGAAAG GAGGAACTAT ATCCGGAT 5248

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 783 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: N19/8 scFv (His Tagged)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
ATG GCC AAT ATT GTG CTG ACC CAA TCT CCA                                  30
Met Ala Asn Ile Val Leu Thr Gln Ser Pro
1               5                   10

GCT TCT TTG GCT GTG TCT CTA GGG CAG AGG                                  60
Ala Ser Leu Ala Val Ser Leu Gly Gln Arg
15                  20

GCC ACC ATA TCC TGC AGA GCC AGT GAA AGT                                  90
Ala Thr Ile Ser Cys Arg Ala Ser Glu Ser
25                  30

GTT GAT AGT TAT GAC AAT AGT TTT ATG CAC                                 120
Val Asp Ser Tyr Asp Asn Ser Phe Met His
35                  40

TGG TAC CAG CAG AAA CCA GGA CAG CCA CCC                                 150
Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro
45                  50

AAA CTC CTC ATC TTT CTT GCA TCC AAC CTA                                 180
Lys Leu Leu Ile Phe Leu Ala Ser Asn Leu
55                  60

GAA TCT GGG GTC CCT GCC AGG TTC AGT GGC                                 210
Glu Ser Gly Val Pro Ala Arg Phe Ser Gly
65                  70

AGT GGG TCT AGG ACA GAC TTC ACC CTC ACC                                 240
Ser Gly Ser Arg Thr Asp Phe Thr Leu Thr
75                  80

ATT GAT CCT GTG GAG GCT GAT GAT GCT GCA                                 270
Ile Asp Pro Val Glu Ala Asp Asp Ala Ala
85                  90

ACC TAT TAC TGT CAG CAA AAT AAT GAG GTT                                 300
Thr Tyr Tyr Cys Gln Gln Asn Asn Glu Val
95                 100

CCG AAC ACG TTC GGA GGG GGG ACC AAG CTG                                 330
Pro Asn Thr Phe Gly Gly Gly Thr Lys Leu
105                 110

GAA ATA AAA CGG ACC GGA GGT GGC GGG TCG                                 360
Glu Ile Lys Arg Thr Gly Gly Gly Gly Ser
115                 120

GGT GGC GGG GGA TCG GGT GGC GGA GGG TCG                                 390
Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
125                 130

GAC GTC AAG CTC GTG GAG TCT GGG GGA GAC                                 420
Asp Val Lys Leu Val Glu Ser Gly Gly Asp
135                 140

TTA GTG AAG CTT GGA GGG TCC CTG AAA CTC                                 450
Leu Val Lys Leu Gly Gly Ser Leu Lys Leu
145                 150

TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT                                 480
Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
155                 160

AGC TAT TAT ATG TCT TGG GTT CGC CAG ATT                                 510
Ser Tyr Tyr Met Ser Trp Val Arg Gln Ile
165                 170

TCA GAG AAG AGG CTG GAG TTG GTC GCA GCC                                 540
Ser Glu Lys Arg Leu Glu Leu Val Ala Ala
175                 180

ATT AAT AGT AAT GGT GAT AGC ACC TAC TAT                                 570
Ile Asn Ser Asn Gly Asp Ser Thr Tyr Tyr
185                 190
```


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```
CCA GAC ACT GTG AAG GGC CGA TTC ACC ATC                                  600
Pro Asp Thr Val Lys Gly Arg Phe Thr Ile
195                 200

TCC AGA GAC AAT GCC AAG AGC ACC CTG GAT                                  630
Ser Arg Asp Asn Ala Lys Ser Thr Leu Asp
205                 210

CTG CAA ATG AGC AGT CTG AAG TCT GAG GAC                                  660
Leu Gln Met Ser Ser Leu Lys Ser Glu Asp
215                 220

ACA GCC TTG TAT TTC TGT GTA AGA GAG ACT                                  690
Thr Ala Leu Tyr Phe Cys Val Arg Glu Thr
225                 230

TAT TAC TAC GGG ATT AGT CCC GTC TTC GAT                                  720
Tyr Tyr Tyr Gly Ile Ser Pro Val Phe Asp
235                 240

GTC TGG GGC ACA GGG ACC ACG GTC ACC GTC                                  750
Val Trp Gly Thr Gly Thr Thr Val Thr Val
245                 250

TCC TCA CTC GAG CAC CAC CAC CAC CAC CAC                                  780
Ser Ser Leu Glu His His His His His His
255                 260

TGA                                                                      783
```

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv C012 (humanized)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                   30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                   60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                                   90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                                  120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                                  150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                                  180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                  210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                  240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                                  270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90
```


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```
CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                                  300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                  100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                                  330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                                  360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                                  390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                                  420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                                  450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                                  480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                                  510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                                  540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                                  570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                                  600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                                  630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                                  660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                                  690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                                  720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                                      747
Gly Thr Leu Val Thr Val Ser Ser
245
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv DO12B
(Humanized scFv)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                          30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                          60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                          90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                         120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                         150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                         180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                         210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                         240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                         270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                         300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                         330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                         360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                         390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                         420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                         450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                         480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                         510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                         540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                         570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
185                 190
```


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```
CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                                  600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                                  630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                                  660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                                  690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                                  720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                                      747
Gly Thr Leu Val Thr Val Ser Ser
245

(2) INFORMATION FOR SEQ ID NO:22:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH:  747 base pairs
          (B) TYPE:  Nucleic Acid
          (C) STRANDEDNESS:  Double
          (D) TOPOLOGY:  linear

    (ii) MOLECULE TYPE:  Other nucleic acid
          (A) DESCRIPTION:5G1.1 scFv DO12C
          (Humanized scFv)

    (xi) SEQUENCE DESCRIPTION:  SEQ ID NO:22:

ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                   30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                   60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                                   90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                                  120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                                  150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                                  180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                  210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                  240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                                  270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                                  300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100
```


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```
GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                                  330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                                  360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                                  390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                                  420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                                  450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                                  480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                                  510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                                  540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                                  570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                                  600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                                  630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                                  660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                                  690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                                  720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                                      747
Gly Thr Leu Val Thr Val Ser Ser
245
```

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv DO12D
(Humanized scFv)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                   30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10
```


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```
TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                  60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                                  90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                                 120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                                 150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                                 180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                 210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                 240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                                 270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                                 300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                                 330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                                 360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                                 390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                                 420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                                 450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                                 480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                                 510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                                 540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                                 570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                                 600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                                 630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210
```


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```
AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                                  660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                                  690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                                  720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                                      747
Gly Thr Leu Val Thr Val Ser Ser
245
```

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv C013 (humanized)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                   30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                   60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                                   90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                                  120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                                  150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                                  180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                  210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                  240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                                  270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                                  300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                                  330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                                  360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120
```


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-continued

```
TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                                  390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                                  420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                                  450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                                  480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                                  510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                                  540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                                  570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                                  600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                                  630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                                  660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                                  690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                                  720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                                      747
Gly Thr Leu Val Thr Val Ser Ser
245
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv C014 (humanized)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                   30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                   60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                                   90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
25                  30
```


Samsung Bioepis Exhibit No. 1002
Page 3291

-continued

```
ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                                  120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                                  150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                                  180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                  210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                  240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                                  270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                                  300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                                  330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                                  360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                                  390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                                  420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                                  450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                                  480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                                  510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                                  540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                                  570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                                  600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                                  630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                                  660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                                  690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230
```


Samsung Bioepis Exhibit No. 1002
Page 3292

-continued

```
CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                                  720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                                      747
Gly Thr Leu Val Thr Val Ser Ser
245
```

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1 scFv C015 (humanized)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                   30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1               5                   10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                   60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                                   90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                                  120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                                  150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                                  180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                  210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                  240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                                  270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                                  300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                                  330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                                  360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                                  390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                                  420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
135                 140
```


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Page 3293

-continued

```
GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                          450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                          480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                          510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                          540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                          570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                          600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                          630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                          660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                          690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                          720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                              747
Gly Thr Leu Val Thr Val Ser Ser
245
```

What is claimed is:

1. An antibody comprising at least one antibody-antigen binding site, said antibody exhibiting specific binding to human complement component C5, said specific binding being targeted to the alpha chain of human complement component C5, wherein the antibody 1) inhibits complement activation in a human body fluid, 2) inhibits the binding of purified human complement component C5 to either human complement component C3 or human complement component C4, and 3) does not specifically bind to the human complement activation product free C5a.

2. The antibody of claim **1** wherein the inhibition of complement activation in the human body fluid is measurable as an increment of blockade of C5a generation and an increment of blockade of complement hemolytic activity in the body fluid, said increment of blockade of C5a generation being substantially equal to said increment of blockade of complement hemolytic activity.

3. The antibody of claim **1** wherein, upon binding to human C5, there is a 60% to 90% reduction in the ability of C5 to bind to human complement component C3.

4. The antibody of claim **1** wherein, upon binding to human C5, there is a 60% to 90% reduction in the ability of C5 to bind to human complement component C4.

5. The antibody of claim **1** wherein the antibody binds specifically to an isolated oligopeptide comprising an amino acid sequence corresponding to amino acid 8 through amino acid 12 of SEQ ID NO:1.

6. The antibody of claim **1** wherein the inhibition of complement activation in the human body fluid is measurable as a substantially complete blockade of C5a generation in the body fluid and a substantially complete blockade of complement hemolytic activity in the body fluid when the antibody is added to the body fluid at a concentration yielding a ratio equal to or less than 10 moles of antibody-antigen binding sites of the antibody to 1 mole of human C5 in the body fluid.

7. The antibody of claim **1** wherein the antibody is a humanized antibody.

8. The antibody of claim **1** wherein the antibody is an scFv.

9. The antibody of claim **1**, wherein, when administered to a human patient via intravenous infusion, the antibody provides complete complement inhibition at dosages below 0.005 g/kg.

10. The antibody of claim **1**, wherein, when administered to a human patient via intravenous infusion, the antibody provides therapeutic benefits at dosages below 0.0022 g/kg.

11. The antibody of claim **10**, wherein the antibody is administered in association with an extracorporeal circulation procedure.

12. The antibody of claim **1** wherein the inhibition of complement activation in the human body fluid is measurable as a substantially complete blockade of C5a generation in the body fluid and a substantially complete blockade of complement hemolytic activity in the body fluid when the

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antibody is added to the body fluid at a concentration yielding a ratio equal to or less than 3 moles of antibody-antigen binding sites of the antibody to 1 mole of human C5 in the body fluid.

13. The antibody of claim **1**, wherein, when administered to a human patient via intravenous infusion, the antibody provides therapeutically effective complement inhibition at dosages below 0.003 g/kg.

14. A sterile non-pyrogenic therapeutic agent comprising the antibody of claim **1** in a formulation suitable for administration to a human.

15. The therapeutic agent of claim **14** wherein the antibody is a humanized immunoglobulin.

16. The therapeutic agent of claim **14** wherein the antibody is an scFv.

17. The therapeutic agent of claim **14** wherein the antibody is made up of two or more heterodimeric subunits each containing one heavy and one light chain.

18. Antibody 5G1.1 scFv CB (humanized) having the amino acid sequence encoded by the nucleic acid sequence of SEQ ID NO:8.

19. An isolated antigen binding protein comprising:

1) a variable light region CDR1 comprising an amino acid sequence corresponding to amino acid residues 26–36 of SEQ ID NO:8,

2) a variable light region CDR2 comprising an amino acid sequence corresponding to amino acid residues 52–58 of SEQ ID NO:8,

3) a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid residues 91 through amino acid 99 of SEQ ID NO:8,

4) a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid residues 152 through amino acid 161 of SEQ ID NO:8,

5) a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid residues 176 through amino acid 192 of SEQ ID NO:8,

6) a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid residues 225 through amino acid 237 of SEQ ID NO:8,

said protein exhibiting specific binding to human complement component C5, said specific binding being targeted to the alpha chain of human complement component C5, wherein the protein inhibits complement activation in a human body fluid and does not specifically bind to the human complement activation product free C5a.

20. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO: 12.

21. Hybridoma 5G1.1 having ATCC designation HB-11625.

22. An antibody produced by the hybridoma of claim **21**.

23. An antibody comprising at least one antibody-antigen binding site, said antibody exhibiting specific binding to human complement component C5, said specific binding being targeted to the alpha chain of human complement component C5, wherein:

(A) the antibody inhibits (i) C5b-9-mediated hemolysis and (ii) C5a generation in a fluid comprising human serum; and

(B) the antibody does not specifically bind to the human complement activation product free C5a.

* * * * *

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UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page, and Column 1, lines 1-3,
Delete the title "**C5-SPECIFIC ANTIBODIES FOR THE TREATMENT OF INFLAMMATORY DISEASES**" and insert therefor -- **ANTIBODIES TO HUMAN COMPLEMENT COMPONENT C5** --.

Column 19,
Line 54, delete "(SEQ ID NO:1)".
Line 55, following "KSSKC peptide", insert -- (SEQ ID NO:1) --.

Column 55,
Please delete lines 50-53, and insert therefor:

```
Val Ile Asp His Gln Gly Thr Lys Ser Ser
                  5                  10

Lys Cys Val Arg Gln Lys Val Glu Gly Ser Ser
                 15                  20
```

Column 57,
Please delete lines 22-37, and insert therefor:

```
                  Met Gly Leu Leu Gly Ile Leu Cys Phe Leu
                             -15                 -10

  Ile Phe Leu Gly Lys Thr Trp Gly Gln Glu Gln Thr Tyr Val
              -5              -1                   5

  Ile Ser Ala Pro Lys Ile Phe Arg Val Gly Ala Ser Glu Asn
             10                  15                  20

  Ile Val Ile Gln Val Tyr Gly Tyr Thr Glu Ala Phe Asp Ala
                  25                  30

  Thr Ile Ser Ile Lys Ser Tyr Pro Asp Lys Lys Phe Ser Tyr
   35                  40                  45

  Ser Ser Gly His Val His Leu Ser Ser Glu Asn Lys Phe Gln
       50                  55                  60

  Asn Ser Ala Ile Leu Thr Ile Gln Pro Lys Gln Leu Pro Gly
            65                  70                  75

  Gly Gln Asn Pro Val Ser Tyr Val Tyr Leu Glu Val Val Ser
             80                  85                  90
```


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Page 3296

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 57,
Please delete lines 38-55, and insert therefor:

```
Lys His Phe Ser Lys Ser Lys Arg Met Pro Ile Thr Tyr Asp
                95                  100

Asn Gly Phe Leu Phe Ile His Thr Asp Lys Pro Val Tyr Thr
105                 110                 115

Pro Asp Gln Ser Val Lys Val Arg Val Tyr Ser Leu Asn Asp
    120                 125                 130

Asp Leu Lys Pro Ala Lys Arg Glu Thr Val Leu Thr Phe Ile
        135                 140                 145

Asp Pro Glu Gly Ser Glu Val Asp Met Val Glu Glu Ile Asp
            150                 155                 160

His Ile Gly Ile Ile Ser Phe Pro Asp Phe Lys Ile Pro Ser
                165                 170

Asn Pro Arg Tyr Gly Met Trp Thr Ile Lys Ala Lys Tyr Lys
175                 180                 185

Glu Asp Phe Ser Thr Thr Gly Thr Ala Tyr Phe Glu Val Lys
    190                 195                 200

Glu Tyr Val Leu Pro His Phe Ser Val Ser Ile Glu Pro Glu
        205                 210                 215
```

Column 59,
Please delete lines 1-4, and insert therefor:

```
Tyr Asn Phe Ile Gly Tyr Lys Asn Phe Lys Asn Phe Glu Ile
            220                 225                 230

Thr Ile Lys Ala Arg Tyr Phe Tyr Asn Lys Val Val Thr Glu
                235                 240
```


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Page 3297

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 59,
Please delete lines 5-28, and insert therefor:

```
Ala Asp Val Tyr Ile Thr Phe Gly Ile Arg Glu Asp Leu Lys
245                 250                 255

Asp Asp Gln Lys Glu Met Met Gln Thr Ala Met Gln Asn Thr
    260                 265                 270

Met Leu Ile Asn Gly Ile Ala Gln Val Thr Phe Asp Ser Glu
        275                 280                 285

Thr Ala Val Lys Glu Leu Ser Tyr Tyr Ser Leu Glu Asp Leu
            290                 295                 300

Asn Asn Lys Tyr Leu Tyr Ile Ala Val Thr Val Ile Glu Ser
                305                 310

Thr Gly Gly Phe Ser Glu Glu Ala Glu Ile Pro Gly Ile Lys
315                 320                 325

Tyr Val Leu Ser Pro Tyr Lys Leu Asn Leu Val Ala Thr Pro
    330                 335                 340

Leu Phe Leu Lys Pro Gly Ile Pro Tyr Pro Ile Lys Val Gln
        345                 350                 355

Val Lys Asp Ser Leu Asp Gln Leu Val Gly Gly Val Pro Val
            360                 365                 370

Ile Leu Asn Ala Gln Thr Ile Asp Val Asn Gln Glu Thr Ser
                375                 380

Asp Leu Asp Pro Ser Lys Ser Val Thr Arg Val Asp Asp Gly
385                 390                 395

Val Ala Ser Phe Val Leu Asn Leu Pro Ser Gly Val Thr Val
    400                 405                 410
```


Samsung Bioepis Exhibit No. 1002
Page 3298

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 59,
Please delete lines 29-52, and insert therefor:

```
Leu Glu Phe Asn Val Lys Thr Asp Ala Pro Asp Leu Pro Glu
        415                 420                 425

Glu Asn Gln Ala Arg Glu Gly Tyr Arg Ala Ile Ala Tyr Ser
            430                 435                 440

Ser Leu Ser Gln Ser Tyr Leu Tyr Ile Asp Trp Thr Asp Asn
                445                 450

His Lys Ala Leu Leu Val Gly Glu His Leu Asn Ile Ile Val
455                 460                 465

Thr Pro Lys Ser Pro Tyr Ile Asp Lys Ile Thr His Tyr Asn
    470                 475                 480

Tyr Leu Ile Leu Ser Lys Gly Lys Ile Ile His Phe Gly Thr
        485                 490                 495

Arg Glu Lys Phe Ser Asp Ala Ser Tyr Gln Ser Ile Asn Ile
            500                 505                 510

Pro Val Thr Gln Asn Met Val Pro Ser Ser Arg Leu Leu Val
                515                 520

Tyr Tyr Ile Val Thr Gly Glu Gln Thr Ala Glu Leu Val Ser
525                 530                 535

Asp Ser Val Trp Leu Asn Ile Glu Glu Lys Cys Gly Asn Gln
    540                 545                 550

Leu Gln Val His Leu Ser Pro Asp Ala Asp Ala Tyr Ser Pro
        555                 560                 565

Gly Gln Thr Val Ser Leu Asn Met Ala Thr Gly Met Asp Ser
            570                 575                 580
```


Samsung Bioepis Exhibit No. 1002
Page 3299

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 61,
Please delete lines 1-26, and insert therefor:

```
Trp Val Ala Leu Ala Ala Val Asp Ser Ala Val Tyr Gly Val
                585                 590

Gln Arg Gly Ala Lys Lys Pro Leu Glu Arg Val Phe Gln Phe
595                 600                 605

Leu Glu Lys Ser Asp Leu Gly Cys Gly Ala Gly Gly Gly Leu
    610                 615                 620

Asn Asn Ala Asn Val Phe His Leu Ala Gly Leu Thr Phe Leu
        625                 630                 635

Thr Asn Ala Asn Ala Asp Asp Ser Gln Glu Asn Asp Glu Pro
            640                 645                 650

Cys Lys Glu Ile Leu Arg Pro Arg Arg Thr Leu Gln Lys Lys
                655                 660

Ile Glu Glu Ile Ala Ala Lys Tyr Lys His Ser Val Val Lys
665                 670                 675

Lys Cys Cys Tyr Asp Gly Ala Cys Val Asn Asn Asp Glu Thr
    680                 685                 690

Cys Glu Gln Arg Ala Ala Arg Ile Ser Leu Gly Pro Arg Cys
        695                 700                 705

Ile Lys Ala Phe Thr Glu Cys Cys Val Val Ala Ser Gln Leu
            710                 715                 720

Arg Ala Asn Ile Ser His Lys Asp Met Gln Leu Gly Arg Leu
                725                 730

His Met Lys Thr Leu Leu Pro Val Ser Lys Pro Glu Ile Arg
735                 740                 745

Ser Tyr Phe Pro Glu Ser Trp Leu Trp Glu Val His Leu Val
    750                 755                 760
```


Samsung Bioepis Exhibit No. 1002
Page 3300

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 61,
Please delete lines 27-52, and insert therefor:

```
Pro Arg Arg Lys Gln Leu Gln Phe Ala Leu Pro Asp Ser Leu
        765                 770                 775

Thr Thr Trp Glu Ile Gln Gly Ile Gly Ile Ser Asn Thr Gly
            780                 785                 790

Ile Cys Val Ala Asp Thr Val Lys Ala Lys Val Phe Lys Asp
                795                 800

Val Phe Leu Glu Met Asn Ile Pro Tyr Ser Val Val Arg Gly
805                 810                 815

Glu Gln Ile Gln Leu Lys Gly Thr Val Tyr Asn Tyr Arg Thr
    820                 825                 830

Ser Gly Met Gln Phe Cys Val Lys Met Ser Ala Val Glu Gly
        835                 840                 845

Ile Cys Thr Ser Glu Ser Pro Val Ile Asp His Gln Gly Thr
            850                 855                 860

Lys Ser Ser Lys Cys Val Arg Gln Lys Val Glu Gly Ser Ser
                865                 870

Ser His Leu Val Thr Phe Thr Val Leu Pro Leu Glu Ile Gly
875                 880                 885

Leu His Asn Ile Asn Phe Ser Leu Glu Thr Trp Phe Gly Lys
    890                 895                 900

Glu Ile Leu Val Lys Thr Leu Arg Val Val Pro Glu Gly Val
        905                 910                 915

Lys Arg Glu Ser Tyr Ser Gly Val Thr Leu Asp Pro Arg Gly
            920                 925                 930

Ile Tyr Gly Thr Ile Ser Arg Arg Lys Glu Phe Pro Tyr Arg
                935                 940
```


Samsung Bioepis Exhibit No. 1002
Page 3301

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 63,
Please delete lines 1-26, and insert therefor:

```
Ile Pro Leu Asp Leu Val Pro Lys Thr Glu Ile Lys Arg Ile
945                 950                 955

Leu Ser Val Lys Gly Leu Leu Val Gly Glu Ile Leu Ser Ala
    960                 965                 970

Val Leu Ser Gln Glu Gly Ile Asn Ile Leu Thr His Leu Pro
        975                 980                 985

Lys Gly Ser Ala Glu Ala Glu Leu Met Ser Val Val Pro Val
            990                 995                 1000

Phe Tyr Val Phe His Tyr Leu Glu Thr Gly Asn His Trp Asn
                1005                1010

Ile Phe His Ser Asp Pro Leu Ile Glu Lys Gln Lys Leu Lys
1015                1020                1025

Lys Lys Leu Lys Glu Gly Met Leu Ser Ile Met Ser Tyr Arg
    1030                1035                1040

Asn Ala Asp Tyr Ser Tyr Ser Val Trp Lys Gly Gly Ser Ala
        1045                1050                1055

Ser Thr Trp Leu Thr Ala Phe Ala Leu Arg Val Leu Gly Gln
            1060                1065                1070

Val Asn Lys Tyr Val Glu Gln Asn Gln Asn Ser Ile Cys Asn
                1075                1080

Ser Leu Leu Trp Leu Val Glu Asn Tyr Gln Leu Asp Asn Gly
1085                1090                1095

Ser Phe Lys Glu Asn Ser Gln Tyr Gln Pro Ile Lys Leu Gln
    1100                1105                1110

Gly Thr Leu Pro Val Glu Ala Arg Glu Asn Ser Leu Tyr Leu
        1115                1120                1125
```


Samsung Bioepis Exhibit No. 1002
Page 3302

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 63,
Please delete lines 27-52, and insert therefor:

```
Thr Ala Phe Thr Val Ile Gly Ile Arg Lys Ala Phe Asp Ile
            1130                1135                1140

Cys Pro Leu Val Lys Ile Asp Thr Ala Leu Ile Lys Ala Asp
                 1145                1150

Asn Phe Leu Leu Glu Asn Thr Leu Pro Ala Gln Ser Thr Phe
1155                1160                1165

Thr Leu Ala Ile Ser Ala Tyr Ala Leu Ser Leu Gly Asp Lys
    1170                1175                1180

Thr His Pro Gln Phe Arg Ser Ile Val Ser Ala Leu Lys Arg
        1185                1190                1195

Glu Ala Leu Val Lys Gly Asn Pro Pro Ile Tyr Arg Phe Trp
            1200                1205                1210

Lys Asp Asn Leu Gln His Lys Asp Ser Ser Val Pro Asn Thr
                 1215                1220

Gly Thr Ala Arg Met Val Glu Thr Thr Ala Tyr Ala Leu Leu
1225                1230                1235

Thr Ser Leu Asn Leu Lys Asp Ile Asn Tyr Val Asn Pro Val
    1240                1245                1250

Ile Lys Trp Leu Ser Glu Glu Gln Arg Tyr Gly Gly Gly Phe
        1255                1260                1265

Tyr Ser Thr Gln Asp Thr Ile Asn Ala Ile Glu Gly Leu Thr
            1270                1275                1280

Glu Tyr Ser Leu Leu Val Lys Gln Leu Arg Leu Ser Met Asp
                 1285                1290

Ile Asp Val Ser Tyr Lys His Lys Gly Ala Leu His Asn Tyr
1295                1300                1305
```


Samsung Bioepis Exhibit No. 1002
Page 3303

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 65,
Please delete lines 1-26, and insert therefor:

```
Lys Met Thr Asp Lys Asn Phe Leu Gly Arg Pro Val Glu Val
    1310                1315                1320

Leu Leu Asn Asp Asp Leu Ile Val Ser Thr Gly Phe Gly Ser
        1325                1330                1335

Gly Leu Ala Thr Val His Val Thr Thr Val Val His Lys Thr
            1340                1345                1350

Ser Thr Ser Glu Glu Val Cys Ser Phe Tyr Leu Lys Ile Asp
                1355                1360

Thr Gln Asp Ile Glu Ala Ser His Tyr Arg Gly Tyr Gly Asn
1365                1370                1375

Ser Asp Tyr Lys Arg Ile Val Ala Cys Ala Ser Tyr Lys Pro
    1380                1385                1390

Ser Arg Glu Glu Ser Ser Ser Gly Ser Ser His Ala Val Met
        1395                1400                1405

Asp Ile Ser Leu Pro Thr Gly Ile Ser Ala Asn Glu Glu Asp
            1410                1415                1420

Leu Lys Ala Leu Val Glu Gly Val Asp Gln Leu Phe Thr Asp
                1425                1430

Tyr Gln Ile Lys Asp Gly His Val Ile Leu Gln Leu Asn Ser
1435                1440                1445

Ile Pro Ser Ser Asp Phe Leu Cys Val Arg Phe Arg Ile Phe
    1450                1455                1460

Glu Leu Phe Glu Val Gly Phe Leu Ser Pro Ala Thr Phe Thr
        1465                1470                1475

Val Tyr Glu Tyr His Arg Pro Asp Lys Gln Cys Thr Met Phe
            1480                1485                1490
```


Samsung Bioepis Exhibit No. 1002
Page 3304

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 65,
Please delete lines 27-50, and insert therefor:

```
Tyr Ser Thr Ser Asn Ile Lys Ile Gln Lys Val Cys Glu Gly
                1495                1500

Ala Ala Cys Lys Cys Val Glu Ala Asp Cys Gly Gln Met Gln
1505                1510                1515

Glu Glu Leu Asp Leu Thr Ile Ser Ala Glu Thr Arg Lys Gln
    1520                1525                1530

Thr Ala Cys Lys Pro Glu Ile Ala Tyr Ala Tyr Lys Val Ser
        1535                1540                1545

Ile Thr Ser Ile Thr Val Glu Asn Val Phe Val Lys Tyr Lys
            1550                1555                1560

Ala Thr Leu Leu Asp Ile Tyr Lys Thr Gly Glu Ala Val Ala
                1565                1570

Glu Lys Asp Ser Glu Ile Thr Phe Ile Lys Lys Val Thr Cys
1575                1580                1585

Thr Asn Ala Glu Leu Val Lys Gly Arg Gln Tyr Leu Ile Met
    1590                1595                1600

Gly Lys Glu Ala Leu Gln Ile Lys Tyr Asn Phe Ser Phe Arg
        1605                1610                1615

Tyr Ile Tyr Pro Leu Asp Ser Leu Thr Trp Ile Glu Tyr Trp
            1620                1625                1630

Pro Arg Asp Thr Thr Cys Ser Ser Cys Gln Ala Phe Leu Ala
                1635                1640

Asn Leu Asp Glu Phe Ala Glu Asp Ile Phe Leu Asn Gly Cys
1645                1650                1655
```


Samsung Bioepis Exhibit No. 1002
Page 3305

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 81,
Please delete lines 18-47, and insert therefor:

```
ATG GCC GAC ATC CAG ATG ACT CAG TCT CCA                    30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1                 5                  10

GCT TCA CTG TCT GCA TCT GTG GGA GAA ACT                    60
Ala Ser Leu Ser Ala Ser Val Gly Glu Thr
                 15                  20

GTC ACC ATC ACA TGT GGA GCA AGT GAG AAT                    90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
                 25                  30

ATT TAC GGT GCT TTA AAT TGG TAT CAG CGG                   120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Arg
                 35                  40

AAA CAG GGA AAA TCT CCT CAG CTC CTG ATC                   150
Lys Gln Gly Lys Ser Pro Gln Leu Leu Ile
                 45                  50

TAT GGT GCA ACC AAC TTG GCA GAT GGC ATG                   180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Met
                 55                  60

TCA TCG AGG TTC AGT GGC AGT GGA TCT GGT                   210
Ser Ser Arg Phe Ser Gly Ser Gly Ser Gly
                 65                  70

AGA CAG TAT TAT CTC AAG ATC AGT AGC CTG                   240
Arg Gln Tyr Tyr Leu Lys Ile Ser Ser Leu
                 75                  80

CAT CCT GAC GAT GTT GCA ACG TAT TAC TGT                   270
His Pro Asp Asp Val Ala Thr Tyr Tyr Cys
                 85                  90

CAA AAT GTG TTA AAT ACT CCT CTC ACG TTC                   300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                 95                 100
```


Samsung Bioepis Exhibit No. 1002
Page 3306

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 81,
Please delete line 48, through column 83, line 21, and insert therefor:

```
GGT GCT GGG ACC AAG TTG GAG CTG AAA CGG                    330
Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg
                105                 110

ACC GGA GGT GGC GGG TCG GGT GGC GGG GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCG GGT GGC GGA GGG TCG CAG GTT CAG CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

CAG CAG TCT GGA GCC GAG CTG ATG AAG CCT                    420
Gln Gln Ser Gly Ala Glu Leu Met Lys Pro
                135                 140

GGG GCC TCA GTG AAG ATG TCC TGC AAG GCT                    450
Gly Ala Ser Val Lys Met Ser Cys Lys Ala
                145                 150

ACT GGC TAC ATA TTC AGT AAC TAC TGG ATA                    480
Thr Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAG TGG ATA AAG CAG AGG CCT GGA CAT GGC                    510
Gln Trp Ile Lys Gln Arg Pro Gly His Gly
                165                 170

CTT GAG TGG ATT GGT GAG ATT TTA CCT GGA                    540
Leu Glu Trp Ile Gly Glu Ile Leu Pro Gly
                175                 180

AGT GGT TCT ACT GAG TAC ACT GAG AAC TTC                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
                185                 190

AAG GAC AAG GCC GCA TTC ACT GCA GAT ACA                    600
Lys Asp Lys Ala Ala Phe Thr Ala Asp Thr
                195                 200
```


Samsung Bioepis Exhibit No. 1002
Page 3307

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 83,
Please delete lines 22-36, and insert therefor:

```
TCC TCC AAC ACA GCC TAC ATG CAA CTC AGC                    630
Ser Ser Asn Thr Ala Tyr Met Gln Leu Ser
                205                 210

AGC CTG ACA TCA GAG GAC TCT GCC GTC TAT                    660
Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr
                215                 220

TAC TGT GCA AGA TAT TTC TTC GGT AGT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCC AAC TGG TAC TTC GAT GTC TGG GGC GCA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Ala
                235                 240

GGG ACC ACG GTC ACC GTC TCC TCA TGA                        747
Gly Thr Thr Val Thr Val Ser Ser
                245
```

Please delete lines 46-57, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                     30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                     60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                     90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CGT                    120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Arg
                35                  40
```


Samsung Bioepis Exhibit No. 1002
Page 3308

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 85,
Please delete lines 1-30, and insert therefor:

```
AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                    150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                 45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                    180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
                 55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                    210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                 65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                    240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                 75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                 85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                 95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140
```


Samsung Bioepis Exhibit No. 1002
Page 3309

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 85,
Please delete lines 31-60, and insert therefor:

```
GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
                185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240
```


Samsung Bioepis Exhibit No. 1002
Page 3310

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 87,

Please delete lines 1-3, and insert therefor:

```
GGA ACC CTG GTC ACT GTC TCG AGC TGA                              747
Gly Thr Leu Val Thr Val Ser Ser
               245
```

Please delete lines 13-36, and insert therefor:

```
ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC                           30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC                           60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10

CAT TCA GGT CAT AGC CTG CAG GAC ATC CAG                           90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                   1               5

ATG ACT CAG TCT CCA GCT TCA CTG TCT GCA                          120
Met Thr Gln Ser Pro Ala Ser Leu Ser Ala
                10                  15

TCT GTG GGA GAA ACT GTC ACC ATC ACA TGT                          150
Ser Val Gly Glu Thr Val Thr Ile Thr Cys
                20                  25

GGA GCA AGT GAG AAT ATT TAC GGT GCT TTA                          180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
                30                  35

AAT TGG TAT CAG CGG AAA CAG GGA AAA TCT                          210
Asn Trp Tyr Gln Arg Lys Gln Gly Lys Ser
                40                  45

CCT CAG CTC CTG ATC TAT GGT GCA ACC AAC                          240
Pro Gln Leu Leu Ile Tyr Gly Ala Thr Asn
                50                  55
```


Samsung Bioepis Exhibit No. 1002
Page 3311

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 87,

Please delete line 37, through column 89, line 9, and insert therefor:

```
TTG GCA GAT GGC ATG TCA TCG AGG TTC AGT                    270
Leu Ala Asp Gly Met Ser Ser Arg Phe Ser
                 60                  65

GGC AGT GGA TCT GGT AGA CAG TAT TAT CTC                    300
Gly Ser Gly Ser Gly Arg Gln Tyr Tyr Leu
                 70                  75

AAG ATC AGT AGC CTG CAT CCT GAC GAT GTT                    330
Lys Ile Ser Ser Leu His Pro Asp Asp Val
                 80                  85

GCA ACG TAT TAC TGT CAA AAT GTG TTA AAT                    360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
                 90                  95

ACT CCT CTC ACG TTC GGT GCT GGG ACC AAG                    390
Thr Pro Leu Thr Phe Gly Ala Gly Thr Lys
                100                 105

TTG GAG CTG AAA CGA ACT GTG GCT GCA CCA                    420
Leu Glu Leu Lys Arg Thr Val Ala Ala Pro
                110                 115

TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG                    450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
                120                 125

CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG                    480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
                130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG                    510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
                140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC                    540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
                150                 155
```


Samsung Bioepis Exhibit No. 1002
Page 3312

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 89,

Please delete lines 10-29, and insert therefor:

```
CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC                    570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
                160                 165

ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC                    600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
                170                 175

AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA                    630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
                180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC                    660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
                190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG                    690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
                200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG                    720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
                210                 215

TGT TAG                                                    726
Cys
```

Please delete lines 39-44, and insert therefor:

```
ATG AAA TGG AGC TGG GTT ATT CTC TTC CTC                     30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
            -15                 -10

CTG TCA GTA ACT GCA GGT GTC CAC TCC CAG                     60
Leu Ser Val Thr Ala Gly Val His Ser Gln
            -5                   1
```


Samsung Bioepis Exhibit No. 1002
Page 3313

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 89,
Please delete line 45, through column 91, line 15, and insert therefor:

```
GTT CAG CTG CAG CAG TCT GGA GCT GAG CTG                    90
Val Gln Leu Gln Gln Ser Gly Ala Glu Leu
                5                   10

ATG AAG CCT GGG GCC TCA GTG AAG ATG TCC                   120
Met Lys Pro Gly Ala Ser Val Lys Met Ser
            15                  20

TGC AAG GCT ACT GGC TAC ATA TTC AGT AAC                   150
Cys Lys Ala Thr Gly Tyr Ile Phe Ser Asn
            25                  30

TAC TGG ATA CAG TGG ATA AAG CAG AGG CCT                   180
Tyr Trp Ile Gln Trp Ile Lys Gln Arg Pro
            35                  40

GGA CAT GGC CTT GAG TGG ATT GGT GAG ATT                   210
Gly His Gly Leu Glu Trp Ile Gly Glu Ile
            45                  50

TTA CCT GGA AGT GGT TCT ACT GAG TAC ACT                   240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr
            55                  60

GAG AAC TTC AAG GAC AAG GCC GCA TTC ACT                   270
Glu Asn Phe Lys Asp Lys Ala Ala Phe Thr
            65                  70

GCA GAT ACA TCC TCC AAC ACA GCC TAC ATG                   300
Ala Asp Thr Ser Ser Asn Thr Ala Tyr Met
            75                  80

CAA CTC AGC AGC CTG ACA TCA GAG GAC TCT                   330
Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser
            85                  90

GCC GTC TAT TAC TGT GCA AGA TAT TTC TTC                   360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
            95                  100
```


Samsung Bioepis Exhibit No. 1002
Page 3314

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 91,
Please delete lines 16-45, and insert therefor:

```
GGT AGT AGC CCC AAC TGG TAC TTC GAT GTC                    390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
            105                 110

TGG GGC GCA GGG ACC ACG GTC ACC GTC TCC                    420
Trp Gly Ala Gly Thr Thr Val Thr Val Ser
            115                 120

TCA GCC TCC ACC AAG GGC CCA TCG GTC TTC                    450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
            125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                    480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
            135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                    510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
            145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                    540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
            155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                    570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
            165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                    600
Val His Thr Phe Pro Ala Val Leu Gln Ser
            175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                    630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
            185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                    660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
            195                 200
```


Samsung Bioepis Exhibit No. 1002
Page 3315

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 91,
Please delete lines 46-54, and insert therefor:

```
ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                    690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
            205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                    720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
            215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                    750
Pro Lys Ser Cys Asp Lys Thr His Thr
            225
```

Column 93
Please delete lines 4-21, and insert therefor:

```
ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC                     30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
                -15                 -10

CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA                     60
Leu Ser Val Thr Ala Gly Val His Ser Gln
                 -5                   1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC                     90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
              5                  10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC                    120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
             15                  20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT                    150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
             25                  30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC                    180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
             35                  40
```


Samsung Bioepis Exhibit No. 1002
Page 3316

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 93,
Please delete lines 22-51, and insert therefor:

```
GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC                    210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
             45                  50

TTA CCG GGC TCT GGT AGC ACC GAA TAT GCC                    240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Ala
             55                  60

CAA AAA TTC CAG GGC CGT GTT ACT ATG ACT                    270
Gln Lys Phe Gln Gly Arg Val Thr Met Thr
             65                  70

GCG GAC ACT TCG ACT AGT ACA GCC TAC ATG                    300
Ala Asp Thr Ser Thr Ser Thr Ala Tyr Met
             75                  80

GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG                    330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
             85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT                    360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
             95                 100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT                    390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
            105                 110

TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG                    420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
            115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC                    450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
            125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                    480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
            135                 140
```


Samsung Bioepis Exhibit No. 1002
Page 3317

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 93,
Please delete line 52, through column 95, line 27, and insert therefor:

```
GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                    510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
            145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                    540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
            155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                    570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
            165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                    600
Val His Thr Phe Pro Ala Val Leu Gln Ser
            175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                    630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
            185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                    660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
            195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                    690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
            205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                    720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
            215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                    750
Pro Lys Ser Cys Asp Lys Thr His Thr
            225                 230
```


Samsung Bioepis Exhibit No. 1002
Page 3318

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 95,

Please delete line 31, through column 97, line 3, and insert therefor:

```
ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC                    30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
                -15                 -10

CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA                    60
Leu Ser Val Thr Ala Gly Val His Ser Gln
                 -5                  1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC                    90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
              5                  10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC                   120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
             15                  20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT                   150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
             25                  30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC                   180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
             35                  40

GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC                   210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
             45                  50

TTA CCG GGC TCT GGT AGC ACC GAA TAT ACC                   240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr
             55                  60

GAA AAT TTT AAA GAC CGT GTT ACT ATG ACG                   270
Glu Asn Phe Lys Asp Arg Val Thr Met Thr
             65                  70

CGT GAC ACT TCG ACT AGT ACA GTA TAC ATG                   300
Arg Asp Thr Ser Thr Ser Thr Val Tyr Met
             75                  80
```


Samsung Bioepis Exhibit No. 1002
Page 3319

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 97,
Please delete lines 4-33, and insert therefor:

```
GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG                    330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
             85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT                    360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
             95                 100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT                    390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
            105                 110

TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG                    420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
            115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC                    450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
            125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                    480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
            135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                    510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
            145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                    540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
            155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                    570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
            165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                    600
Val His Thr Phe Pro Ala Val Leu Gln Ser
            175                 180
```


Samsung Bioepis Exhibit No. 1002
Page 3320

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 97,
Please delete lines 34-48, and insert therefor:

```
TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                    630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
            185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                    660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
            195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                    690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
            205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                    720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
            215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                    750
Pro Lys Ser Cys Asp Lys Thr His Thr
            225                 230
```

Column 99,
Please delete lines 2-13, and insert therefor:

```
ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC                     30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC                     60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10

CAT TCA GGT CAT AGC CTG CAG GAT ATC CAG                     90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                   1                  5

ATG ACC CAG TCC CCG TCC TCC CTG TCC GCC                    120
Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
                10                  15
```


Samsung Bioepis Exhibit No. 1002
Page 3321

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 99,
Please delete lines 14-43, and insert therefor:

```
TCT GTG GGC GAT AGG GTC ACC ATC ACC TGC                    150
Ser Val Gly Asp Arg Val Thr Ile Thr Cys
                  20                  25

GGC GCC AGC GAA AAC ATC TAT GGC GCG CTG                    180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
                  30                  35

AAC TGG TAT CAA CGT AAA CCT GGG AAA GCT                    210
Asn Trp Tyr Gln Arg Lys Pro Gly Lys Ala
                  40                  45

CCG AAG CTT CTG ATT TAC GGT GCG ACG AAC                    240
Pro Lys Leu Leu Ile Tyr Gly Ala Thr Asn
                  50                  55

CTG GCA GAT GGA GTC CCT TCT CGC TTC TCT                    270
Leu Ala Asp Gly Val Pro Ser Arg Phe Ser
                  60                  65

GGA TCC GGC TCC GGA ACG GAT TAC ACT CTG                    300
Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu
                  70                  75

ACC ATC AGC AGT CTG CAA CCT GAG GAC TTC                    330
Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe
                  80                  85

GCT ACG TAT TAC TGT CAG AAC GTT TTA AAT                    360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
                  90                  95

ACT CCG TTG ACT TTC GGA CAG GGT ACC AAG                    390
Thr Pro Leu Thr Phe Gly Gln Gly Thr Lys
                 100                 105

GTG GAA ATA AAA CGA ACT GTG GCT GCA CCA                    420
Val Glu Ile Lys Arg Thr Val Ala Ala Pro
                 110                 115
```


Samsung Bioepis Exhibit No. 1002
Page 3322

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 99,
Please delete line 44, through column 101, line 17, and insert therefor:

```
TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG                    450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
                120                 125

CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG                    480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
                130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG                    510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
                140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC                    540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
                150                 155

CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC                    570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
                160                 165

ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC                    600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
                170                 175

AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA                    630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
                180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC                    660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
                190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG                    690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
                200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG                    720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
                210                  215

TGT TAG                                                    726
Cys
```


Samsung Bioepis Exhibit No. 1002
Page 3323

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 101,

Please delete lines 28-57, and insert therefor:

```
ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC                    30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC                    60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10

CAT TCA GGT CAT AGC CTG CAG GAT ATC CAG                    90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                   1               5

ATG ACC CAG TCC CCG TCC TCC CTG TCC GCC                   120
Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
                10                  15

TCT GTG GGC GAT AGG GTC ACC ATC ACC TGC                   150
Ser Val Gly Asp Arg Val Thr Ile Thr Cys
                20                  25

GGC GCC AGC GAA AAC ATC TAT GGC GCG CTG                   180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
                30                  35

AAC TGG TAT CAA CGT AAA CCT GGG AAA GCT                   210
Asn Trp Tyr Gln Arg Lys Pro Gly Lys Ala
                40                  45

CCG AAG CTT CTG ATT TAC GGT GCG ACG AAC                   240
Pro Lys Leu Leu Ile Tyr Gly Ala Thr Asn
                50                  55

CTG GCA GAT GGA GTC CCT TCT CGC TTC TCT                   270
Leu Ala Asp Gly Val Pro Ser Arg Phe Ser
                60                  65

GGA TCC GGC TCC GGA ACG GAT TTC ACT CTG                   300
Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu
                70                  75
```


Samsung Bioepis Exhibit No. 1002
Page 3324

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 103,
Please delete lines 1-30, and insert therefor:

```
ACC ATC AGC AGT CTG CAG CCT GAA GAC TTC                    330
Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe
                 80                  85

GCT ACG TAT TAC TGT CAG AAC GTT TTA AAT                    360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
                 90                  95

ACT CCG TTG ACT TTC GGA CAG GGT ACC AAG                    390
Thr Pro Leu Thr Phe Gly Gln Gly Thr Lys
                100                 105

GTG GAA ATA AAA CGA ACT GTG GCT GCA CCA                    420
Val Glu Ile Lys Arg Thr Val Ala Ala Pro
                110                 115

TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG                    450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
                120                 125

CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG                    480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
                130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG                    510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
                140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC                    540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
                150                 155

CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC                    570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
                160                 165

ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC                    600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
                170                 175
```


Samsung Bioepis Exhibit No. 1002
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CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 103,
Please delete lines 31-44, and insert therefor:

```
AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA                    630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
                180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC                    660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
                190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG                    690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
                200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG                    720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
                210                 215

TGT TAG                                                    726
Cys
```

Please delete line 55, through column 105, line 9, and insert therefor:

```
ATG GAC ATG AGG GTC CCC GCT CAG CTC CTG                     30
Met Asp Met Arg Val Pro Ala Gln Leu Leu
            -20                 -15

GGG CTC CTG CTA CTC TGG CTC CGA GGT GCC                     60
Gly Leu Leu Leu Leu Trp Leu Arg Gly Ala
            -10                  -5

AGA TGT GAT ATC CAG ATG ACC CAG TCC CCG                     90
Arg Cys Asp Ile Gln Met Thr Gln Ser Pro
          1                  5

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                    120
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
     10                  15
```


Samsung Bioepis Exhibit No. 1002
Page 3326

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 105,

Please delete lines 10-39, and insert therefor:

```
GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                    150
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
     20                  25

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                    180
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
     30                  35

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                    210
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
     40                  45

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                    240
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
     50                  55

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                    270
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
     60                  65

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                    300
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
     70                  75

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    330
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
     80                  85

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    360
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
     90                  95

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGA                    390
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
    100                 105

ACT GTG GCT GCA CCA TCT GTC TTC ATC TTC                    420
Thr Val Ala Ala Pro Ser Val Phe Ile Phe
    110                 115
```


Samsung Bioepis Exhibit No. 1002
Page 3327

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 105,
Please delete line 40, through column 107, line 9, and insert therefor:

```
CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA                    450
Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
    120                 125

ACT GCC TCT GTT GTG TGC CTG CTG AAT AAC                    480
Thr Ala Ser Val Val Cys Leu Leu Asn Asn
    130                 135

TTC TAT CCC AGA GAG GCC AAA GTA CAG TGG                    510
Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp
    140                 145

AAG GTG GAT AAC GCC CTC CAA TCG GGT AAC                    540
Lys Val Asp Asn Ala Leu Gln Ser Gly Asn
    150                 155

TCC CAG GAG AGT GTC ACA GAG CAG GAC AGC                    570
Ser Gln Glu Ser Val Thr Glu Gln Asp Ser
    160                 165

AAG GAC AGC ACC TAC AGC CTC AGC AGC ACC                    600
Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr
    170                 175

CTG ACG CTG AGC AAA GCA GAC TAC GAG AAA                    630
Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys
    180                 185

CAC AAA GTC TAC GCC TGC GAA GTC ACC CAT                    660
His Lys Val Tyr Ala Cys Glu Val Thr His
    190                  195

CAG GGC CTG AGC TCG CCC GTC ACA AAG AGC                    690
Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
    200                 205

TTC AAC AGG GGA GAG TGT TAG                               711
Phe Asn Arg Gly Glu Cys
    210
```


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Page 3328

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 107,
Please delete lines 20-49, and insert therefor:

```
ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC                    30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
               -15                 -10

CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA                    60
Leu Ser Val Thr Ala Gly Val His Ser Gln
                -5                   1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC                    90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
              5                   10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC                   120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
              15                  20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT                   150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
              25                  30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC                   180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
              35                  40

GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC                   210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
              45                  50

TTA CCG GGC TCT GGT AGC ACC GAA TAT GCC                   240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Ala
              55                  60

CAA AAA TTC CAG GGC CGT GTT ACT ATG ACT                   270
Gln Lys Phe Gln Gly Arg Val Thr Met Thr
              65                  70

CGT GAC ACT TCG ACT AGT ACA GTA TAC ATG                   300
Arg Asp Thr Ser Thr Ser Thr Val Tyr Met
              75                  80
```


Samsung Bioepis Exhibit No. 1002
Page 3329

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 107,
Please delete line 50, through column 109, line 21, and insert therefor:

```
GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG                    330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
             85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT                    360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
             95                 100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT                    390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
            105                 110

TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG                    420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
            115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC                    450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
            125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT                    480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
            135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC                    510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
            145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG                    540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
            155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC                    570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
            165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC                    600
Val His Thr Phe Pro Ala Val Leu Gln Ser
            175                 180
```

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 109,

Please delete lines 22-36, and insert therefor:

```
TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG                    630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
            185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG                    660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
            195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC                    690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
            205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG                    720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
            215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA                    750
Pro Lys Ser Cys Asp Lys Thr His Thr
            225                 230
```

Please delete lines 47-58, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                     30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                     60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                     90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                    120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                35                  40
```


Samsung Bioepis Exhibit No. 1002
Page 3331

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 111,
Please delete lines 1-30, and insert therefor:

```
AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                    150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                 45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                    180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
                 55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                    210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                 65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                    240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                 75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                 85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                 95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140
```


Samsung Bioepis Exhibit No. 1002
Page 3332

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 111,
Please delete lines 31-60, and insert therefor:

```
GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                    570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
                185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240
```


Samsung Bioepis Exhibit No. 1002
Page 3333

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 113,
Please delete lines 1-3, and insert therefor:

```
GGA ACC CTG GTC ACT GTC TCG AGC TGA                                    747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Column 119,
Please delete lines 2-25, and insert therefor:

```
ATG GCC AAT ATT GTG CTG ACC CAA TCT CCA                                 30
Met Ala Asn Ile Val Leu Thr Gln Ser Pro
  1               5                  10

GCT TCT TTG GCT GTG TCT CTA GGG CAG AGG                                 60
Ala Ser Leu Ala Val Ser Leu Gly Gln Arg
                15                  20

GCC ACC ATA TCC TGC AGA GCC AGT GAA AGT                                 90
Ala Thr Ile Ser Cys Arg Ala Ser Glu Ser
                25                  30

GTT GAT AGT TAT GAC AAT AGT TTT ATG CAC                                120
Val Asp Ser Tyr Asp Asn Ser Phe Met His
                35                  40

TGG TAC CAG CAG AAA CCA GGA CAG CCA CCC                                150
Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro
                45                  50

AAA CTC CTC ATC TTT CTT GCA TCC AAC CTA                                180
Lys Leu Leu Ile Phe Leu Ala Ser Asn Leu
                55                  60

GAA TCT GGG GTC CCT GCC AGG TTC AGT GGC                                210
Glu Ser Gly Val Pro Ala Arg Phe Ser Gly
                65                  70

AGT GGG TCT AGG ACA GAC TTC ACC CTC ACC                                240
Ser Gly Ser Arg Thr Asp Phe Thr Leu Thr
                75                  80
```


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CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 119,
Please delete lines 26-55, and insert therefor:

```
ATT GAT CCT GTG GAG GCT GAT GAT GCT GCA                    270
Ile Asp Pro Val Glu Ala Asp Asp Ala Ala
                 85                  90

ACC TAT TAC TGT CAG CAA AAT AAT GAG GTT                    300
Thr Tyr Tyr Cys Gln Gln Asn Asn Glu Val
                 95                 100

CCG AAC ACG TTC GGA GGG GGG ACC AAG CTG                    330
Pro Asn Thr Phe Gly Gly Gly Thr Lys Leu
                105                 110

GAA ATA AAA CGG ACC GGA GGT GGC GGG TCG                    360
Glu Ile Lys Arg Thr Gly Gly Gly Gly Ser
                115                 120

GGT GGC GGG GGA TCG GGT GGC GGA GGG TCG                    390
Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
                125                 130

GAC GTC AAG CTC GTG GAG TCT GGG GGA GAC                    420
Asp Val Lys Leu Val Glu Ser Gly Gly Asp
                135                 140

TTA GTG AAG CTT GGA GGG TCC CTG AAA CTC                    450
Leu Val Lys Leu Gly Gly Ser Leu Lys Leu
                145                 150

TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT                    480
Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
                155                 160

AGC TAT TAT ATG TCT TGG GTT CGC CAG ATT                    510
Ser Tyr Tyr Met Ser Trp Val Arg Gln Ile
                165                 170

TCA GAG AAG AGG CTG GAG TTG GTC GCA GCC                    540
Ser Glu Lys Arg Leu Glu Leu Val Ala Ala
                175                 180
```


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Page 3335

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 119,
Please delete lines 56, through column 121, line 22, and insert therefor:

```
ATT AAT AGT AAT GGT GAT AGC ACC TAC TAT                    570
Ile Asn Ser Asn Gly Asp Ser Thr Tyr Tyr
                185                 190

CCA GAC ACT GTG AAG GGC CGA TTC ACC ATC                    600
Pro Asp Thr Val Lys Gly Arg Phe Thr Ile
                195                 200

TCC AGA GAC AAT GCC AAG AGC ACC CTG GAT                    630
Ser Arg Asp Asn Ala Lys Ser Thr Leu Asp
                205                 210

CTG CAA ATG AGC AGT CTG AAG TCT GAG GAC                    660
Leu Gln Met Ser Ser Leu Lys Ser Glu Asp
                215                 220

ACA GCC TTG TAT TTC TGT GTA AGA GAG ACT                    690
Thr Ala Leu Tyr Phe Cys Val Arg Glu Thr
                225                 230

TAT TAC TAC GGG ATT AGT CCC GTC TTC GAT                    720
Tyr Tyr Tyr Gly Ile Ser Pro Val Phe Asp
                235                 240

GTC TGG GGC ACA GGG ACC ACG GTC ACC GTC                    750
Val Trp Gly Thr Gly Thr Thr Val Thr Val
                245                 250

TCC TCA CTC GAG CAC CAC CAC CAC CAC CAC                    780
Ser Ser Leu Glu His His His His His His
                255                 260

TGA                                                       783
```


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CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 121,
Please delete lines 32, through column 123, line 3, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                    30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                    60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                    90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                   120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                   150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                   180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
                55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                   210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                   240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                   270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                   300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                95                 100
```


Samsung Bioepis Exhibit No. 1002
Page 3337

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 123,
Please delete lines 4-33, and insert therefor:

```
GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
                185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
                195                 200
```


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CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 123,
Please delete lines 34-48, and insert therefor:

```
TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                        747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Column 125,
Please delete lines 2-13, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                     30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                     60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                     90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                    120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                35                  40
```


Samsung Bioepis Exhibit No. 1002
Page 3339

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 125,
Please delete lines 14-43, and insert therefor:

```
AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                    150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                 45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                    180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
                 55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                    210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                 65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                    240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                 75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                 85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                 95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140
```


Samsung Bioepis Exhibit No. 1002
Page 3340

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 125,
Please delete line 44, through column 127, line 15, and insert therefor:

```
GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                    570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
                185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240
```


Samsung Bioepis Exhibit No. 1002
Page 3341

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 127,

Please delete lines 16-18, and insert therefor:

```
GGA ACC CTG GTC ACT GTC TCG AGC TGA                                    747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Please delete lines 29-52, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                                 30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                                 60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                                 90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                                120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                35                  40

AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                                150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                                180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
                55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                                210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                                240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                75                  80
```


Samsung Bioepis Exhibit No. 1002
Page 3342

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 127,
Please delete line 53, through column 129, line 24, and insert therefor:

```
CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                 85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                 95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180
```


Samsung Bioepis Exhibit No. 1002
Page 3343

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 129,
Please delete lines 25-45, and insert therefor:

```
TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                    570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
                185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                        747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Please delete line 56, through column 131, line 3, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                     30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                     60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20
```


Samsung Bioepis Exhibit No. 1002
Page 3344

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 131,
Please delete lines 4-33, and insert therefor:

```
GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                    90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
                 25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                   120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                 35                  40

AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT                   150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                 45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                   180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
                 55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                   210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                 65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                   240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                 75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                   270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                 85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                   300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                 95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                   330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                   360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120
```


Samsung Bioepis Exhibit No. 1002
Page 3345

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 131,
Please delete line 34, through column 133, line 3, and insert therefor:

```
TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC                    570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
                185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220
```


Samsung Bioepis Exhibit No. 1002
Page 3346

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 133,

Please delete lines 4-12, and insert therefor:

```
TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                        747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Please delete lines 22-45, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                     30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                     60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                     90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                    120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                    150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC                    180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
                55                  60
```


Samsung Bioepis Exhibit No. 1002
Page 3347

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 133,
Please delete line 46, through column 135, line 12, and insert therefor:

```
CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                    210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                 65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                    240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                 75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                 85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                 95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160
```


Samsung Bioepis Exhibit No. 1002
Page 3348

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 135,

Please delete lines 13-39, and insert therefor:

```
CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
                185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                        747
Gly Thr Leu Val Thr Val Ser Ser
                245
```


Samsung Bioepis Exhibit No. 1002
Page 3349

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 135,
Please delete line 49, through column 137, line 21, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                    30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                    60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC                    90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                   120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                35                  40

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                   150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                   180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
                55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                   210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                   240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                   270
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                85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                   300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                95                 100
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Samsung Bioepis Exhibit No. 1002
Page 3350

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 137,
Please delete lines 22-51, and insert therefor:

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GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
                185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
                195                 200
```


Samsung Bioepis Exhibit No. 1002
Page 3351

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 137,
Please delete line 52, through column 139, line 6, and insert therefor:

```
TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA                        747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Column 139,
Please delete lines 16-27, and insert therefor:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG                     30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
  1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG                     60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC CGT GCT AGC GAA AAC                     90
Val Thr Ile Thr Cys Arg Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG                    120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                35                  40
```


Samsung Bioepis Exhibit No. 1002
Page 3352

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 139,
Please delete lines 28-57, and insert therefor:

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AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT                    150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                 45                  50

TAC GGT GCG ACG AGC CTG CAG TCT GGA GTC                    180
Tyr Gly Ala Thr Ser Leu Gln Ser Gly Val
                 55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA                    210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                 65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG                    240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                 75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT                    270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                 85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC                    300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                 95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT                    330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA                    360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG                    390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA                    420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140
```


Samsung Bioepis Exhibit No. 1002
Page 3353

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 141,
Please delete lines 1-30, and insert therefor:

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GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT                    450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT                    480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC                    510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC                    540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT                    570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
                185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT                    600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC                    630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT                    660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC                    690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA                    720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240
```


Samsung Bioepis Exhibit No. 1002
Page 3354

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,355,245 B1
DATED : March 12, 2002
INVENTOR(S) : Mark J. Evans et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 141,
Please delete lines 31-33, and insert therefor:

```
GGA ACC CTG GTC ACT GTC TCG AGC TGA                    747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

Signed and Sealed this

Nineteenth Day of November, 2002

Attest:

Attesting Officer

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

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Page 3355

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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ORIGINAL ARTICLE

Effect of Eculizumab on Hemolysis and Transfusion Requirements in Patients with Paroxysmal Nocturnal Hemoglobinuria

Peter Hillmen, M.B., Ph.D., Claire Hall, M.B., Ch.B., Judith C.W. Marsh, M.B., M.D., Modupe Elebute, M.B., M.D., Michael P. Bombara, B.S., Beth E. Petro, B.S., Matthew J. Cullen, B.Sc., Stephen J. Richards, Ph.D., Scott A. Rollins, Ph.D., Christopher F. Mojcik, M.D., Ph.D., and Russell P. Rother, Ph.D.

ABSTRACT

BACKGROUND

Paroxysmal nocturnal hemoglobinuria (PNH) arises from a somatic mutation of the *PIG-A* gene in a hematopoietic stem cell and the subsequent production of blood cells with a deficiency of surface proteins that protect the cells against attack by the complement system. We tested the clinical efficacy of eculizumab, a humanized antibody that inhibits the activation of terminal complement components, in patients with PNH.

METHODS

Eleven transfusion-dependent patients with PNH received infusions of eculizumab (600 mg) every week for four weeks, followed one week later by a 900-mg dose and then by 900 mg every other week through week 12. Clinical and biochemical indicators of hemolysis were measured throughout the trial.

RESULTS

Mean lactate dehydrogenase levels decreased from 3111 IU per liter before treatment to 594 IU per liter during treatment (P=0.002). The mean percentage of PNH type III erythrocytes increased from 36.7 percent of the total erythrocyte population to 59.2 percent (P=0.005). The mean and median transfusion rates decreased from 2.1 and 1.8 units per patient per month to 0.6 and 0.0 units per patient per month, respectively (P=0.003 for the comparison of the median rates). Episodes of hemoglobinuria were reduced by 96 percent (P<0.001), and measurements of the quality of life improved significantly.

CONCLUSIONS

Eculizumab is safe and well tolerated in patients with PNH. This antibody against terminal complement protein C5 reduces intravascular hemolysis, hemoglobinuria, and the need for transfusion, with an associated improvement in the quality of life in patients with PNH.

From the Department of Haematology, Leeds Teaching Hospitals National Health Science Trust, Leeds, United Kingdom (P.H., C.H., M.J.C., S.J.R.); the Department of Haematology, St. George's Hospital Medical School, London (J.C.W.M., M.E.); and Alexion Pharmaceuticals, Cheshire, Conn. (M.P.B., B.E.P, S.A.R., C.F.M., R.P.R.). Address reprint requests to Dr. Hillmen at the Department of Haematology, Leeds General Infirmary, Great George St., Leeds LS1 3EX, United Kingdom, or at peter.hillmen@panp-tr.northy.nhs.uk.

N Engl J Med 2004;350:552-9.

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Page 3357

THE MAJOR CLINICAL SIGNS OF PAROXYSMAL nocturnal hemoglobinuria (PNH) are intravascular hemolysis, venous thrombosis, and hemoglobinuria.[1] The disease arises from a somatic mutation of the *PIG-A* gene in a pluripotent hematopoietic stem cell. *PIG-A* encodes a protein that is essential for the synthesis of glycosylphosphatidylinositol (GPI), a lipid moiety that is embedded in the plasma membrane, where it serves to anchor a wide variety of proteins to the cell surface. The mutant stem cell subsequently expands to form a hematopoietic clone with a deficiency in proteins that are normally attached to the cell surface by the GPI anchor.[2,3] The mature blood cells derived from the hematopoietic clone can have a complete deficiency (type III) or a partial deficiency (type II) of GPI-linked proteins and almost always coexist with residual cells with a normal expression of these proteins (previously identified as type I).

The clinical features of PNH result from the lack of one or more GPI-linked proteins that protect cells from complement-mediated attack. Two such proteins — CD55 and CD59 — are absent from PNH type III erythrocytes, platelets, and other blood cells.[4-6] CD55 regulates early complement activation by inhibiting C3 convertases,[7] whereas CD59 inhibits the assembly of the membrane-attack complex C5b–C9 by interacting with C8 and C9.[4,5] The lack of CD59 is probably responsible for the increased sensitivity of PNH erythrocytes and platelets to complement.[4,8-13]

Eculizumab is a recombinant humanized monoclonal antibody that was designed to block the activation of terminal complement components.[14,15] It binds specifically to the terminal complement protein C5, inhibiting its cleavage into C5a and C5b, thereby preventing the release of the inflammatory mediator C5a and the formation of the cytolytic-pore C5b–C9. Blockade of the complement cascade at C5 preserves the early components of complement that are essential for the opsonization of microorganisms and clearance of immune complexes.[16] In this trial, we investigated whether eculizumab could reduce the incidence of intravascular hemolysis, hemoglobinuria, and transfusion requirements in patients with PNH.

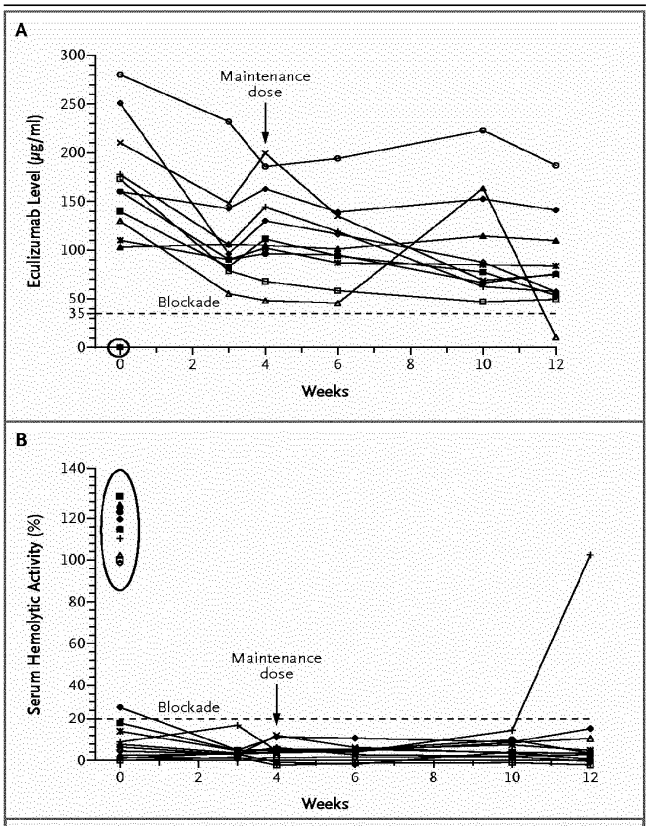


Figure 1. **Pharmacokinetic and Pharmacodynamic Analysis of Eculizumab in 11 Patients with Paroxysmal Nocturnal Hemoglobinuria (PNH).**

Panel A depicts serum levels of eculizumab during the 12-week treatment period. Eculizumab levels just before a patient's first dose of eculizumab are circled on the x axis. Also shown at time zero are levels of eculizumab one hour after the initial dose. Other data points indicate trough levels of eculizumab for each patient at weeks 3, 4, 6, 10, and 12. The beginning of the 900-mg maintenance dose is indicated by an arrow. The level of eculizumab required to block complement activity (≥35 μg per milliliter) is indicated by a dashed line. Panel B shows hemolytic activity in serum during the 12-week treatment period, as assessed in a presensitized chicken-erythrocyte hemolytic assay. Pretreatment serum hemolytic activity values are circled at time zero. Also shown at time zero are values for hemolytic activity one hour after the initial dose. Other data points indicate trough hemolytic activity at weeks 3, 4, 6, 10, and 12. The dashed line indicates the level of hemolytic activity that is considered to represent complete complement blockade (≤20 percent).

METHODS

PATIENTS

The study was conducted from May through December 2002. Men and women (18 years of age and older) who had received a diagnosis of PNH at least six months earlier, had a detectable GPI-deficient hematopoietic clone, and had received at least four red-cell transfusions in the preceding 12 months were

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eligible. Patients were required to have a negative throat culture for *Neisseria meningitidis* and *N. gonorrhoeae*. All patients were vaccinated against *N. meningitidis* (Mengivac (A+C), Aventis Pasteur) before treatment. One patient had a stroke after consent but never received eculizumab and was excluded. Patients who were taking stable doses of immunosuppressive drugs (e.g., cyclosporine), warfarin, and iron supplements were permitted to continue them.

The trial was approved by the local research ethics committee and was performed according to the International Conference on Harmonisation and Good Clinical Practice Standards. Eleven patients gave written informed consent and were treated with eculizumab.

TREATMENT SCHEDULE

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.

INVESTIGATIONS

In this open-label pilot study, we obtained data on the pharmacokinetics, pharmacodynamics, and immunogenicity of eculizumab and observed its clinical effects by measuring the following: lactate dehydrogenase, haptoglobin, bilirubin, and hemoglobin levels; reticulocyte counts; the proportion of GPI-deficient cells, as assessed by flow cytometry[17]; the rate of transfusion with packed red cells; the rate of occurrence of hemoglobinuria (assessed by daily comparison of the first morning urine sample with a standardized color chart before and during treatment); and the quality of life, as reflected by the scores on the European Organization for Research and Treatment of Cancer (EORTC) QLQ-C30 instrument. The trigger for transfusion during the study period remained unchanged for each patient, as compared with their care before entry into the study: patients received blood transfusions when they had symptoms resulting from anemia.

Assessment of the safety of eculizumab included ascertainment of treatment-related adverse events, electrocardiography, and routine laboratory tests (e.g., serum chemical analyses and complete blood counts).

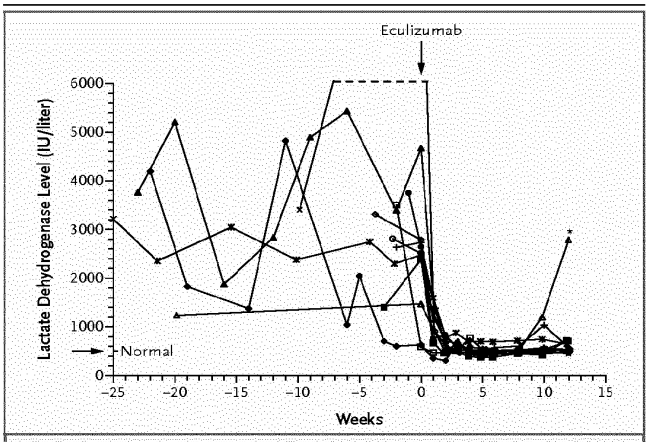


Figure 2. Analysis of Lactate Dehydrogenase Levels, a Biochemical Indicator of Hemolysis, in 11 Patients with Paroxysmal Nocturnal Hemoglobinuria up to 25 Weeks before and during 12 Weeks of Eculizumab Treatment.

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.

ASSAY METHODS

The pharmacokinetics of eculizumab were determined with an enzyme-linked immunosorbent assay that detects both free and C5-bound eculizumab.[15] The pharmacodynamics of eculizumab were determined by measuring the capacity of the patient's serum to lyse chicken erythrocytes in a standard total human serum-complement hemolytic assay.[18] The presence or absence of antibodies against eculizumab was assessed by an enzyme-linked immunosorbent assay.[19]

STATISTICAL ANALYSIS

Biochemical values were compared with the use of a paired Student's t-test, quality-of-life measurements with the use of a mixed-effect analysis of covariance, the median rate of transfusions with the use of a Wilcoxon signed-rank test, and the comparison of the number of days with paroxysms with the use of Fisher's exact test.

The corresponding author and the sponsor were jointly responsible for the design of this trial and the development of the protocol. Data were collected and analyzed by a clinical research organization, Kendle International, which maintained the trial data base and provided statistical support. The manuscript was prepared by the corresponding author, with substantial review and comments by the other authors and the sponsor. Final decisions on

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the content of the manuscript rested with the corresponding author in consultation with the other authors. All authors had access to the primary data.

RESULTS

DEMOGRAPHIC CHARACTERISTICS

Six men and five women (median age, 48 years; range, 21 to 67) with a median duration of PNH of 8.6 years (range, 1.7 to 37.4) participated in the trial. Five of the patients had platelet counts at base line of less than 150,000 per cubic millimeter. Eight patients had previously received a diagnosis of aplastic anemia, two were concomitantly receiving cyclosporine for aplastic anemia, and six were receiving warfarin.

SAFETY

All patients completed the 12-week study. There were no deaths or thrombotic events, and all patients subsequently entered a 12-month extension study. Each patient reported one or more adverse events during the trial. Events reported by three patients included headache and upper respiratory tract infection. Events reported by two patients included influenza-like symptoms, rigors, dizziness, nausea, nasal congestion, and joint aches. None of these events were attributed to the study medication. Serious adverse events occurred in two patients. The first was hospitalized with a viral chest infection. The second reported nausea, vomiting, and headache after the first infusion, with dizziness and shivering the following day. The patient was hospitalized overnight, and subsequent infusions were well tolerated.

PHARMACOKINETICS, PHARMACODYNAMICS, AND IMMUNOGENICITY OF ECULIZUMAB

Peak and trough levels of eculizumab were well above 35 μg per milliliter from one hour after the first dose through the completion of the 600-mg weekly dose period (Fig. 1A). In 10 patients, serum trough levels of eculizumab remained above 35 μg per milliliter for the entire study.

The hemolytic activity of serum from these 10 patients was completely blocked (less than 20 percent in the chicken–red-cell assay) for essentially the entire treatment period (Fig. 1B). In 1 of the 11 patients, the trough level of eculizumab fell below 35 μg per milliliter at week 12 and serum hemolytic activity returned. In no case were antibodies against eculizumab detected.

BIOCHEMICAL INDICATORS OF HEMOLYSIS

Levels of lactate dehydrogenase in serum were markedly elevated in all patients before eculizumab treatment. Mean (±SD) lactate dehydrogenase levels fell from 3111±598 IU per liter during the 12 months before enrollment to 594±32 IU per liter (normal range, 150 to 480) during treatment ($P=0.002$) (Fig. 2).

The decrease in lactate dehydrogenase began after a single dose of eculizumab in all patients. Lactate dehydrogenase levels remained within or just above the normal range for the duration of the study (Fig. 2). In the one patient in whom eculizumab levels fell below 35 μg per milliliter at week 12 (Fig. 1A), hemolytic activity returned (Fig. 1B) and lactate dehydrogenase levels increased transiently (Fig. 2).

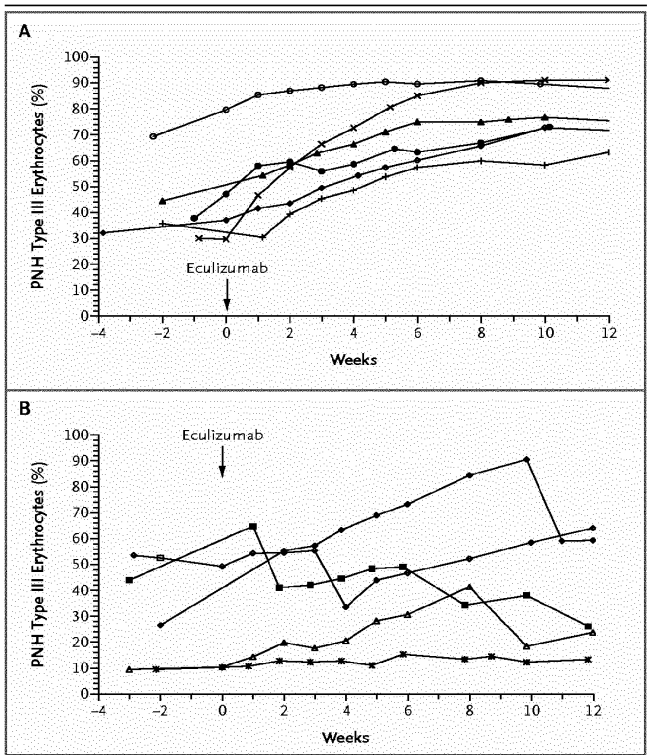


Figure 3. **Changes in the Percentage of Paroxysmal Nocturnal Hemoglobinuria (PNH) Type III Erythrocytes during Eculizumab Treatment in Six Patients Who Received No Transfusions during Treatment (Panel A) and Five Patients Who Received at Least One Transfusion after Starting Treatment (Panel B).** The initial dose of eculizumab is indicated by the arrow in each panel.

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The dosing frequency was increased from 900 mg every 14 days to 900 mg every 12 days, reestablishing complete complement blockade during the ongoing 12-month extension study (data not shown).

Haptoglobin became detectable in the serum of 5 of the 11 patients after two weeks of eculizumab treatment but returned to undetectable levels soon thereafter (data not shown). Bilirubin levels were also elevated in most patients at base line and did not change significantly during treatment (data not shown).

EFFECT ON PNH CLONES

Type III erythrocytes are highly sensitive to lysis by complement and as a result have a short life span. In our study, the percentage of type III erythrocytes increased significantly from a mean of 36.7±5.9 percent before treatment to 59.2±8.0 percent at the end of 12 weeks of treatment (P=0.005) (Fig. 3). The increase was particularly consistent in six patients who remained transfusion-independent during the study (Fig. 3A). In patients who received transfusions during the study, sudden drops in the proportion of type III cells were seen as the transfused red cells diluted the type III population (Fig. 3B). There were no significant changes in the percentages of PNH type III neutrophils, monocytes, or platelets during treatment with eculizumab; in most cases, these percentages were 90 to 100 percent before the study (data not shown).

TRANSFUSION REQUIREMENTS, HEMOGLOBIN LEVELS, AND RETICULOCYTE COUNTS

During the year preceding enrollment, the range of red-cell transfusions received by the 11 patients was 12 to 55 units, whereas during the three months of the study, the range was 0 to 8 units. Before eculizumab treatment, the mean and median transfusion rates were 2.1 and 1.8 units per patient per month, respectively (Table 1). These transfusion rates decreased to 0.6 and 0.0 unit per patient per month, respectively, during the three months of treatment with eculizumab (P=0.003). Hemoglobin levels did not increase significantly during the treatment period, although hemoglobin values in six patients stabilized without transfusions (Table 1). Similarly, the numbers of reticulocytes remained relatively constant during eculizumab treatment.

HEMOGLOBINURIA

Our patients recorded the color of their urine each morning using a color chart designed to assess the

Table 1. **Transfusion Rates, Hemoglobin Levels, and Reticulocyte Counts 12 Months before and after 3 Months of Eculizumab Treatment.**

Patient No.	12 Mo before Eculizumab Treatment				After 3 Mo of Eculizumab Treatment			
	Transfusions		Hemoglobin	Reticulocytes	Transfusions		Hemoglobin	Reticulocytes
	no. of units	*rate**	*g/dl*	*×10⁻³/mm³*	*no. of units*	*rate†*	*g/dl*	*×10⁻³/mm³*
1	22	1.8	10.3	77.5	2	0.7	10.0	100.7
2	23	1.9	8.3	200.0	8	2.9	8.8	182.6
3	20	1.6	10.1	169.5	0	0.0	10.7	175.9
4	28	2.3	9.3	282.0	0	0.0	9.4	333.3
5	12	1.0	11.9	96.3	2	0.7	10.6	121.8
6	14	1.2	9.8	346.8	0	0.0	10.6	259.0
7	34	2.8	12.8	100.6	0	0.0	13.5	166.8
8	21	1.7	9.5	164.5	0	0.0	9.8	239.6
9	55	4.5	10.7	138.0	3	1.1	11.4	285.8
10	41	3.4	8.5	108.7	5	1.8	8.8	140.1
11	14	1.2	8.5	91.4	0	0.0	10.0	97.4
Median‡		1.8				0.0		
Mean		2.1	10.0	161.4		0.6	10.3	191.2

* The rate (in units per month) was calculated as (number of units÷365 days)×30.
† The rate (in units per month) was calculated as (number of units÷84 days)×30.
‡ P=0.003 for the change in the median rate of transfusion by the Wilcoxon signed-rank test.

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degree of hemoglobinuria (Fig. 4A) during both the 2-to-4-week screening period and the 12-week treatment period. Paroxysms of hemoglobinuria were prospectively defined as dark-colored urine with a colorimetric level of 6 or more. In nine patients for whom urine scores were assessed, the mean incidence of paroxysms was reduced from 2.9 days to 0.12 day per patient per month ($P<0.001$) (Fig. 4B).

QUALITY OF LIFE

The quality of life was assessed with the use of the EORTC QLQ-C30 instrument. When responses at base line were compared with responses during 12 weeks of eculizumab treatment, there were significant improvements in the domains of global health status ($P=0.02$), physical functioning ($P<0.001$), emotional functioning ($P<0.001$), cognitive functioning ($P=0.002$), fatigue ($P<0.001$), dyspnea ($P=0.002$), and insomnia ($P=0.049$) (Table 2).

DISCUSSION

Patients with PNH have chronic, often disabling symptoms of fatigue and intermittent episodes of dysphagia, abdominal pain, and hemoglobinuria. These symptoms are thought to be related to the intravascular destruction of PNH type III erythrocytes, which are deficient in complement inhibitors, by autologous complement. The hemolytic anemia frequently renders the patients transfusion-dependent. In addition, patients have an extremely high risk of potentially life-threatening thrombosis, particularly thrombosis of the hepatic and cerebral veins. Approximately 50 percent of patients with PNH die of the disease; the median duration of survival after diagnosis is 10 years.[1]

We found that the defect in the membrane-bound inhibitor of terminal complement components in PNH was ameliorated by the administration of eculizumab. This antibody specifically prevents cleavage of C5, which is necessary for assembly of the membrane-attack complex. Blockade of terminal complement components presumably prolongs the survival of type III erythrocytes (since there was no simultaneous increase in reticulocytes), which are highly sensitive to lysis by complement, thereby increasing the proportion of these cells in the blood and reducing signs of hemolysis in most patients. In some patients, the percentage of type III erythrocytes increased to more than 80 percent of the total erythrocyte population. This interpretation of the mechanism of action of eculizumab is consistent with the report of an asymptomatic patient with PNH who had more than 80 percent type III erythrocytes and a concomitant deficiency in the terminal complement protein C9.[20] Thus, inhibition of the assembly of C5b–C9 by an antibody or by a congenital deficiency of a terminal component of the complement system can protect type III erythrocytes from complement-mediated lysis.

The long-term effects of protecting PNH type III erythrocytes from complement are not known. For example, will removing the negative pressure

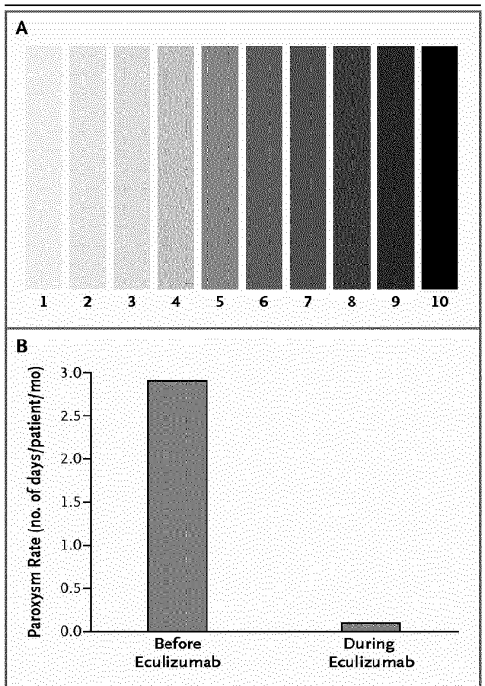


Figure 4. **Incidence of Paroxysms during Eculizumab Treatment.**

Panel A shows a urine color scale devised to monitor the incidence of paroxysms of hemoglobinuria in patients with paroxysmal nocturnal hemoglobinuria before and during treatment. A paroxysm was prospectively defined as a urine score of 6 or greater in this study. Panel B depicts the change in the paroxysm rate (defined as the mean number of days in paroxysm per patient per month) in nine patients 1 month before and during 12 weeks of eculizumab therapy. Pretreatment data on paroxysms were inadvertently not collected for two patients, and these two were therefore excluded from the analysis.

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Table 2. **Change in the Quality of Life during Eculizumab Treatment.***

Domain	Mean Base-Line Score†	Change from Base Line‡§	P Value§
Global health status	56.1	13.7	0.02
Physical functioning	70.9	13.0	<0.001
Emotional functioning	70.5	12.7	<0.001
Cognitive functioning	77.3	11.8	0.002
Fatigue	47.5	−15.3	<0.001
Dyspnea	39.4	−12.4	0.002
Insomnia	30.3	−10.8	0.049

* The quality of life was assessed with the European Organization for Research and Treatment of Cancer QLQ-C30 instrument.
† Numbers represent mean values of linearly transformed scores.
‡ Values for change from base line represent least-square means. A positive value indicates an improvement in the score for global health status, physical functioning, emotional functioning, and cognitive functioning, whereas a negative value indicates an improvement in the score for fatigue, dyspnea, and insomnia.
§ Values are from a mixed analysis-of-covariance model with visit as a fixed effect, patient as a random effect, and base line as a covariate.

on type III hematopoietic cells alter the rate of expansion of the PNH clone? What might occur in a patient with an increased population of PNH type III erythrocytes if treatment with eculizumab is stopped? Two of the patients who entered the eculizumab extension study had transient breakthroughs (lasting two to three days) in complement blockade until the dosing interval was adjusted. Both patients had hemoglobinuria with mild symptoms, but the episodes were not life threatening and were easily managed (data not shown). Definitive answers to these questions will require further study.

In this trial, lactate dehydrogenase levels declined rapidly and remained reduced as long as the serum level of eculizumab exceeded 35 μg per milliliter. The importance of maintaining this level of antibody was demonstrated in a single patient, in whom the eculizumab level transiently dropped below 35 μg per milliliter at week 12, resulting in a return of serum complement activity and an increase in lactate dehydrogenase levels. Subsequent administration of eculizumab reestablished complement blockade and rapidly reduced lactate dehydrogenase levels. Interestingly, lactate dehydrogenase levels were reduced in most patients to just above the upper limit of normal. The slightly elevated levels of this enzyme during treatment with eculizumab could reflect persistent, low-level C3b-mediated extravascular hemolysis or, possibly, undefined mechanisms of hemolysis that are unrelated to complement.

We also found that eculizumab treatment significantly reduced transfusion requirements, even though the levels of hemoglobin did not change significantly. However, the hemoglobin level in an individual patient before study entry was artificially maintained as a result of the transfusion of normal red cells. The transfused red cells survive far longer than PNH cells. Therefore, the stabilization of hemoglobin levels with a reduced need or no need for transfusion is a result of the protection of PNH red cells from complement-mediated lysis by eculizumab.

The decrease in transfusion requirements was most apparent in the six patients without a clinically significant degree of bone marrow failure (as defined by a normal platelet count). All but one of these patients were transfusion-independent during the study and remained so during an extension study. The remaining patient received a single 3-unit transfusion during the study, as compared with the receipt of 55 units in the 12 months preceding enrollment.

There was a rapid improvement in the quality of life during eculizumab therapy, as measured by the EORTC QLQ-C30. These clinical observations support the hypothesis that many of the important coexisting clinical conditions in patients with PNH are directly related to chronic and acute episodes of hemolysis, possibly through the scavenging of nitric oxide by plasma free hemoglobin.[21-25]

Eculizumab was safe and well tolerated during this open-label pilot study. The adverse events reported by patients were similar in type and frequency to those reported with either eculizumab or placebo in other controlled trials. All patients are currently participating in a one-year extension study in which the drug continues to be well tolerated. Furthermore, the rates of intravascular hemolysis, as measured by lactate dehydrogenase levels and hemoglobinuria, remain reduced in all patients, with 5 of 11 patients having been transfusion-independent for at least one year since starting eculizumab treatment.

The *PIG-A* mutation in patients with PNH causes deficiencies in the membrane-bound complement inhibitors CD55 and CD59, resulting in intravascular hemolysis.[2,3] However, patients who have genetic deficiencies in the surface expression of CD55 (Inab phenotype) with normal levels of CD59 have no clinical signs of hemolysis.[8,9] Conversely, a pa-

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tient with a genetic deficiency in the expression of CD59 but normal levels of CD55 had symptoms indistinguishable from those of PNH.[10,11] Therefore, the somatic mutation in the *PIG-A* gene that causes the deficiency of the membrane-bound terminal complement inhibitor CD59 is critical to the pathogenesis of PNH. We found that terminal complement inhibition with eculizumab ameliorated the untoward effects of this deficiency.

In summary, eculizumab appears to enhance the survival of type III PNH erythrocytes, improving the quality of life and reducing the extent of hemolysis, hemoglobinuria (the clinical hallmark of PNH), and the need for blood transfusions in patients with PNH. This study confirms that terminal complement activation is the key mediator of erythrocyte destruction in PNH.

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Dr. Hillmen reports serving as a consultant to Alexion Pharmaceuticals and receiving grant support from the company; Drs. Rollins, Mojcik, and Rother, Mr. Bombara, and Ms. Petro report having equity ownership in Alexion Pharmaceuticals; and Drs. Rollins and Rother have assigned to Alexion Pharmaceuticals their inventions made as employees of the company and have received no royalties from Alexion for these inventions. Dr. Rollins receives royalties for inventions he made before becoming an employee of Alexion.

We are indebted to Drs. Leonard Bell, Stephen Squinto, and Paul Finnegan for critical review of the manuscript; to Karen Hannon for excellent technical support; and to Jan Farish, David Buchanon, Pilar Hernandez-Campo, and the nurses of Healthcare-at-Home for their assistance in carrying out the study.

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β-glucan released in the BM by Mϕ functions to prime the CR3 of HSPC that have been tethered to the iC3b deposited onto BM cells, thereby enhancing the normal rate of HSPC expansion and differentiation. The oral WGP preparation is currently under study as a possible therapeutic to be given to individuals at risk for exposure to harmful radiation as the result of a nuclear power plant accident or nuclear terrorism. These data indicate an important role in hematopoiesis of both the C3a–C3aR axis and the iC3b–CR3 receptor axis, and do not exclude the participation of other components or receptors.

Supported by NIH CA84612 and US Army DAMD 17-02-1-0445.

Keywords: Bone marrow recovery; β-Glucan

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Eculizumab, a C5 complement-blocking antibody, abolishes hemolysis and reduces transfusion dependency in patients with paroxysmal nocturnal hemoglobinuria (PNH)

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Paroxysmal nocturnal hemoglobinuria (PNH) is characterized by intravascular hemolysis, venous thrombosis and is associated with aplastic anemia. The complement-mediated hemolysis is due to the deficiency of complement-regulatory proteins on the surface of PNH red cells, particularly the terminal complement inhibitor CD59. Eculizumab is a humanized monoclonal antibody that binds to C5 and inhibits terminal complement activation. 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. All 11 patients completed the study and are currently participating in an open-label extension study. Eculizumab was well tolerated with no patients reporting a serious adverse event. The biochemical parameters of hemolysis dramatically improved or normalized for all patients within a week of starting therapy. Mean LDH decreased from 3111 ± 598 U/l during the 12 months prior to treatment to 594±32 U/l following treatment ($P = 0.002$) while mean AST decreased from 76 ± 16 U/l at baseline to 28 ± 4 U/l following treatment ($P = 0.032$). Paroxysms, as defined by episodes of hemoglobinuria on a urine colorimetric scale, decreased by 96% ($P < 0.001$). In addition, PNH type III red cells increased 65% from 36.7 ± 5.9 to 59.2 ± 8.0% during the treatment period ($P = 0.005$), presumably due to a prolongation of PNH red cell survival. Transfusions were significantly reduced in the 3 months of eculizumab compared to the previous 12-month historical data from 2.03 to 0.65 units per patient per month ($P = 0.023$). Quality of life (EORTC QLC30) improved significantly post-eculizumab compared to pre-eculizumab. There was no change in the proportion of PNH neutrophils, monocytes or platelets in any of the patients while on eculizumab. No episodes of thrombosis occurred in these 11 patients although follow-up is short and 4 of 6 patients are on warfarin. In summary, the results from this pilot study demonstrate that eculizumab was well tolerated, decreased hemolysis and improved symptoms in PNH patients. This study suggests that blocking complement at C5 is an effective therapeutic strategy for hemolytic PNH resulting in control of hemolysis and a reduction in transfusion dependency.

Keywords: Paroxysmal nocturnal hemoglobinuria (PNH); C5

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DX-88 a recombinant inhibitor of human plasma kallikrein. Efficacy and safety in hereditary and acquired angioedema

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DX-88 is a recombinant inhibitor of plasma kallikrein. It was designed as a Kunitz domain protein and selected by phage display to be highly specific for human plasma kallikrein. Safety and efficacy of DX-88 in reverting symptoms in patients with hereditary and acquired angioedema (HAE and AAE) has been tested.

The study was designed to have nine different HAE/AAE patients treated with DX-88 for a non-lifethreatening angioedema. The three ascending dose regimens, (10, 40, and 80 mg) given as a short intravenous infusion, were used. Time to relief of symptoms, time to resolution and adverse events have been recorded. The following biochemical parameters have been assessed: plasma levels of C1INH and kallikrein function, plasma concentrations of C1INH and C4, cleavage of HK pharmacokinetic of DX-88.

Seven patients with HAE and two with AAE (five women and four men, age range 31–67 years old) have been treated. Angioedema was localized to the face (four patients), to the hands (one patients) to the genitals (one patient) and

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Eculizumab, a C5 complement blocking antibody, is the first therapy to reduce transfusion requirements in patients with paroxysmal nocturnal haemoglobinuria (PNH)

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Paroxysmal nocturnal haemoglobinuria (PNH) is characterized by intravascular haemolysis and venous thrombosis due to the unopposed effect of complement on PNH red cells and platelets, respectively. Eculizumab is a humanized monoclonal antibody that binds C5 and inhibits terminal complement activation. Eleven transfusion-dependent PNH patients have 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. Adverse events were relatively minor and self-limiting. The biochemical parameters of haemolysis dramatically improved for all patients within a week. Mean LDH decreased from 3089 ± 599 U/L before to 597 ± 32 U/L after treatment ($P = 0.002$) and AST from 77 ± 20 to 26 ± 3 U/L ($P = 0.04$). Episodes of haemoglobinuria reduced by 90% ($P < 0.001$). Quality of life (EORTC QLC30) improved significantly post-eculizumab compared to pre-eculizumab. One patient began treatment during a severe acute haemolytic episode with almost black urine and within 24 h there was a complete resolution of her haemoglobinuria and symptoms. Transfusions for all patients were significantly reduced in the 3 months of eculizumab compared with the previous 12 months from 2.2 to 0.7 units/month ($P = 0.003$). The reduction in transfusions was most dramatic in the seven patients without thrombocytopenia from 2.3 to 0.2 units/month ($P = 0.001$). There was an increase in PNH type III red cells from a mean of 35.6% pre-eculizumab to 58.7% post-eculizumab ($P = 0.005$) due to their protection from complement-mediated lysis and the reduction in transfusions. There was no change in the proportion of PNH neutrophils, monocytes or platelets. All patients have continued on eculizumab in an extension study. In summary, eculizumab is well tolerated, decreases haemolysis, improves symptoms and significantly reduces transfusions in PNH patients. Eculizumab appears to be the first effective therapy for haemolysis in PNH and merits further development. This is the first trial of an anti-complement antibody therapy in PNH. The responses were remarkable both in reduction in haemolysis and transfusions and in the improvement in quality of life. Thus it is the first effective therapy for haemolysis in PNH and would also be expected to reduce the risk of thrombosis (this study could not address this). The trial was performed at two centres in the UK only.

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The induction of tolerance to RhD in HLA DR15 transgenic mice

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The RhD protein is a clinically important red cell alloantigen that induces the production of anti-D in RhD-negative individuals. Because of the immunogenicity of RhD, all blood donations are typed and matched for this antigen, and RhD-negative women are managed for the prevention of haemolytic disease of the newborn with prophylactic anti-D immunoglobulin. Understanding the mechanisms by which helper T-cells control the antibody response to RhD would enable the design of alternative strategies such as active toleration. We have shown that CD4+ helper T cells from alloimmunized donors can proliferate and produce both IFN-gamma and IL-4 in response to synthetic peptides derived from the RhD sequence, indicating a Th0 balanced cytokine response. The responses are HLA class II restricted, with HLA-DR15 being over-represented in good responders. Certain peptides are immunodominant, and induce proliferative responses in most alloimmunized donors. These peptides are candidates for the induction of tolerance to the RhD protein by the mucosal route. On the other hand, some peptides from the RhD protein preferentially induce IL-10 production by putative Tr1 regulatory cells *invitro*, and these peptides inhibit T-cell proliferation in response to stimulation with purified RhD protein *in vitro*. These peptides may be used to induce tolerance by the parenteral route. We have used a HLA-DR15 transgenic mouse model to show that T-cells from mice immunized with the RhD protein proliferate in response to purified RhD protein and to RhD peptides, and serum antibodies specific for human erythrocytes are also produced. Mucosal administration of immunodominant RhD peptides, or parenteral administration of IL-10 inducing peptides prior to immunization with RhD protein, inhibits both T-cell proliferation and antibody production. These results suggest that synthetic RhD peptides can induce tolerance to the RhD protein and may be of value in the prevention of immunization in RhD-negative individuals.

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A humanized monoclonal antibody against vWF A1 domain inhibits vWF:RiCof activity and platelet adhesion in human volunteers

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AJW200 (Ajinomoto), a humanized IgG4 monoclonal antibody directed against the A1 domain of von Willebrand factor (vWF), specifically inhibits vWF:platelet GPIb receptor interactions. At

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Eculizumab Could Be Paroxysmal Nocturnal Hemoglobinuria Breakthrough

Dec 13, 2002

Jane Salodof MacNeil

Dec. 13, 2002 (Philadelphia) - Preliminary results from an ongoing open-label phase Ib clinical trial suggest an experimental drug called eculizumab could be the first to alleviate symptoms of paroxysmal nocturnal hemoglobinuria (PNH).

Data on the first 11 patients show that three months of eculizumab therapy reduced the need for blood transfusions by 68%. An 81% reduction in biochemical parameters of hemolysis was also reported, and duration of clinical paroxysms went down by 90% from 2.1 days per patient per month to 0.2.

"With leukemia trials you have to wait months and years to see if there's a benefit. You knew immediately with this drug," principal investigator Peter Hillmen, MB, ChB, PhD, told Medscape. "[The patients] came in the next week, and they were fine."

Dr. Hillmen, a clinical hematologist at The General Infirmary at Leeds in the U.K., presented the results here at the American Society of Hematology annual meeting.

The paper was chosen because eculizumab would be the first targeted therapy with relevance to PNH, according to Russell Ware, MD, PhD, of Duke University Medical Center, co-chair of the session on acquired disorders.

Dr. Ware called the work "very exciting" in an interview with Medscape, but cautioned, "There's still a lot of work to be done. They need to study dosing, side effects — both short and long term — and risks as well as benefits to patients."

An acquired genetic disorder, PNH occurs in roughly one in 200,000 people, according to Dr. Hillmen.

Patients with PNH are deficient in the PIG-A enzyme and consequently their cells cannot protect themselves from complements in the blood. As a result, red blood cells are prone to lysis, producing blood in the person's plasma and urine, which often becomes dark red.

Patients typically become anemic, suffer significant fatigue, and have a high risk of dying from thrombosis. They are usually treated with steroids such as prednisone, immunosupressive drugs, and blood transfusions.

Eculizumab is a long-acting C5 inhibitor that Alexion Pharmaceuticals Inc. of Cheshire, Connecticut, is also testing in rheumatoid arthritis. Inhibition of complements 5-9 is associated with slightly higher risk of meningococcal infection, according to Dr. Hillmen. Only one case has been observed among 500 patients who have taken the drug so far, he said, including the rheumatoid arthritis trials.

"That's pretty good, and yet a case of meningitis might not be trivial," Dr. Ware noted.

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.

Ten patients have completed the 12-week treatment, and all chose to enter a 12-month extension trial. Another trial was at 10 weeks when the data was reported. All patients had at least four transfusions in the 12 months prior to the trial (average, 9 transfusions). The seven patients whose platelets levels were above 100 had the best response. Those with platelets levels below 100 did not improve as dramatically, Dr. Hillmen said, suggesting the drug is less effective in patients with bone marrow failure.

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Quality-of-life measures showed improvement in physical functioning, global health status, fatigue, and dyspnea parameters. Dr. Hillmen said some patients in the study had been so debilitated by fatigue that they had not worked for as long as 30 years.

"He overtook me on the stairs last week," Dr. Hillmen said of one of the most debilitated patients prior to treatment. "It had that dramatic an effect,"

The investigators reported that the drug was well tolerated. Muscle and joint aches and pains, upper respiratory infection, and headache were the most frequent adverse events.

A spokesman for Alexion Pharmaceuticals told Medscape that the company plans to work with the U.S. Food and Drug Administration's orphan drug program to design what will probably be a phase II/III trial.

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Reviewed by Gary D. Vogin, MD

Jane Salodof MacNeil is a freelance writer for Medscape.

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Immune-Mediated Hemolytic Anemia

Wendell F. Rosse, Peter Hillmen, and Alan D. Schreiber

Hemolytic anemia due to immune function is one of the major causes of acquired hemolytic anemia. In recent years, as more is known about the immune system, these entities have become better understood and their treatment improved. In this section, we will discuss three areas in which this progress has been apparent.

In Section I, Dr. Peter Hillmen outlines the recent findings in the pathogenesis of paroxysmal nocturnal hemoglobinuria (PNH), relating the biochemical defect (the lack of glycosylphosphatidylinositol [GPI]-linked proteins on the cell surface) to the clinical manifestations, particularly hemolysis (and its effects) and thrombosis. He discusses the pathogenesis of the disorder in the face of marrow dysfunction insofar as it is known. His major emphasis is on innovative therapies that are designed to decrease the effectiveness of complement activation, since the lack of cellular modulation of this system is the primary cause of the pathology of the disease. He recounts his considerable experience with a humanized monoclonal antibody against C5, which has a remarkable effect in controlling the manifestations of the disease. Other means of controlling the action of complement include replacing the missing modulatory proteins on the cell surface; these studies are not as developed as the former agent.

In Section II, Dr. Alan Schreiber describes the biochemistry, genetics, and function of the Fcγ receptors and their role in the pathobiology of autoimmune hemolytic anemia and idiopathic thrombocytopenic purpura due to IgG antibodies. He outlines the complex varieties of these molecules, showing how they vary in genetic origin and in function. These variations can be related to three-dimensional topography, which is known in some detail. Liganding IgG results in the transduction of a signal through the tyrosine-based activation motif and Syk signaling. The role of these receptors in the pathogenesis of hematological diseases due to IgG antibodies is outlined and the potential of therapy of these diseases by regulation of these receptors is discussed.

In Section III, Dr. Wendell Rosse discusses the forms of autoimmune hemolytic anemia characterized by antibodies that react preferentially in the cold–cold agglutinin disease and paroxysmal cold hemoglobinuria (PCH). The former is due to IgM antibodies with a common but particular structure that reacts primarily with carbohydrate or carbohydrate-containing antigens, an interaction that is diminished at body temperature. PCH is a less common but probably underdiagnosed illness due to an IgG antibody reacting with a carbohydrate antigen; improved techniques for the diagnosis of PCH are described. Therapy for the two disorders differs somewhat because of the differences in isotype of the antibody. Since the hemolysis in both is primarily due to complement activation, the potential role of its control, as by the monoclonal antibody described by Dr. Hillmen, is discussed.

I. Paroxysmal Nocturnal Hemoglobinuria: Current Understanding of the Biology of PNH

*Peter Hillmen, PhD**

Paroxysmal nocturnal hemoglobinuria (PNH)[1,2] is characterized by chronic intravascular hemolysis that is punctuated by episodes, or paroxysms, during which there is a marked increase in the intensity of hemolysis with macroscopic hemoglobinuria. The other elements of the clinical triad of PNH are venous thrombosis and an association with aplastic anemia. Recent therapeutic developments promise to radically alter the natural history of hemolytic PNH.

PNH is unique because it is an acquired hemolytic anemia due to an intrinsic red cell defect. In 1970 Oni et al first demonstrated that PNH red cells are monoclonal and that the remaining normal red cells are

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polyclonal. It was clear by the 1980s that the PNH cells were deficient in a large number of cell surface antigens but it was unclear how a single mutation could lead to the deficiency of such a variety of antigens nor how this resulted in the hemolysis characteristic of PNH.

Glycosylphosphatidylinositol-Linked Antigens and PNH

In the 1980s it became clear that a variety of antigens were attached to the cell membrane by a glycolipid structure. This structure is highly preserved throughout evolution with the same basic "backbone" consisting of a phosphatidylinositol, a single glucosamine, three mannoses and an ethanolamine—a glycosylphosphatidylinositol (GPI) structure. The antigens missing from PNH cells are all GPI-anchored to the cell membrane and thus a single mutation disrupting GPI biosynthesis would result in the PNH phenotype. The biosynthetic defect in PNH always affects the transfer of glucosamine from UDP-*N*-acetyl glucosamine onto phosphatidyl inositol. Kinoshita and his group first cloned the *pig-a* gene, which was subsequently found to be mutated in all cases of PNH reported to date.[3-5]

Intravascular Hemolysis in PNH

The work of both Ham and Dacie in the 1930s first revealed that the hemolysis in PNH was due to the effect of a serum factor on abnormal PNH red cells. Rosse proceeded to show that this factor was complement that when activated led to the intravascular hemolysis of PNH red cells. The characteristic symptoms of PNH—abdominal pain, dysphagia, erectile failure and severe lethargy—can be attributed to the intense intravascular hemolysis and the resulting free plasma hemoglobin. This appears to be due to the absorption of nitric oxide by free hemoglobin and since nitric oxide is critical for smooth muscle function this results in the symptoms described (see below).

The functions of the GPI-linked antigens are extremely varied. At least two are important in the control of complement. Decay accelerating factor (DAF or CD55) controls the early part of the complement cascade by regulating the activity of the C3 and C5 convertases. Thus CD55 deficiency initially appeared to explain the sensitivity of PNH red cells to complement. However, the observation that individuals with inherited CD55 deficiency (Inab- phenotype) did not suffer from hemolysis proved that deficiency of CD55 does not cause the hemolysis in PNH. Membrane inhibitor of reactive lysis (MIRL or CD59) was identified in 1989 and is also GPI-linked. CD59 inhibits terminal complement by preventing the incorporation of C9 onto C5b-8 and therefore preventing the formation of the membrane attack complex (MAC). In 1990 an individual with inherited isolated deficiency of CD59 was described with many features similar to classic PNH, such as intravascular hemolysis with hemoglobinuria and thrombosis of the cerebral veins.[6] Thus CD59 deficiency is the abnormality that is responsible for the hemolysis and thrombosis classic of PNH.

Venous thrombosis in PNH

The most feared complication of PNH is venous thrombosis, which has a predilection for the intra-abdominal and cerebral veins. In two historical series of patients, approximately 50% experienced venous thrombosis at some time during their disease and a third of patients died as a result of thrombosis.[7,8] The risk of thrombosis is greater in patients from Europe and the United States than in patients from the Far East.[9] A possible explanation is that aplastic anemia (AA) is more prevalent in the Far East and therefore patients are more likely to have PNH/AA rather than hemolytic disease. Alternatively patients from different ethnic groups may have different additional inherited prothrombotic traits—however, no correlation between the inherited thrombophilia and thrombosis in PNH has been demonstrated.[10] The cause of the thrombotic tendency in PNH is not entirely clear. The overwhelming evidence implicates the GPI-deficient platelets, which are more easily activated by complement than normal platelets. Thus PNH platelets, which comprise the vast majority of platelets in almost all hemolytic patients, similar to the proportion of PNH neutrophils, undergo microvesiculation at far lower concentrations of activated complement leading to greater prothrombinase activity and to thrombus formation.[11] Alternative mechanisms have been suggested, such as deficiency of urokinase plasminogen activator receptor from PNH neutrophils or directly due to intravascular hemolysis, but the platelet abnormalities appear to be the most important factor.[11,12]

Nitric Oxide and the Symptoms of PNH

It is likely that the symptoms of PNH during a paroxysm, such as esophageal spasm and abdominal pain, are caused by smooth muscle dysfunction due to disturbances in the metabolism of nitric oxide (NO). These symptoms are identical to those observed when free hemoglobin was given to normal individuals during the early attempts to produce artificial blood and can be induced by the inhibition of NO synthetase in normal individuals. Haptoglobin efficiently removes free hemoglobin and is necessary because the function of hemoglobin depends upon its correct compartmentalization in red cells. Free hemoglobin, as is seen in hemolytic PNH, is an extremely efficient scavenger of NO with

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10^6 times greater affinity of the heme moiety for NO than that for oxygen. Thus intravascular hemolysis absorbs NO, disturbs smooth muscle function and causes the symptoms seen during a paroxysm.

Conventional Therapeutic Strategies in PNH

The principal conventional therapy for PNH is supportive care with transfusions as required and the treatment of complications, such as thrombosis, when they occur. The only curative strategy is allogeneic stem cell transplantation but this carries a considerable risk of mortality from the reported series[13] and, in view of the fact that a proportion of patients will eventually experience a spontaneous remission of PNH (occurs in approximately 15% of hemolytic patients)[7] and with the advent of potentially effective novel therapies, this should only be considered in selected cases, such as those with a syngeneic donor or with associated bone marrow failure. In these patients the indications for transplantation are similar to those for AA. Patients with cytopenias due to associated AA will often respond to immunosuppressive therapy with antilymphocyte globulin and/or cyclosporine. The occurrence of venous thrombosis affecting a major vessel is a life-threatening occurrence and the advent of reduced-intensity conditioning allogeneic stem cell transplantation, which carries a lower risk than conventional bone marrow transplantation (BMT), has led to this approach being used in some patients with life-threatening thromboses.

Diagnosis of PNH

Flow cytometry

The gold standard diagnostic test for PNH is the analysis of GPI-linked molecules on the surface of hematopoietic cells by flow cytometry (see **Figure 1**).[14] An unusual feature of flow cytometry for PNH diagnosis is that a positive test is defined by an absence of antigens rather than the aberrant expression of antigens as seen in most other flow cytometric applications. However in almost all PNH patients there is at least a small residuum of normal cells, which acts as an internal control to demonstrate that the staining and gating strategy are correct. The minimum requirement to diagnose PNH is the demonstration that a proportion of red cells are deficient in at least two different GPI-linked molecules. The proportion of GPI-linked deficient granulocytes is not affected by either hemolysis or transfusions and therefore gives the best estimate of the actual proportion of PNH hematopoiesis. In addition to establishing the diagnosis of PNH, flow cytometry also provides information regarding the severity of deficiency of the antigens from PNH red cells. PNH Type III red cells

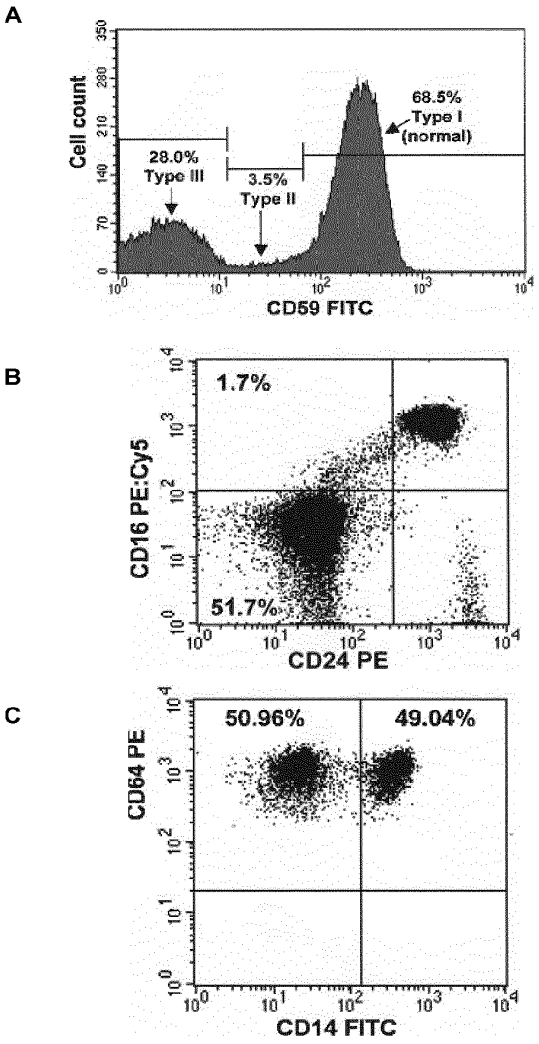


Figure 1. Flow cytometry to diagnose paroxysmal nocturnal hemoglobinuria (PNH) and to quantitate the clone.

A. Demonstrates a population comprising 28% of the red cells that are completely deficient in CD59 (and other glycosylphosphatidylinositol (GPI)–linked antigens) and a second abnormal population of partially deficient red cells comprising 3.5% of the total.

B. Demonstrates the lack of more than one GPI-linked antigen (CD16 and CD24) from the same patient's granulocytes.

C. Demonstrates that the proportion of PNH monocytes usually closely corresponds to the granulocyte analysis. (Courtesy of Dr. S.J. Richards)

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are completely deficient in GPI-linked molecules and are 15 to 25 times more sensitive to activated complement (compared to normal) whereas PNH Type II red cells have a partial deficiency (PNH Type II cells) and are only 3 to 5 times as sensitive. The size of the PNH clone and type of PNH cells are important determinants of the clinical presentation. For example, patients with hemolytic anemia and macroscopic hemoglobinuria will almost always have over 10% PNH Type III red cells and a majority, often over 95%, of PNH neutrophils. The risk of thrombosis is directly related to the proportion of PNH neutrophils, and this information has been used to stratify patients when deciding who should receive warfarin as primary prophylaxis against venous thrombosis.

Tests of complement sensitivity

Other tests, such as the demonstration that PNH red cells have an increased sensitivity to complement (the Ham test), are less sensitive than flow cytometry. These may be useful screening tests but can no longer be relied on to establish the diagnosis. The diagnosis should always be confirmed by flow cytometry. An alternative diagnostic approach is to utilize the toxin Aerolysin, which is produced by the bacteria *Aeromonas hydrophila* and binds to the GPI structure.[15] Aerolysin has been fluorescently labeled (FLAER), and therefore any nucleated cell that expresses GPI-anchored antigens is positive, whereas the GPI-deficient cells remain negative. The FLAER test is currently under investigation as a diagnostic test for PNH.

PNH Pathophysiology:

Relative Growth Advantage of the PNH Clone

PNH is a unique disorder in which an abnormal clone or small number of clones expand to replace almost the entire hematopoietic stem cell pool, but these clones do not have any "malignant" tendency in that they appear to be regulated in a normal manner with no tendency to metastasize beyond the normal hematopoietic compartment. Also GPI-deficient cells with *pig-a* mutations occur very frequently at low levels in normal individuals but do not expand in competition with the normal hematopoietic cells. Dacie first proposed that in order to develop PNH two things were required: first, the occurrence of a GPI-deficient clone arising in a multipotent hematopoietic stem cell; second, a second event that encourages the expansion of the PNH clone over the residual normal hematopoiesis—a relative growth advantage for the PNH cells.[16] The clue to this second event is the close relationship between aplastic anemia and PNH. It appears that normal hematopoiesis is suppressed by the immune system, presumably either directly or indirectly through one or more GPI-linked antigens, and therefore this attack spares the GPI-deficient PNH clone. Thus, in an environment where there is intense pressure for hematopoiesis (aplastic anemia), the PNH clone is driven to produce mature hematopoietic cells and expands to fill the void left by the aplastic process. The exact mechanism for the relative growth advantage of PNH cells remains unclear but is the subject of considerable scientific activity.

Novel Therapeutic Strategies in PNH

Prevention and treatment of thrombosis

The first thrombosis in a patient with PNH heralds a significant deterioration in the patient's health and a worsening prognosis. Reports of tissue plasminogen activator (tPA) for intra-abdominal venous thrombosis in PNH show that the thrombus can be cleared effectively in a proportion of patients.[17] The standard of care after established venous thrombosis in PNH is life-long full anticoagulation. In view of the high risk of thrombosis and the fact that patients have decreased quality of life following the first thrombosis, selected patients could be considered for warfarin as primary prophylaxis prior to the thrombosis. Hall et al[18] recently reported a retrospective analysis comparing 39 patients at high risk of venous thrombosis treated with warfarin as primary prophylaxis to 56 patients with similar sized clones who were not treated with warfarin. The incidence of thrombosis at 10 years in the group of patients not on warfarin was 36.5%, which was statistically significantly higher than the warfarin group in whom no patients had a thrombosis. However, 2 patients in the warfarin group had major hemorrhages, with 1 dying as a direct result of an intracranial bleed. Despite this being a retrospective analysis and the fact that the patients were not randomized the results suggest that primary prophylaxis may have a role (**Figures 2** and **3**). The data are compelling, particularly in view of the fact that because the patients in the warfarin group were selected for anticoagulation they would be expected to have at least as high a risk of thrombosis as the non-warfarin group. PNH patients with neutrophil clone sizes of over 50%, platelet counts above 100×10^9/L and no other contraindication for warfarin therapy should be considered for primary prophylaxis. There are no studies of antiplatelet drugs, such as aspirin or clopidogrel, in PNH.

Inhibition of the complement cascade

Complement is the principal effector of the innate immune system. Complement activation is a complex cascade leading to the production of anaphylatoxins,

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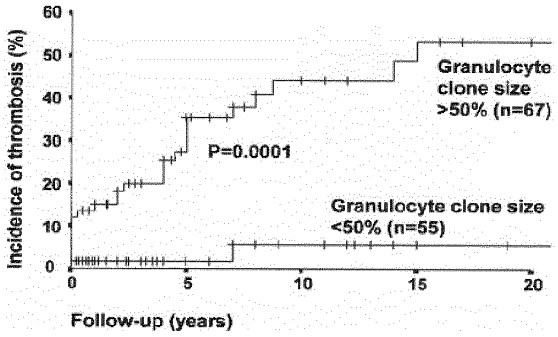


Figure 2. Effect of glycosylphosphatidylinositol (GPI)-deficient granulocyte clone size on incidence of venous thrombosis (primary prophylaxis patients excluded).

When primary prophylaxis patients are excluded, the 10 year cumulative incidence rate of thrombosis in patients with PNH granulocyte clone size of > 50% is 44%, compared with a thrombosis rate of 5.8% in those with clone size of < 50% (P < 0.01*).

* P value calculated with use of the log-rank test; Hall et al. Blood. 2003;102:3587-3591.

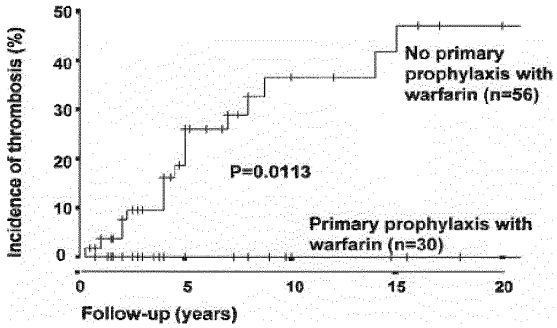


Figure 3. Effect of warfarin prophylaxis on venous thrombosis in patients with paroxysmal nocturnal hemoglobinuria (PNH) granulocyte clone sizes of > 50% (patients presenting with thrombosis excluded).

The 10-year cumulative incidence rate of venous thrombosis in patients with PNH granulocyte clones of > 50%, not presenting with thrombosis and not taking warfarin is 36.5%. In comparison, the current thrombosis rate is 0% in patients taking primary prophylaxis (P = 0.01*).

Thirty-two of the 39 patients on primary prophylaxis had granulocyte clone sizes > 50% and could therefore be included in this analysis. A further 2 of these patients were excluded because, having stopped warfarin (one through personal choice and one because of warfarin-associated hemorrhage), they went on to suffer venous thrombosis.

Time 0 was the time of presentation with PNH.

* P value calculated with use of the log-rank test; Hall et al. Blood. 2003;102:3587-3591.

chemotaxins and the membrane attack complex (MAC). Three pathways activate the cascade (see **Figure 4**): the classical (initiated by antigen/antibody complex), alternative (initiated by microbial membranes or immune complexes), and lectin pathways. These pathways all converge to cleave C5 into C5a, a potent anaphylatoxin, and C5b. C5b is the initial molecule of terminal complement and binds C6, then C7, and then C8. C5b-8 forms the scaffold for C9 molecules, which bind to each other to form the MAC. The MAC forms a pore in the cell membrane, which results in cell lysis. CD59 prevents the incorporation of C9 onto C5b-8. A remarkable feature of the complement cascade is that the MAC appears to be largely redundant in normal adults. Individuals with inherited deficiency of any of the complement molecules prior to C5 are vulnerable to both pyogenic organisms and to autoimmune disorders. In contrast, deficiency of any of the molecules after C5 remarkably has little phenotype. The only apparent se-

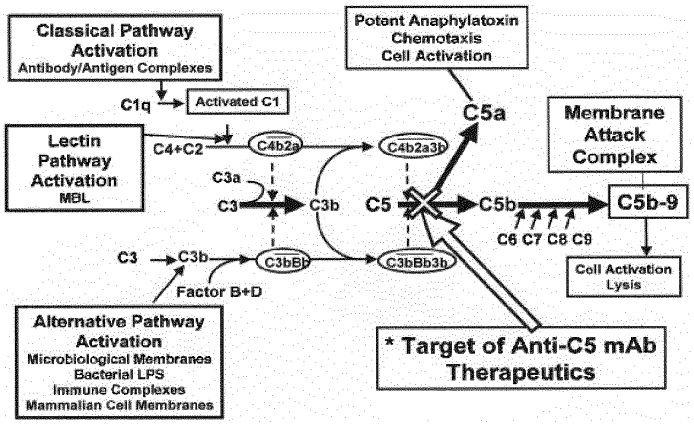


Figure 4. The complement cascade and eculizumab.

The complement cascade culminating in the production of the membrane attack complex that results in the lysis of the cell. The cleavage of C5 is the pivotal point of the pathway. Inherited deficiencies prior to C5 result in recurrent pyogenic infections and autoimmune disorders, whereas deficiencies after C5 have remarkably little effect except for an increased risk of infection by encapsulated organisms. The site of blockage of eculizumab is demonstrated.

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quela is an increased risk of infection by encapsulated organisms, namely *Haemophilus influenzae, Neisseria meningitides* and *N gonorrhoea*. In the case of *N meningitides,* although the deficient individuals have an increased risk of suffering infection they also have a significantly lower mortality from infection when compared to complement-replete individuals. C5 is a good therapeutic target, because blockade here would not only prevent the creation of MAC but would also prevent the release of the potent anaphylatoxin C5a. Eculizumab is a humanized chimeric antibody against C5 with a completely nonfunctional Fc domain. Eculizumab has a high affinity for C5 and thus when bound remains so until the complex is removed from the circulation. Recently a pilot study of eculizumab in 11 patients with transfusion-dependent PNH was reported.[19] The antibody was given at a dose of 600 mg every week for 4 weeks and then at a dose of 900 mg every other week. The drug is given by intravenous infusion over 30 minutes and was extremely well tolerated. The parameters of intravascular hemolysis, namely lactate dehydrogenase (LDH) and aspartate transaminase (AST), immediately fell to normal or near normal levels. The mean LDH prior to eculizumab was 3111 ± 598 IU/L (normal range 150–480) and this fell by the first week to 594 ± 32 IU/L. There was a significant improvement in the quality of life when comparing before the start of eculizumab and at week 12. There was also a significant reduction in mean transfusion requirement for the whole group from 2.1 units per month to 0.6 units per month. The benefit was most evident in patients with normal or near normal platelet counts and therefore no evidence of co-existent clinically significant marrow failure. There was also a complete cessation of hemoglobinuria immediately after commencing eculizumab. The proportion of PNH red cells in patients not on eculizumab is usually significantly lower than the proportion of PNH neutrophils because of the effect of transfusions and also the selective hemolysis of PNH red cells compared to their normal counterparts. The reduction or cessation of transfusions and control of hemolysis in patients on eculizumab leads to an increase in the proportion of PNH red cells toward the level of the PNH neutrophils. The median proportion of PNH type III red cells increased from 36.7% ± 5.9% prior to eculizumab to 59.2% ± 8.2% by week 12 of therapy. This raises the concern that stopping eculizumab for any reason might render the patient susceptible to a massive hemolytic attack and profound anemia. Two of the patients on eculizumab have had a breakthrough from complement blockade immediately prior to the next dose of eculizumab with a return of hemoglobinuria. Although both suffered hemolysis, the severity of the attack was not too extreme and in both the complement was reblocked immediately after the next dose of eculizumab and remained so on a slightly more frequent dosing regimen. Although eculizumab appears to almost completely stop the intravascular hemolysis, the patients' hemoglobin does not return to normal (it usually plateaus between 10 and 12 g/dL), the bilirubin and reticulocytes remain elevated and the haptoglobin initially becomes detectable but reverts to undetectable within a few weeks of the start of therapy in almost all patients. Thus a continuing low level of well-compensated extravascular hemolysis persists, suggesting that the hemolysis in PNH is not only due to terminal complement activity but that there is also a component of extravascular hemolysis due to complement activity prior to C5. Eculizumab appears to be a highly promising therapy for PNH and is now the subject of a Phase III randomized clinical trial.

Replacement of complement regulatory proteins on PNH cells

An alternative therapeutic strategy in PNH could be to replace CD59, the deficient complement regulatory protein. In the first instance a gene therapeutic[20] approach might appear attractive. However, introducing the *pig-a* gene into the PNH hematopoietic stem cell is far from trivial and would simply render the "corrected" cell as a target for the aplastic process that was the reason for the proliferation of the PNH clone. An alternative strategy would be to simply replace CD59 on the PNH red cell surface. It is impractical to extract GPI-linked CD59 from cell membranes as it is extremely tightly bound and does not re-attach well to cells in the presence of albumin. CD59 is functional when bound to the cell by alternative mechanisms, such as a transmembrane linkage, but again this approach is impractical.[21] Sah et al[22] have recently reported the use of an alternative artificial glycolipid anchor (Prodaptin) to anchor CD59 into the cell. Prodaptin-CD59 has been shown to effectively coat PNH cells in vitro and murine cells in vivo and to restore the cell's resistance to complement. This approach is attractive because the complement system is not blocked and the increased risk of *N meningitides,* which is seen in complement deficient individuals, is no longer an issue.

Global PNH Registry

In view of the infrequency of PNH it is extremely difficult to perform large or randomized trials of therapeutic interventions. In addition, the advent of complement inhibitors, such as eculizumab, or the routine use of prophylactic anticoagulation promises to alter the natural history of PNH. In fact, the approval process of

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eculizumab as a therapeutic agent will be on the basis of relatively small and short clinical trials. Thus few patients are likely to have received more than 3 years of eculizumab. It is possible that any complications of such a novel agent might take several years to develop. These factors have led to the recent development of a Global Registry for PNH. It is hoped that a large number of PNH patients will be registered on the Global PNH Registry in order that a more thorough insight into the effect of these changes on the natural history of PNH can be established (www.PNHregistry.org).

Conclusion

The understanding of the biology and natural history of PNH has altered dramatically over the last 10 years. This has facilitated major steps forward in our ability to diagnose the disorder and in our therapeutic strategies for PNH. There is now initial evidence that we may be able to prevent the most feared complication of PNH, namely thrombosis, with the use of primary prophylaxis with anticoagulants for selected patients. The recent description of a small pilot study of the complement inhibitor eculizumab offers the first promise that we may have a "targeted" therapy capable of controlling the hemolysis of PNH. Other novel approaches to therapy in PNH are also being explored. The development of the Global PNH Registry offers the potential to further our understanding and therefore therapy of PNH.

II. Fcγ Receptor Structure/Function and Role in Immune Complex–Mediated Autoimmune Disease

*Randall G. Worth, PhD, Brian A. Jones, PhD, and Alan D. Schreiber, MD**

Antigen recognition by cells of the immune system occurs via several mechanisms. One important family of receptors involved in the recognition of immunoglobulin (Ig)-coated particles and complexes are Fc receptors. Fc receptors recognize the Fc portion of Ig and are accordingly grouped into subfamilies. They are named depending upon which class of Ig they bind. The major Fc receptors are Fcγ receptors that bind IgG, FcαR that bind IgA, and FcεR that bind IgE.[1] Fc receptors are responsible for such functions as endocytosis, phagocytosis, granule release, reactive mediator release and cell activation/cytotoxicity. Fc receptors are found on specific cell types corresponding to their ability to recognize Ig. As such, Fcγ receptors are found primarily on neutrophils, macrophages and monocytes where they can detect and phagocytose IgG-coated particles, and on B lymphocytes.

Fc Receptor Subtypes and Expression Patterns

Fc receptors can be divided into two groups based on their signaling capability and structure. The first and most common type of Fc receptor is a multichain heterocomplex composed of a ligand-binding α-chain and one or more signal-transducing γ-chains. The second type of Fc receptor is a single-chain transmembrane receptor containing a signal-generating motif(s) in the cytoplasmic domain and, thus, not requiring another signal-transducing subunit.

Receptors for a specific isotype of IgG can vary in structure and can differ in ligand affinity and signaling ability.[1] Fcγ receptors are categorized into three classes: Fcγ RI, Fcγ RII, and Fcγ RIII (**Figure 5**; see Color Figures, page 514). Fcγ RI is a high affinity receptor that can be subdivided into three groups (A, B and C) that are encoded by three different genes. Fcγ RI is expressed on monocytes, macrophages, neutrophils and some dendritic cells. The receptor can be upregulated upon stimulation with interferon (IFN)-γ, tumor necrosis factor (TNF)-α or granulocyte colony-stimulating factor (G-CSF).[2,3] Similar to Fcγ RI, Fcγ RII has been divided into three families encoded by three genes, named A, B and C, which are of relatively low avidity for monomeric IgG but of high avidity for complexed IgG. Fcγ RII is expressed on various cell types and contributes to cell function based on the subclass of Fcγ RII. Fcγ RIIA is expressed on neutrophils, monocytes, macrophages, natural killer cells and platelets. Fcγ RIIB is expressed in two forms following alternative splicing, forming the Fcγ RIIB1 and Fcγ RIIB2 isoforms, and is limited in expression to B cells, neutrophils, macrophages and monocytes. Fcγ RIIC is expressed on human natural killer cells. Fcγ RIII is divided into two families, the transmembrane form Fcγ RIIIa and the GPI-linked Fcγ RIIIB found on neutrophils and natural killer cells. Fcγ RIIIA is found on monocytes and macrophages, and has also been shown to be expressed and upregulated on eosinophils.[4]

Crystal Structure/Affinity for IgG

Both recent immunotherapy using IgG antibodies as well as certain antibody-mediated autoimmune diseases utilize Fcγ receptors for the clearance of IgG-coated cells from circulation. While this may be beneficial in immunotherapy, it is detrimental in autoimmune disease. Understanding the interaction between the Fc domain of IgG and the Fcγ receptors is necessary in order to develop better therapy. There are two extracellular

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immunoglobulin-like domains for Fcγ RII and Fcγ RIII, while Fcγ RI has three immunoglobulin-like domains. The third domain of Fcγ RI has been attributed to the high affinity binding of IgG by this receptor; however, since the extracellular region of Fcγ RI has not been crystallized this has not been confirmed experimentally. The crystal structures of the extracellular region of Fcγ RIIA, Fcγ RIIB, Fcγ RIIIA, and Fcγ RIIIB have been determined.[5] The two immunoglobulin-like domains of these receptors, D1 (membrane distal) and D2 (membrane proximal), are bent at an approximately 50–70° angle relative to each other depending on the algorithm used. All Fcγ receptors are structurally very homologous, with their domains oriented in a steep angle to each other. While most studies have shown Fcγ receptors existing in the membrane as a monomer, some conflicting reports have suggested that Fcγ RIIa forms a homodimer, with a 2:1 stoichiometry of receptor to IgG.[6] However, the crystallization of Fcγ RIIIa in complex with the Fc region of IgG1 suggests a 1:1 stoichiometry of receptor to IgG.[7] It is believed that the other Fcγ receptors display similar binding stoichiometry due to their high degree of homology.[8] Also, as a dimer is able to crosslink Fc receptors to induce signaling, it may not be efficient for cells to be able to induce signaling by binding a single IgG molecule. However, the conformation of both bound and unbound Fcγ RIIa remains a matter of debate.

The binding sites of the Fcγ receptors for IgG are fairly well established. From the crystallization studies as well as some mutational studies, the D2, or membrane proximal, domain of the Fcγ receptor is important for the binding of IgG. The binding of an IgG by the receptor induces approximately a 10° shift in the angle between the D1 and D2 domains.[7] The Fcγ receptor binds asymmetrically to an IgG molecule in the lower hinge region of the Cγ 2 domains of an IgG heavy chain. The asymmetrical binding may result from a conformational change in one of the Cγ 2 domains of IgG in relation to the other. A factor that could affect the IgG binding ability of the Fcγ receptor is the glycosylation state of the receptor and IgG. One example of this is found in the crystal structure of Fcγ RIIIa/IgG-Fc, whereby the glycosylation of the IgG1 at 297N was not involved in the binding site. However, two groups have shown that glycosylation of an IgG may be involved with the formation of a stable Cγ 2-Cγ 2 interaction of the two heavy chains of IgG1. When the carbohydrates are removed, there is decreased binding of IgG1 to both Fcγ RIII and Fcγ RIIb (**Table 1**).[9,10] The glycosylation of Fcγ receptors can also affect binding of IgG. Expression of Fcγ RIIIa on different cell types leads to different glycosylation patterns. The differences in the ability to bind monomeric IgG by Fcγ RIIIa have been attributed to the cell type specific glycosylation of the receptor.[11] Also, one particular glycosylation site in the D2 domain of Fcγ RIIIa and Fcγ RIIIb, 163N, is located in the IgG-binding site. Receptors lacking glycosylation at this amino acid have an increased affinity for IgG.[12] Thus, the glycosylation state of the receptor, which may vary in cases of inflammation, could affect the binding of IgG.

Another example of affinity modulation is through polymorphism. As such, a polymorphism in Fcγ RIIa (131R or 131H) affects the binding affinity of Fcγ RIIa for human IgG2; Fcγ RIIa (131H) binds IgG2 but Fcγ RIIa (131R) does not.[13] Results of a recent meta-analysis show that the Fcγ RIIa polymorphism is important for genetic susceptibility for systemic lupus erythematosus (SLE).[14] There is also a polymorphism in Fcγ RIIIa (158V or 158F) that affects binding to human IgG1 and human IgG3. NK cells from patients with Fcγ RIIIa (158F) are unable to bind IgG3 and bind IgG1 with lower affinity, whereas NK cells from patients with Fcγ RIIIa (158V) are able to do so.[15] In addition, a triallelic polymorphism in Fcγ RIIIa (48 H, R, or L) was originally reported to affect the binding affinity for human IgG. Subsequently, this difference in binding affinity was determined to be due to the polymorphism at amino acid 158.[16] This is consistent with structural studies as 48 H/R/L is located in the D1 immunoglobulin-like domain whereas 158 V/F is located in the D2, IgG-binding domain. Finally, in Fcγ RIIIb, the GPI-linked Fcγ receptor expressed on neutrophils, there are two alleles (NA1 and NA2). These two alleles have minimal differences in binding affinity for IgG, but patients homozygous for the NA1 allele have a higher level of phagocytosis of IgG-coated particles.[17]

Table 1. Relative affinities of IgG subclasses for various Fcγ receptors.

	Fcγ RI	Fcγ RIIa	Fcγ RIIb	Fcγ RIII
Molecular Mass	70 kDa	40 kDa	40 kDa	50-80 kDa
IgG Subclass Specificity	1 ≥ 3 > 4 > 2	3 > 1 > 2 > 4	3 ≥ 1 > 4 > 2	1 = 3 > 2 = 4
IgG Affinity	10^{-7}–10^{-9} M	> 10^{-7} M	> 10^{-7} M	> 2 × 10^{-7} M

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Signal Transduction from Fcγ Receptors

Given that most Fcγ receptors are transmembrane proteins, the first step of receptor activation and subsequent phagocytosis is binding of IgG containing complexes to the receptor extracellular domain. Binding and phagocytosis by human Fcγ receptors has been observed both in normal human leukocytes and in model systems such as transfected COS-1 cells.[18] Initial Fcγ receptor activation takes place upon ligand binding to the extracellular domain. Since each Fcγ receptor has a structurally distinct extracellular domain, the receptor binds to IgG with varying affinity, as shown in **Table 1**.

Fcγ receptors traditionally signal through an immunoreceptor tyrosine–based activation motif (ITAM) or through inhibitory residues found in an immunoreceptor tyrosine–based inhibitory motif (ITIM).[19,20] ITIMs are composed of an I/VxYxxL/I sequence that recruits tyrosine phosphatases to the signaling complex. Therefore, the presence of ITIM-bearing receptors imposes a negative effect. As such, the presence of Fcγ RIIB, an ITIM-bearing receptor, inhibits phagocytic signaling mediated by activating Fcγ receptors.[21]

The classic ITAM motif consists of two YxxL sequences separated by 7 amino acids.[22] Fcγ receptor ITAM sequences in the Fc receptor associated γ -chain abide by this structure. However, the cytoplasmic domain of Fcγ RIIa contains an ITAM-like domain. This ITAM-like domain contains the two YxxL motifs but a spacer sequence containing 12 amino acids instead of the usual 7.[20] ITAM tyrosine residues have been shown to be crucial for mediating the phagocytic response. When either of the ITAM tyrosines are mutated to phenylalanine, phagocytosis is inhibited by ~70%–80%. However, if both tyrosine residues are mutated phagocytosis is abolished.[23-25]

It has been proposed that ITAM tyrosines are phosphorylated by Src family kinases after crosslinking. Several members of the Src family have been shown to associate with specific Fcγ receptors. However, which Src kinase is responsible for phosphorylation of a specific Fc receptor is not established. Studies have been somewhat inconclusive in elucidating which kinase is responsible for phagocytic signaling through each Fcγ receptor. An example of these observations can be found in knockout experiments where phagocytosis is not abolished in Hck, Lyn, or Fgr single knockouts.[26] In addition, in triple knockout mice, phagocytosis by macrophages and neutrophils is still partially intact, suggesting other kinases may play a redundant role in phagocytosis.[27]

Phosphorylation of ITAM tyrosines creates Src homology 2 (SH2)-binding sites required for signal transduction involving other members of the tyrosine kinase family.[28-30] Most importantly, Syk tyrosine kinase, which contains two SH2-binding sites, is recruited to phosphorylated ITAM residues (**Figure 6**). Fcγ R phagocytosis is dependent upon Syk signaling, and has been observed to be enhanced upon the overexpression of Syk in model systems.[31] In addition, when Syk kinase expression is inhibited with antisense oligonucleotides both in vitro and in vivo, phagocytosis and inflammation are abolished.[32,33]

SH2-containing proteins are important in signaling complexes. For example, recent studies have emphasized the role of adaptor proteins in phagocytic signaling. Adaptor molecules such as SLP-76, LAT, Cbl and others have the ability to recruit SH2-containing proteins to sig-

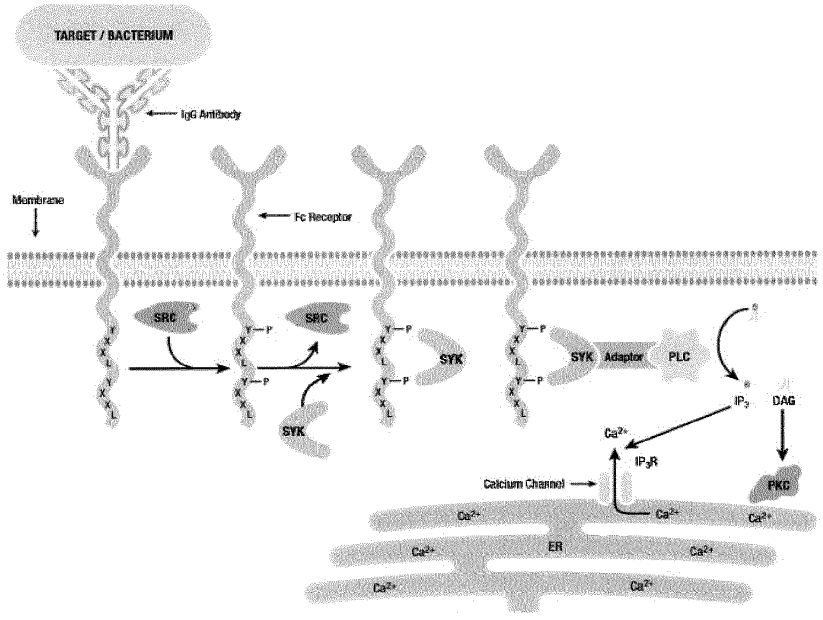


Figure 6. Proximal signal transduction by activating Fcγ receptors.

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naling complexes, notably in lipid rafts (**Figure 6**). These adaptor proteins play a significant role in recruiting such secondary signaling molecules as phospholipase C (PLC), Grb2, Shc and others. The ability for adaptor molecules to recruit proteins to the site of signal propagation by Fcγ receptors is important for efficiently triggering the downstream signaling leading to target internalization and mediator release.

Fcγ Receptor–Associated Diseases

As described above, the isotype of IgG determines the class of Fcγ receptor ligated and the nature of the signal propagated. Of particular interest are the myriad of diseases based upon IgG-Fcγ receptor interaction leading to inflammation, phagocytosis and endocytosis. Two particular diseases mediated by Fcγ receptors are immune thrombocytopenic purpura (ITP) and autoimmune hemolytic anemia (AIHA). Both of these diseases are caused by production of self-reactive antibodies against either platelet antigens or erythrocyte antigens. ITP is mediated by production of IgG against one or more antigens exposed on platelets. Specifically, antibodies reactive to platelet GPIIb-IIIa, GPIb-IX, GPIb and GPIIIa among others have been shown to be potentiators of ITP.[34] AIHA has been shown to be due in large part to self-reactive antibodies against erythrocyte Band 3, an ion transporter found in erythrocyte membranes also shown to be involved in erythrocyte senescence. Both of these diseases are mediated by Fcγ receptors found on phagocytes (macrophages) as part of the reticuloendothelial system (RES) in the spleen and liver and may lead to thrombocytopenia or anemia, respectively.[34]

Various observations by multiple laboratories have determined the role of Fcγ receptors in these diseases most conclusively in murine models lacking the common γ-chain (γ KO). Experimentally induced AIHA and ITP are markedly decreased in mice lacking the γ-chain.[35] Additionally, administration of monoclonal antibody 2.4G2, which binds to and blocks mouse FcγRII and FcγRIII, allows a rapid recovery after induction of AIHA or ITP. Similarily, administration of GM-CSF has been shown by various groups to accelerate AIHA potentially due to upregulation of FcγRI.[36] These reports show that altering the balance of stimulatory to inhibitory Fcγ receptors has a marked effect on disease progression and susceptibility. As such, viral infection has been shown to stimulate or increase susceptibility to both AIHA and ITP. However, the mechanism(s) by which viral infection does so has yet to be fully elucidated. Potential scenarios of viral infection that could lead to this outcome include stimulated production of IFNγ during viral infection that causes upregulation of FcγRI. Alternatively, viral infection may cause a change in the expression pattern of Fcγ receptors due to transcriptional activation or other mechanisms. For example, childhood ITP has been described after infection with varicella zoster virus (VZV), causing molecular mimicry of antibodies against VZV to crossreact with platelet antigens, thus causing ITP.[37] ITP in some patients has been shown to be due to production of predominantly self-reactive IgG1.[38]

Therapy for ITP or AIHA

Treatment for ITP and AIHA has recently been reviewed elsewhere.[39,40] Many of the therapies used involve altering Fcγ receptor expression or phagocytosis. One of the first treatments is the administration of glucocorticoids. Prednisone and dexamethasone have been shown to downregulate Fcγ receptor expression, thus potentially decreasing the phagocytosis of IgG-coated particles. Other treatments involve administration of anti-D IgG to Rh-D positive patients. The rationale behind this treatment is that the IgG will coat erythrocytes with subsequent binding by Fcγ receptors. Binding of the IgG-coated red blood cells will prevent the binding and phagocytosis of the IgG-coated platelets. However, self-limited hemolytic anemia is to be expected after anti-D treatment.

High-dose intravenous immunoglobulin (IVIG) is also used as a treatment for both ITP and AIHA. However, the mechanism by which this inhibits the clearance of the IgG-coated platelets or IgG-coated red blood cells is still unknown. It is believed to work through a number of different mechanisms, which may include anti-idiotypic antibodies and decreased autoantibody production,[41] and, of interest here, its effects on Fcγ receptor function. One principal theory for a mechanism of IVIG's function is through a blockade of Fcγ receptors. At high local concentrations of IgG, the IVIG may either bind to the low-affinity Fcγ receptors, preventing the binding of IgG-coated cells, or outcompete binding of the high avidity immune complexes. Also, the possibility of IgG dimers existing in IVIG preparations would even further enhance this blockade.[42] Another possibility involves the inhibitory FcγRIIb, which is able to inhibit phagocytosis mediated by activating Fcγ receptors.[21] Injection of IVIG into a mouse model of ITP increased circulating platelet counts.[43] Also in this model was an increased percentage of non B cell splenocytes expressing FcγRIIb. Increased expression of this receptor relative to the expression of activating receptors could decrease the phagocytosis of IgG-coated platelets. The mechanism for the IVIG-mediated increase in Fcγ receptor–expressing cells is still unknown.

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Summary
Fcγ receptors are integral for the removal of IgG-coated cells from the circulation. In autoimmune diseases such as autoimmune hemolytic anemia and immune thrombocytopenia the removal of the IgG-coated erythrocytes or IgG-coated platelets is detrimental to a patient. Much is known regarding the structure and signaling properties of the Fcγ receptor family. As the two autoimmune diseases mentioned involve phagocytosis of IgG-coated cells, interference at either the Fcγ receptor binding of these complexes or interference of the phagocytosis process could possibly improve on the current treatments available for these disorders. Understanding the crystal structure of the Fcγ receptors may lead to the development of molecules that inhibit the binding to an IgG-coated cell. Small molecules might inhibit Fcγ receptor signaling at many different steps leading to phagocytosis. Finally, treatment to decrease expression of the activating Fcγ receptor or increasing expression of the inhibitory Fcγ receptor may also prove to be an effective therapy.

III. Cold-Induced Immune Hemolytic Anemia

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The antibodies that cause immune hemolytic anemia can be classified by isotype (IgG, IgM, or IgA) and by the temperature at which they react maximally with the antigen on the red cell: warm reacting if that temperature is 37°, cold reacting if that temperature is less. Much of the pathophysiology and many of the clinical manifestations are determined by these characteristics. For the most part, the isotype and the temperature of reaction coincide: most frequently, cold-reacting antibodies are IgM and warm-reacting antibodies are IgG, but important exceptions exist. Two clinical entities due to cold-reacting antibodies are defined by differences in isotype: cold agglutinin disease is caused by IgM antibodies and paroxysmal cold hemoglobinuria by IgG antibodies.

The isotype is important because the isotypic characteristics of the antibody direct the mechanisms of destruction. As described in Section II, phagocytic cells have receptors specific for IgG molecules of specific subtypes (IgG1, IgG2, and IgG3) but not for IgM molecules; therefore, Fc-receptor–induced destructive processes apply only when IgG molecules are attached to the red cell in the presence of the phagocytic cells. On the other hand, IgM antibodies fix complement much more readily than IgG molecules because the two necessary attachment sites for C1q are present on a single molecule; therefore, complement plays a primary role in the destruction of the red cells by these antibodies.[1,2]

Why Are Cold-Reacting Antibodies Cold Reacting?

The interaction of antigen and antibody is reversible and the degree of attachment depends upon the forces interacting between the two. For all cold-reacting antibodies, the antigen with which they react is polysaccharide or the polysaccharide parts of glycoproteins. In the case of cold agglutlinins, the antigen is one of the following: the straight-chain paragloboside basic to the expression of the ABH antigens (the i antigen), a branched version of that molecule (the I antigen), polysaccharides resident on glycoproteins (Pr antigens), and rare sialylated polysaccharides. Since the branching enzyme responsible for the I antigen is not activated until after birth, cord cells (and the cells of individuals genetically lacking the branching enzyme) express the i antigen whereas the red cells of adult express the I antigen.

The interaction of antibody and polysaccharide is dependent upon weak forces that are easily disrupted by molecular activity that occurs at higher temperatures. The cold agglutinins of anti-I or anti-i specificity are strikingly similar to one another in the structure of the antigen binding site. These antibodies all react with a monoclonal antibody that identifies the product of the VH4-34 (VH4-21) gene segment.[3] Other antibodies (monoclonal anti-Rh system antibodies, etc.) have been shown to use this same gene segment for the variable portion of the heavy chain and many of them also have cold agglutinin activity against the I/i antigens.[4] The characteristic that leads to such activity is a relative hydrophobicity, and it has been shown by detailed studies that a hydrophobic patch in framework region 1 contributed to by two β-strands is important in binding to the polysaccharide antigen and that this binding is modified by sequences in the complementarity-determining region H 3.[5] (This specific structure does not pertain to cold agglutinins of other specificity, and the reason for their specificity and reactivity, while probably similar, is unknown.)

How Do Cold-Reacting Antibodies Arise?

Cold agglutinins arise in two settings, both perhaps due in part to the fact that B cells utilizing the VH4-34 gene segment normally represent a relatively large percentage of the population (6%–13% of all mature B cells). These cells probably account for the low titers (< 1/10) of cold agglutinin that can be found in the serum of normal individuals. Oligoclonal antibodies, usually in

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elevated titers, appear routinely in moderate titers that are not sufficient to cause significant hemolysis in infectious mononucleosis (with anti-i specificity) and mycoplasma (with anti-I specificity) infections and less commonly in cases of cytomegalovirus, chicken pox, etc. Monoclonal cold-reacting antibodies are an expression of paraneoplasia (benign monoclonal gammopathy) or immunocyte neoplasia including chronic lymphocytic leukemia (CLL) and a variety of lymphomas.[6] Trisomy (complete or partial) of chromosome 3 has been recorded in patients in whom cold agglutinin disease progressed to a lymphoproliferative disorder.[7] The titer of the antibody is, in all cases, a relative measure of the number of abnormal cells.

The Donath-Landsteiner antibody of paroxysmal cold hemoglobinuria (PCH) formerly arose as a cross-reacting antibody to an antigen on *Treponema pallidum* (or so it is thought) and was frequently seen in secondary and tertiary syphilis; this is now rare. It is most commonly encountered in children as a response to viral illness or immunization, much like immune thrombocytopenia in this group; in these cases, it does not persist, but the clinical syndrome it produces may be severe enough to cause death. In adults, it is usually an autoimmune antibody and may be associated with other evidences of autoimmunity.[8]

Diagnosis of Diseases Due to Cold-Reacting Antibodies

Cold agglutinins are readily detected as, being IgM, they agglutinate red cells directly. Three characteristics should be determined: specificity, titer, and thermal amplitude (the highest temperature at which the antibody reacts with red cells). These are readily done with standard techniques.

The detection of Donath-Landsteiner antibodies is much more problematical. Direct agglutination is often present but in relatively low titers (usually less than 1/64). The direct antiglobulin test (direct Coombs' test) with anti-IgG is negative as the antibody elutes from the cells during their preparation. The indirect antiglobulin test is usually ineffective as well but occasionally is positive.[9] The test attributed to Donath and Landsteiner (the bithermic hemolysis of normal red cells) is positive only when the titer in the serum is relatively high. The sensitivity of the test can be increased greatly by the use of red cells from patients with PNH, which are more sensitive to the hemolytic action of complement. The best technique, not generally available, is the use of radiolabeled anti-IgG, which is affixed at 4°C but removed by raising the temperature to 37°C.[10] By using this technique, we were able to identify two patients not detected by the PNH variant of the Donath-Landsteiner test. Many patients with unexplained immune hemolytic anemia, often with a negative direct antiglobulin test, probably have undiagnosed PCH.

Why Is the Degree of Hemolysis So Different Among Patients?

The degree of hemolysis varies among patients because the characteristics of the antibodies responsible for it are so highly variable.[11] In the case of cold agglutinins, the amount of antibody (roughly measured by the titer) and the affinity of the antibody (roughly measured by its thermal amplitude) are the most important determinants of hemolysis. These should be measured both in saline and with albumin added. Some antibodies are less hemolytic because they fix complement inefficiently; these antibodies are usually cryoprecipitable and the effect may have to do with structural changes that result in that characteristic.[12] Some antibodies are not inhibited by the residual C3 on the red cell surface, as most are, and this results in increased hemolysis. Although most antibodies are pentameric IgM, some patients produce considerable quantities of hexameric IgM, which is more efficient in fixing complement.[13]

The same general principles of titer and thermal amplitude apply to Donath-Landsteiner antibodies. Several explanations have been offered to explain why these IgG antibodies result in intravascular hemolysis when the supposedly more efficient IgM antibodies generally do not. Certainly, the agglutination effected by IgM antibodies inhibits the fixation of complement components in vitro, but how much of a role this plays in vivo is unclear. D-L antibodies are able to fix C4 (but not C3) in the cold phase, but again the in vivo importance of this is questionable. The antigens of the D-L antibodies are said to lie closer to the membrane surface, thus facilitating the fixation of complement. In fact, the reason for the greater lysis of D-L antibodies compared to cold agglutinins is not clear.

What Is the Treatment of Diseases Due to Cold-Reacting Antibodies?

A primary treatment of any syndrome of cold-reacting antibodies is keeping the patient warm. This sometimes involves discomfort and is difficult for many patients to maintain. Where possible, avoidance of winter by migration is advisable.

In the case of IgM cold agglutinins, antibody production is not suppressed by prednisone and it should be used only when the simultaneous presence of IgG antibodies is found. Antibody production can sometimes be suppressed by chemotherapy, but the rate of proliferation of benign clones is so small that this is

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often ineffective; obviously, if malignancy is present, its treatment will treat the cold agglutinin problem. More recently, the use of anti-CD20 (rituximab) has found some success[14] as has the use of fludarabine.[15] Some patients appear to benefit by small to moderate doses of erythropoietin (personal observation). Plasmapheresis can be used to remove antibody temporarily but is difficult to maintain for chronic treatment; great caution is required to be sure that the blood does not get cold during the procedure.[16]

The treatment of PCH is like that of any other autoimmune hemolytic anemia due to IgG, except that splenectomy has no place in the regimen as the spleen is not involved in hemolysis. Prednisone, chemotherapy, and anti-CD20 treatment have all been used.

The hemolysis in the hemolytic anemia of cold-reacting antibodies is entirely dependent upon the activation and fixation of complement. In the future, the use of anti-C5 reagents, as described in Section I, or other complement modifying measures should provide relief of the most taxing symptom.

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Eculizumab

Humanized Anti-C5 Monoclonal Antibody

Prop Inn; USAN

h5G1.1
5G1.1

Immunoglobulin, anti-(human complement C5 α-chain) (human-mouse monoclonal 5G1.1 heavy chain), disulfide with human-mouse monoclonal 5G1.1 light chain, dimer

CAS: 219685-50-4

EN: 239304

Abstract

Eculizumab (5G1.1) is a recombinant, fully humanized monoclonal antibody that inhibits the activation of terminal complement components. Eculizumab binds specifically to the terminal human complement protein C5, preventing the release of the inflammatory mediator C5a and the formation of C5b-9 by inhibiting the cleavage of human complement C5 to C5a and C5b. Preclinical studies have demonstrated that the antibody has high binding affinity for C5, blocks the formation of C5a and C5b-9, and protects mammalian cells from C5b-9-mediated damage. Favorable clinical results have been reported in patients with membranous nephritis, rheumatoid arthritis and paroxysmal nocturnal hemoglobinuria (PNH) and the antibody is in phase II clinical development for these disorders; phase I trials have also been conducted in dermatomyositis.

Introduction

There is increasing evidence that activated components of the complement system contribute significantly to the pathogenesis of a large number of acute and chronic inflammatory diseases including rheumatoid arthritis, atherosclerosis, acute myocardial infarction and vascular injury after cardiopulmonary bypass (1-5). Furthermore, complement plays a significant role in the pathogenesis of paroxysmal nocturnal hemoglobinuria (PNH), characterized by intravascular hemolysis and venous thrombosis, and associated with aplastic anemia. Patients with PNH have a deficiency in CD59 and CD55 proteins on the surface of erythrocytes, platelets and other blood cells, which normally protect cells from complement-mediated injury (6-8). Patients with PNH experience fatigue, dysphagia, pain and hemoglobinuria, and the disease is associated with about 50% mortality (9).

Strategies that inhibit the damaging effects of complement on disease pathogenesis have therefore become a major focus in pharmacological research. In particular, inhibition of the complement cascade at C5, the cleavage of which results in the generation of the potent inflammatory mediators C5a and C5b-9 (10), while allowing the upstream generation of C3b, essential for combatting infections and preventing the development of autoimmune diseases (11), would appear to represent a promising approach.

Eculizumab is a recombinant, fully humanized monoclonal antibody that inhibits the activation of terminal complement components. Eculizumab binds specifically to the terminal complement protein C5 and prevents the release of the inflammatory mediator C5a and the formation of C5b-9 by inhibiting the cleavage of human complement C5 to C5a and C5b (12). The antibody is being developed for chronic indications including rheumatoid arthritis, membranous nephritis, dermatomyositis, lupus nephritis and paroxysmal nocturnal hemoglobinuria (13).

Pharmacological Actions

Eculizumab, as well as Fab and scFv fragments thereof, was evaluated for binding to complement and inhibition of complement activation *in vitro*. Eculizumab had similar binding affinity for human C5 compared to the murine antibody (IC_{50} ~ 2 nM), as determined in an ELISA, and it inhibited human serum complement-induced lysis of porcine aortic endothelial cells (PAEC) at the same concentration as the murine antibody (12).

Pharmacokinetics

A double-blind, randomized, placebo-controlled study was conducted with eculizumab (8 mg/kg i.v. once or

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twice monthly) to evaluate the pharmacokinetics/pharmacodynamics in 117 patients with idiopathic membranous nephropathy (mean creatinine clearance = 111 ml/min; 24-h proteinuria = 9 g; protein/creatinine ratio = 5; serum cholesterol = 270 mg/dl; hemoglobin = 12.0 g/dl; serum albumin = 2.8 g/dl). Preliminary results were reported for 11 patients. Mean steady-state trough serum eculizumab levels were 18 μg/ml in the twice-monthly treatment group and 8 μg/ml in the monthly treatment group. Mean steady-state mid-dose interval levels (1 week postdose on day 119 of the study) were 47 μg/ml for patients treated twice monthly and 35 μg/ml for those treated once monthly. Urine levels of eculizumab at midtrial and at the end of the trial were below the level of quantification in 8 patients, and 3 patients excreted 65-400 μg/24 h of eculizumab. All eculizumab-treated patients demonstrated < 10% of the hemolytic activity of placebo-treated patients at 1 h postinfusion. Serum eculizumab levels > 38 μg/ml inhibited hemolysis in all patients. Treatment with eculizumab was found to be safe and well tolerated, with no hypersensitivity reactions noted (14).

Pharmacokinetics and pharmacodynamics were also examined in an open-label pilot study in 11 transfusion-dependent patients with PNH who received treatment with eculizumab (600 mg weekly for 4 weeks, then 900 mg every 2 weeks for 2 months). Peak and trough serum levels of eculizumab were measured 1 h after the initial dose and then at weeks 3, 4, 6, 10 and 12, and in 10 patients eculizumab levels were > 35 μg/ml from 1 h after the first dose to the end of the study period. In 1 patient, eculizumab levels were > 35 μg/ml for the initial 10 weeks but < 35 μg/ml at week 12. In 10 patients, serum hemolytic activity was 20% or less for the entire treatment period, representing complete blockade, with only 1 patient showing increased hemolytic activity at week 12, which corresponded with a decline in eculizumab levels to < 35 μg/ml (15).

Clinical Studies

The safety and biological activity of a single i.v. dose of eculizumab (0.1-8 mg/kg) were investigated in 40 patients with mild to moderate rheumatoid arthritis. Mean C-reactive protein (CRP) levels were significantly reduced at day 7 compared to baseline after the highest dose and returned to baseline by day 14. Patients treated at this dose showed a trend for reductions in the signs and symptoms of rheumatoid arthritis. Doses of 2 mg/kg and above resulted in > 80% inhibition of human complement C5 for at least 10 h, and a single dose of 8 mg/kg provided > 80% inhibition for 10 days, with a return to baseline levels after 17 days. Eculizumab was safe and well tolerated across patient groups (16).

Another study examined the safety, efficacy and pharmacokinetics of eculizumab following multiple doses in 209 patients with rheumatoid arthritis. Patients were randomized to receive placebo, eculizumab 8 mg/kg every 2 weeks, eculizumab 8 mg/kg every week x 5 weeks, then every 2 weeks, or eculizumab 8 mg/kg every week x 5 weeks, then monthly, and evaluated both after 3 months of treatment and 3 months after terminating treatment. After 3 months of treatment, the induction/monthly group met the primary endpoint of improvement in ACR20 score (44% *vs.* 18% on placebo), and both induction groups met the secondary endpoint of decrease in CRP. Baseline C5b-9 levels were correlated with increased CRP levels and therefore systemic inflammation. Baseline C5b-9 levels > 200 mg/ml were associated with clinical response to eculizumab. In cases of elevated C5b-9, an ACR20 response was obtained in 9% of patients in the placebo group, 33% of patients in the twice-monthly eculizumab treatment group, 57% of patients in the induction/monthly treatment group and 50% of patients in the induction/twice-monthly treatment group. The induction regimens completely suppressed complement-mediated serum hemolytic activity up to week 4. The half-life of eculizumab was 10-14 days (17-20).

A randomized, double-blind, placebo-controlled phase IIb clinical trial evaluated the efficacy and safety of eculizumab in 368 adult patients with mild to moderate active rheumatoid arthritis undergoing treatment with methotrexate and/or leflunomide. The patients were enrolled at 69 sites in the U.S. and Canada and were randomized to receive placebo, twice-monthly eculizumab (600 mg i.v. weekly for 5 weeks, then 600 mg once every 2 weeks) or monthly eculizumab (600 mg i.v. weekly for 5 weeks, then 600 mg monthly). The primary endpoint was improvement in ACR20 score after 6 months of treatment. Monthly administration of eculizumab following an induction period was associated with a statistically significant, moderate improvement in the ACR20 score compared to placebo at 6 months. The percentage of patients who achieved an ACR20 response was 34%, 24% and 22%, respectively, on monthly eculizumab, twice-monthly eculizumab and placebo. Monthly therapy was also associated with evidence of a long-term antiinflammatory effect, with a significant reduction in erythrocyte sedimentation rate at 6 months. The incidence of adverse events was slightly higher among patients given eculizumab every 2 weeks, although the incidence of injection-site reactions remained low (21).

A double-blind, placebo-controlled phase I pilot study was performed to determine the safety and tolerability of eculizumab (8 mg/kg weekly for 5 weeks, then once every 2 weeks) in 10 patients with dermatomyositis compared with placebo (n=3). After 2 months of treatment, results showed moderate improvements in skin score, physician global score and manual muscle test in the eculizumab group compared with controls. The placebo group showed deterioration of physician global score, but improvements in skin score and manual muscle test compared to baseline. Disease intensity measured by magnetic resonance imaging (MRI) and various parameters on skin biopsy were also improved in the treatment group compared with placebo. Eculizumab was safe and well

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tolerated, with no significant differences in adverse events between treatment groups (22).

Alexion has sponsored phase II trials in patients with membranous nephritis, with results showing good tolerance. One trial did not reach the primary clinical efficacy endpoint of reduction in proteinuria after 4 months, while in the other trial an increase in remission rate and significant improvements in proteinuria, hyperlipidemia, hypertriglyceridemia and hypoalbuminemia were obtained after 12 months. The company also began a phase II study in lupus nephritis several years ago following the observation of positive effects on proteinuria in a study in patients with systemic lupus erythematosus (13, 23).

Further results regarding efficacy and safety were reported from the open-label pilot study in 11 transfusion-dependent patients with PNH treated with eculizumab (600 mg weekly for 4 weeks, then 900 mg every 2 weeks for 2 months). After 3 months of initial treatment, all patients were enrolled into an open-label extension study and went on to receive eculizumab 900 mg every 2 weeks for over a year. In the 12 months prior to entrance into the study, patients had received over 4 red blood cell transfusions, had detectable glycosylphosphatidylinositol (GPI)-deficient hematopoietic clone, and had a negative throat culture for *Neisseria meningitidis* and *Neisseria gonorrhoeae*. After the first 3 months of treatment, serum hemolytic activity was completely inhibited in the 10 patients who showed serum trough levels of eculizumab above 35 μg/ml; quality-of-life markers were also significantly improved, transfusion rates decreased and serum levels of lactate dehydrogenase (LDH), an indicator of hemolysis, were significantly reduced. Increased levels of PNH type III erythrocytes were thought to be due to prolongation of PNH red cell survival and confirmed that treatment with eculizumab inhibits complement-mediated hemolysis. The proportion of PNH neutrophils, monocytes and platelets did not change. The reduction in transfusions was greatest for patients (n=7) without thrombocytopenia. Biochemical markers of hemolysis showed dramatic improvement 1 week after beginning treatment with eculizumab. The most common adverse events were headache and upper respiratory tract infections, followed by influenza-like symptoms, rigors, dizziness, nausea, nasal congestion and joint aches. No episodes of thrombosis were reported. No adverse events were considered to be drug-related and although 2 patients had serious adverse events, all patients completed the 12-week study (15, 24-26).

Reports of the cumulative results after 6 and 12 months of eculizumab treatment showed that patients with purely hemolytic disease (macroscopic hemoglobinuria with a normal platelet count; n=6) had the best response to treatment, with type III erythrocytes increased from 39% to 67%, median transfusions reduced from 2.0 units/patient/month to 0 units/patient/month, and suppression of hemolysis. Four patients who did not become transfusion-independent were treated with additional subcutaneous erythropoietin with some improvement. Two patients reported breakthroughs in complement activity, demonstrated by increased lactate dehydrogenase levels, hemoglobinuria and symptoms. These symptoms resolved immediately after the next dose of eculizumab and patients were managed by reducing the dosing interval. The most commonly reported adverse events after 12 months of eculizumab treatment were flu-like symptoms, sore throat, headache and upper respiratory infection. Four serious adverse events were reported, but there were no study discontinuations as a result of adverse events and all patients elected to continue eculizumab treatment. It was concluded that eculizumab was well tolerated, decreased hemolysis and transfusion requirements, and improved symptoms of PNH for over 12 months (27, 28).

Conclusions

Eculizumab is a promising new monoclonal antibody that binds to human complement C5 and inhibits its cleavage by C5 convertase. Eculizumab has been granted orphan drug status for the treatment of PNH in both the U.S. and Europe. A pilot program has been completed in patients with PNH and plans are being finalized with the FDA for the next stage of development. Pilot programs have also been completed in patients with dermatomyositis and eculizumab is in phase II trials for rheumatoid arthritis and membranous nephritis.

Source

Alexion Pharmaceuticals, Inc. (US).

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CLINICS

Cumulative results from an ongoing extension study showed that Alexion Pharmaceuticals Inc.'s eculizumab reduced hemolysis and the need for transfusions in patients with paroxysmal nocturnal hemoglobinuria (PNH). Eleven patients were treated in an initial, 12-week pilot study, and all elected to participate in two consecutive one-year extension studies. Maintenance doses of 900 mg eculizumab every 12-14 days were sufficient to completely and consistently block complement activity in all patients, as evidenced by the lack of hemolysis. The significant reductions in red blood cell destruction seen in the pilot study were maintained throughout the extension phases. The mean and median transfusion rates decreased from 2.1 and 1.8 units/patient/month, respectively, during the 12 months prior to eculizumab therapy to 0.4 and 0.3 units/patient/month, respectively, through the 23- to 26-month treatment period. These reductions were significant. Among 11 patients with aplastic PNH, seven have been transfusion independent for at least the last six months of the study period following treatment with eculizumab alone or in combination with erythropoeitin. **Details:** Leonard Bell, CEO, Alexion Pharmaceuticals Inc., 352 Knotter Dr., Cheshire, CT 06410. Ph: (203) 272-2596.

Novadel Pharma Inc. began a pilot feasibility trial of a lingual spray version of zolpidem, a drug for treating insomnia. The study will test the pharmacokinetics of a range of doses of the drug. If it is successful, Novadel intends to file an investigational new drug application to commence an abbreviated clinical development program designed to support a 505(b)(2) submission with the FDA. The product utilizes the company's patented lingual spray drug delivery technology, which is intended to provide for more rapid onset of therapeutic activity. **Details:** Barry Cohen, VP bus. & new prod. dvlpt., Novadel Pharma Inc., 25 Minneakoning Rd., Flemington, NJ 08822. Ph: (908) 782-3431, ext. 2160.

Baxter International Inc. has suspended enrollment in its Phase II/III clinical trial of its Preflucel influenza vaccine, currently underway in Europe. Preliminary data from the study showed a higher than expected rate of mild fever and associated symptoms in subjects who received the vaccine. The incidence of fever and associated symptoms was also higher than that seen with other vaccines available on the market. Baxter has put further clinical studies, which were planned to begin in 2005, on hold until analysis of data from this study is complete. **Details:** Deborah Spak, Baxter International Inc., One Baxter Pkwy., Deerfield, IL 60015-4625 . Ph: (847) 948-2349.

Intercell AG and Scigen Ltd. will collaborate on the development of a new therapeutic vaccine for hepatitis B virus (HBV). The proposed vaccine will combine Scigen's already licensed PreS1-, PreS2- hepatitis B virus antigen (Sci-B-Vac) with Intercell's novel synthetic Immunizer IC 31 as an adjuvant to induce the type of immune response needed to overcome the infection. Under terms of the agreement, Intercell will formulate the vaccine for clinical development and both parties will collaborate in the development and commercialization of the product. The two companies will share development costs, resulting in joint ownership of any future product. Further financial details were not disclosed. **Details:** Katharina Wieser, Intercell AG, Campus Vienna Biocenter 2, 1030 Vienna, Austria. Ph: 44 0 20 7404 5959. Email: kwieser@intercell.com. Mark Compton, mng. dir. & CEO, Scigen Ltd., 152 Beach Rd., #26-07/08 Gateway East, Singapore 189721. Ph: 61 2 9234 1700.

The Vaccine Research Center at the National Institute of Allergy and Infectious Diseases (NIAID) is starting trials of an experimental vaccine against severe acute respiratory (SARS) virus. The vaccine will be tested on 10 healthy volunteers at the NIH Clinical Center (Bethesda, MD). The clinic will do periodic follow-up exams on each volunteer for 32 weeks. The primary goal of the study is to determine if the experimental vaccine is safe in people. A secondary goal is to assess how well the vaccine stimulates the immune system to produce antibodies and cellular immunity, in this case, focusing on the SARS spike protein. The spike protein protrudes from the virus' outer envelope and helps it bind to cells it infects. Instead of using weakened or inactivated virus, which is typical for vaccine development, the new vaccine is composed of a small circular piece of DNA that encodes the viral spike protein. Scientists modified the DNA to minimize the risk of it combining with the SARS virus or other viruses of the SARS type, called coronaviruses. Scientists expect that the DNA will direct human cells to produce proteins very similar to the SARS spike protein. The immune system should recognize these proteins as foreign and then mount a defense against them. If the vaccinated person ever encounters the actual SARS virus, his or her immune system will be primed to neutralize it. **Details:** Linda Joy, med. rel., National Institute of Allergy and Infectious Diseases, NIAID Office of Communications & Public Liaison, 6610 Rockledge Dr., MSC 6612, Bethesda, MD 20892-6612. Ph: (301) 402-1663.

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of FLVCR was also increased in cell lines that are used to study heme transport (e.g., intestinal (Caco-2, 13696 copies/ng, MFI 35), and hepatic cells (HepG2, 504 copies/ng, MFI 56)), or have increased heme utilization (e.g., cancer cell lines (n=5, mRNA range 502-1845 copies/ng; MFI 28-54)).

This is the first description of a mammalian heme transporter. FLVCR exports heme to protect CFU-E from heme toxicity. Our expression studies suggest that hFLVCR may also be important for heme transport in other tissues, e.g., the small intestine, where heme serves as the predominant source of human dietary iron.

Abstract# 153

Imatinib Mesylate Reduces Phlebotomy Requirements in Polycythemia Vera. Richard T. Silver. *Hematology/Oncology, New York-Presbyterian Hospital Weill Cornell Medical University, New York, NY, USA.*

Erythrocytosis is the clinical feature that separates polycythemia vera (PV) phenotypically from the other chronic myeloproliferative diseases and is the cause of many of its most serious complications. The mechanism for autonomous erythropoiesis in PV remains unknown, but a number of growth factors other than erythropoietin including stem cell factor (SCF) are also essential for erythroid progenitor cell proliferation and differentiation. SCF signals by inducing tyrosine phosphorylation of its receptor, c-kit, and cross-talk may also exist between c-kit and the erythropoietin receptor. We therefore sought to determine if the tyrosine kinase inhibitor, imatinib mesylate (IM), which is known to block SCF signaling through c-kit could inhibit the autonomous erythropoiesis of PV. Seven patients, four women and three men, with PV as established by the diagnostic criteria of the Polycythemia Vera Study Group, were studied. All had an increased red blood cell mass by direct measurement, and all causes of secondary erythrocytosis were excluded. Their ages ranged from 32 to 76 yrs, (median 55 yrs). Before IM therapy, disease duration ranged from two months to 2.5 yrs (median 1.5yrs, mean 1.4yrs) and the number of phlebotomies per patient ranged from 2 to 36 (mean 11.6, median 6). Four patients had been treated with phlebotomy alone, and three others additionally with either hydroxyurea (HU), interferon or anagrelide. Two of the seven patients had palpable splenomegaly (10cm below the LCM size). All patients were initially phlebotomized to a Hct less than 42%. The starting dose of IM was 400mg daily with a dose escalation of 100mg daily each week to a maximum of 800mg if the platelet count was above 600,000/μl at two weeks. All seven patients had suppression of erythropoiesis. One patient with palpable splenomegaly had a reduction in spleen size by 50% and one had complete regression after only one month of IM therapy. All patients had cessation of phlebotomy requirements within the first 2 months of IM therapy except for one who required one additional phlebotomy. The median duration on IM has been 87 days (mean 84). To date, three of the seven patients have had a reduction in platelet count below 600,000/μl. There was no significant change in the leukocyte count. Two patients developed dry scaly skin; in one, it was sufficiently severe to require discontinuation of IM. In summary, IM therapy suppressed erythropoiesis in seven PV patients, reduced spleen size in two of two and lowered platelet counts in three of seven. A longer duration of treatment may be necessary to influence thrombopoiesis and normalize spleen size. However, the present results suggest that IM by blocking c-kit (and/or PDGF-R) may be a useful agent in the management of PV and warrants a more extensive clinical trial.

Abstract# 154

Eculizumab, a C5 Complement-Blocking Antibody, Abolishes Hemolysis and Renders Hemolytic Patients with Paroxysmal Nocturnal Hemoglobinuria (PNH) Transfusion Independent. Peter Hillmen,[1] Claire Hall*,[1] Judith Marsh*,[2] Dupe Elebute*,[2] Christopher F. Mojcik*,[3] Scott A. Rollins*,[3] Stephen Richards*,[1] Russell P. Rother*,[3] *[1]Haematology, Leeds Teaching Hospitals NHS Trust, Leeds, United Kingdom; [2]Haematology, St. George's Hospital Medical School, London, United Kingdom; [3]Alexion Pharmaceuticals, Inc., Cheshire, CT, USA.*

Paroxysmal nocturnal hemoglobinuria (PNH) is characterised by intravascular hemolysis, venous thrombosis and is associated with aplastic anemia. The complement-mediated hemolysis is due to the deficiency of complement-regulatory proteins on the surface of PNH red cells, particularly the terminal complement inhibitor CD59. The high risk of venous thrombosis results from PNH platelet activation by complement. Therefore complement inhibition in PNH should control the hemolysis and reduce the risk of venous thrombosis. Eculizumab is a humanized monoclonal antibody that binds to C5 and inhibits terminal complement activation. 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. To date, 4 patients have completed therapy and all have been entered into an extension study. Response data for at least 4 weeks of therapy is available for 6 of the 10 patients. Eculizumab has been well tolerated with no serious adverse events. No episodes of thrombosis have occurred although follow-up is short and 4 of 6 patients are on warfarin. The biochemical parameters of hemolysis dramatically improved or normalised for all patients within a week of starting therapy. Mean LDH decreased from 4494 +/- 3380 U/L prior to treatment to 636 +/- 166 U/L following treatment while mean AST decreased from 106 +/- 66 U/L to 24 +/- 9 U/L during the same interval. In addition, a progressive increase in the PNH type III red cells occurred during the treatment period. While hemoglobinuria was common in this patient group before treatment, no patient has had an episode of hemoglobinuria since commencing eculizumab therapy and patients report a marked improvement in symptoms related to hemolysis, such as dysphagia and lethargy. One patient began treatment during a severe acute hemolytic episode when she was passing nearly black urine and within 24 hours there was a complete resolution of her hemoglobinuria. Patients who had purely hemolytic disease (macroscopic hemoglobinuria with a normal platelet count; n=3) appeared to have the best response to eculizumab with cessation of hemolysis and an increase in mean hemoglobin from 9.4 +/- 1.6 g/dL to 10.3 +/- 0.6 g/dL at four weeks, obtained without transfusion. These hemolytic patients without cytopenias also showed a more pronounced increase in the proportion of PNH red cells (mean increase equal to 62%) presumably due to a prolongation of PNH red cell survival. None of these patients have required transfusions to date. Cytopenic patients with laboratory evidence of hemolysis but minimal hemoglobinuria (n=3) had a marked improvement in hemolytic parameters but less effect on transfusion requirements. There was no change in the proportion of PNH neutrophils, monocytes or platelets in any of the patients whilst on eculizumab. In summary, the preliminary results from this pilot study demonstrate that eculizumab was well tolerated, decreased hemolysis and improved symptoms in PNH patients. This study suggests that blocking complement at C5 is an effective therapeutic strategy for hemolytic PNH resulting in control of hemolysis and a reduction in transfusion dependency.

Abstract# 155

TCR Sequencing: The Molecular Signature of Autoimmunity in Aplastic Anemia. Antonio M. Risitano*,[1] Spencer Green*,[1] Susan Sharpe*,[1] Christine O'Keefe*,[2] Weihua Zeng*,[1] Jaroslaw P. Maciejewski*,[2] Neal S. Young.[1] *[1]Hematology Branch, NHLBI - NIH, Bethesda, MD, USA; [2]Experimental Hematology and Hematopoiesis Division, Taussig Cancer Center, Cleveland, OH, USA.*

Acquired aplastic anemia (AA) is a bone marrow failure syndrome characterized by immune-mediated destruction of hematopoietic progenitors. Because the specific, pathogenic antigens in AA are unknown, our research strategy has included analysis of the variable beta (Vβ)-chain to determine T-cell clonality and as a surrogate for an antigen-driven immune response. A major goal has been the molecular identification of disease-specific T-cell clones. In AA, the distribution of Vβ family usage is altered, with predominance or even expansion of particular Vβ subfamilies. Using flow cytometry, we have identified CD8+ Vβ-family expansions in 40 AA patients. As assessed by CDR3-length analysis, oligoclonally distributed Vβ families were selected, and their CDR3 cloned and sequenced in order to create a library of AA-specific clonotypes. As proof of principle, three frequently expanded Vβ families were initially selected (Vβ2, Vβ3 and Vβ17) for further study, including inter-patient comparisons. RT-PCR was performed using a common constant beta (Cβ) primer and a Vβ-family specific oligonucleotide. Corresponding Vβ regions from healthy donors were cloned and sequenced to determine the normal level of CDR3 diversity. As anticipated from the enormous repertoire of TCR-β chains, in controls almost all individual colonies obtained from a single Vβ PCR product had different sequences. In contrast, PCR products from AA patients showed in all cases a high level of redundancy, with one or two CDR3 sequences accounting for more than 50% of the entire Vβ-specific repertoire, confirming the oligoclonal nature of the expansion. Major clones showed unique sequences for each patient; in some cases small subclones with limited amino acid substitutions from the major clonotype were also present. Perfect correspondence between CDR3 length analysis and sequence was observed. All clonotypic sequences were absent from normal controls, consistent with a putative pathogenic role for patient-specific Vβ expanded cells. In two patients, analysis of serial samples obtained in different stages showed good correlation between the quantity of the identified clonotypes and activity of disease, with the frequency much lower after successful immunosuppressive treatment. A PCR using clonotype-specific CDR3 primers was used to screen and compare a large cohort of patients sharing HLA-class I antigen or the same Vβ expansion (3 or 2). Cloning and sequencing of selected samples led to the identification of some new clonotypes; sequences with high homology to the original patient-specific clonotypes were also found in other cases but at a lower frequency. In conclusion, probably pathogenic T-cell clones are present in most AA patients, strong evidence for an underlying immune pathophysiology. Moreover, their frequency within the entire T-cell pool appears to correlate with disease activity. The significance of highly homologous CDR3 sequences among patients of similar HLA-class I antigen background deserves further investigation but is suggestive of common inciting antigens. Our data support a view of most acquired AA as marrow cell destruction due to antigen-driven oligoclonal T cell immune response, involving limited numbers of Vβ clonotypes, which may differ in antigen affinity, killing efficiency, and susceptibility to immunosuppressive therapy, but which likely share the same hematopoietic target.

HEMOGLOBINOPATHIES II

Abstract# 156

Anti-Inflammatory Therapy Ameliorates Endothelial Activation and Improves Microvascular Blood Flow in Transgenic Sickle Mice. Dhananjay K. Kaul,[1] Xiao-du Liu*,[1] Chris Bryant*,[2] John D. Belcher*,[2] Gregory M. Vercellotti,[2] Robert P. Hebbel.[2] *[1]Medicine, Albert Einstein College of Medicine, Bronx, NY, USA; [2]Medicine, University of Minnesota, Minneapolis, MN, USA.*

In transgenic sickle (β^s) mice, but not normal mice, hypoxia followed by reoxygenation (H/R) causes a distinct inflammatory response characterized by endothelial oxidant generation, leukocyte recruitment and microvascular flow abnormalities (Kaul and Hebbel, J Clin Invest 106, 2000), indicating that hypoxia-induced sickling is etiologic in this response. This is consistent with our hypothesis that endothelial activation and leukocyte recruitment secondary to reperfusion injury would play an important role in the pathophysiology of sickle cell disease. The leukocyte recruitment is secondary to up-regulation of specific adhesion molecules on vascular endothelium. Previous studies have shown that sulfasalazine, a powerful inhibitor of nuclear factor (NF)-κB, a transcription factor, causes a significant decrease in endothelial adhesion molecules, i.e., vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin in both sickle cell anemia patients and β^s mice (Solovey et al. Blood 97, 2001). However, the consequences of NF-κB inhibition on inflammatory response (i.e., leukocyte recruitment) to hypoxia/reoxygenation (H/R) have not been ascertained under in vivo microcirculatory

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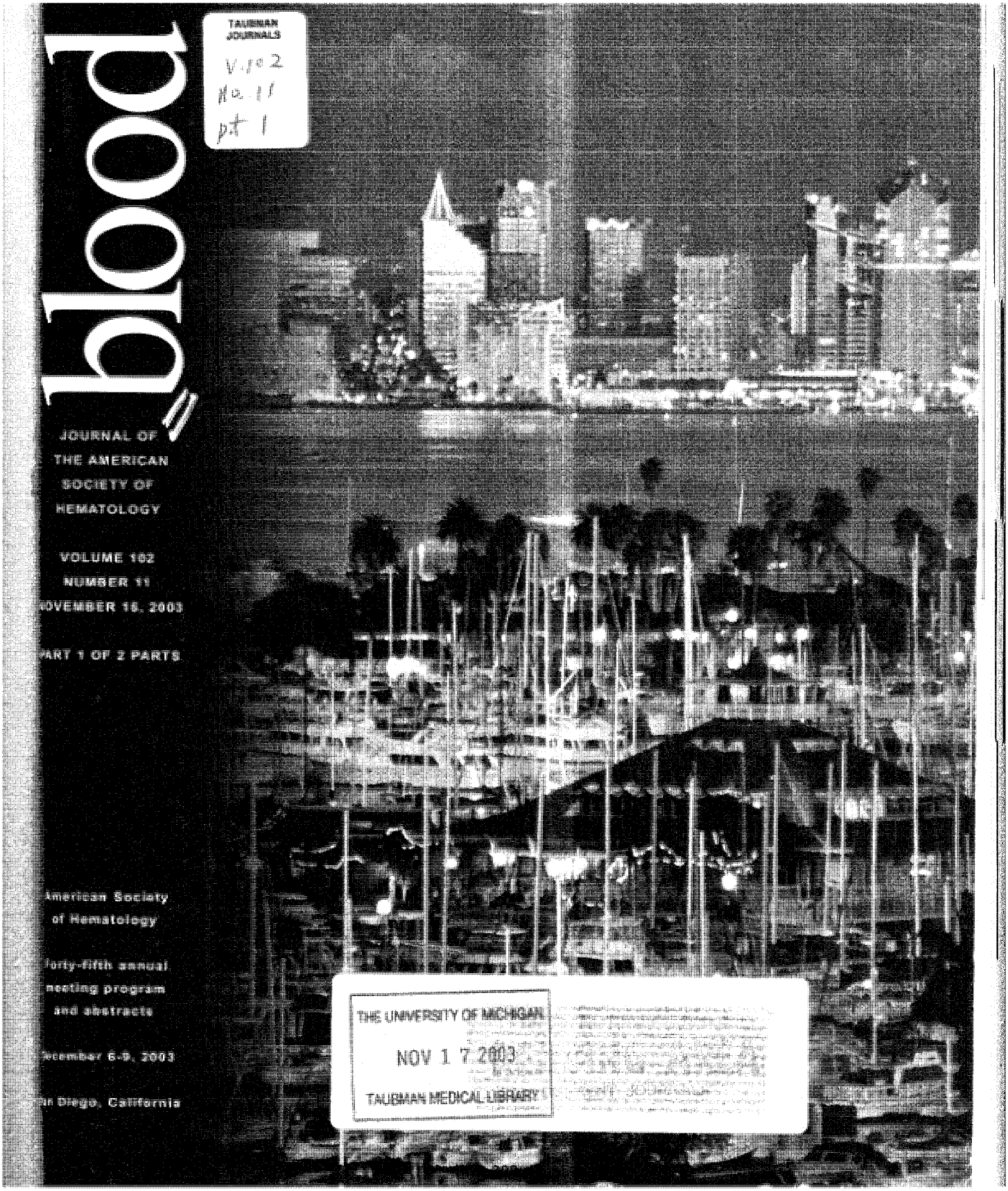
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vs. 8.7%, $P<0.001$) displayed significantly higher frequencies among AA patients than among a general Japanese population. The percentage of patients displaying DR15 was significantly higher in the 128 patients ≥30-years-old (77.3%) than in the 54 patients <30-years-old (37.0%, $P<0.001$). When all patients were divided into PNH$^+$ and PNH$^-$ groups, comprising 95 patients with a minor population of PNH-type cells and 62 patients without PNH-type cells, respectively, the increased frequency of DRB1*1502 was observed in both PNH$^+$ (23.9%) and PNH$^-$ patients (20.7%), while the increased frequency of DRB1*1501 was only observed in PNH$^+$ patients (21.2% vs. 6.7%). Both DRB1*1501 and the presence of PNH-type cells predicted response to monotherapy with cyclosporin A (CsA; odds ratios (ORs)=12.2 and 13.6, respectively), in addition to response to antithymocyte globulin (ATG)±CsA therapy (OR=9.26 and 4.27, respectively). DRB1*1502 was not predictive of response to either therapy (OR=0.72 and 0.66, respectively). Multivariate analysis confirmed the predictive values of DRB1*1501 and the presence of PNH-type cells in both CsA therapy (P=0.047 and P=0.023, respectively) and ATG±CsA therapy (P=0.048 and P=0.034, respectively). These data indicate that although both DRB1*1501 and DRB1*1502 contribute to the development of AA, their contributions may differ. Apparent age-dependent differences in HLA-DR15 frequency suggest that the pathophysiology of AA in adult patients may differ from that in pediatric patients. Furthermore, DRB1*1501 and the presence of a minor population of PNH-type cells represent independent factors favorably affecting response to IST in AA patients.

Abstract# 1855 **Poster Board #-Session: 26-II**

Apoptosis and Proliferation of CD34 Positive Bone Marrow Cells of Polycythemia Vera Patients. Jie Bai*, Zonghong Shao, Hong Liu*, Jun Shi*, Guangsheng He*, Yanran Cao*, Meifeng Tu*, Yuhong Wu*, Liping Jing*, Tiejun Qin*, Hairong Jia*, Juan Sun*, Zhenzhu Cui*, Yanping Xue*, Huishu Chen*, Linsheng Qian*, Tanying Yang*, Chongli Yang*. *Institute of Hematology and Blood Disease Hospital, CAMS and PUMC, Tianjin, China.*

Objective To study the apoptosis and proliferation of CD34 positive(CD34$^+$) bone marrow cells(BMC) of patients with polycythemia vera(PV) **Method** The expression of Annexin V and proliferation related protein Ki67 in the CD34+ BMC of 20 PV patients and control cases[10 primary thrombocythemia(ET), 12 normal persons] were assessed by Bicolor flow cytometry(FCM), then the correlation between apoptosis and clinical situation was analysed in PV patients. **Result** The expression of Annexin V were (15.96%±1.45%)in PV patients' CD34$^+$ BMC(15.53%±1.76%)in ET patients'CD34$^+$ BMC which were lower than the expression in normal persons'.(23.61%±3.89%)(P<0.05). The expression of Ki67 were(48.79%±11.68%) in PV patients' CD34$^+$ BMC and (49.60%±9.98%) in ET patients' CD34$^+$BMC which were significantly higher than that of normal conrol's(33.87%±6.82%). The ratio of apoptosiss/proliferation of PV patients was(0.33±0.104)and that of ET patients(0.32±0.02)which was significantly lower than that of normal control's. The apoptosis of CD34$^+$ BMC was negatively correlated with the level of hemoglobin(Hb)(r=-0.481,p=0.037), white blood cell(WBC)(r=-0.538,p=0.026) and the numbers of endogenous erythroid colony(EEC) of PV patients. The ratio of apoptosiss/proliferation of PV patients was negatively correlated with their Hb(r=-0.537, p=0.018)and WBC(r=-0.667, p=0.003). **Conclusion** There were lower apoptosis and higher proliferation in CD34$^+$ BMC of PV patients. Lower apoptosis was correlated with the disease severity of PV.

Abstract# 1856 **Poster Board #-Session: 27-II**

Flow Cytometric Analysis of Medullary Hematopoiesis in Tumor Necrosis Factor-α (Tg197) Mice: A Potential Model of Anemia of Chronic Disease. Renold J. Capocasale*,[1] J. O'Brien*,[1] D. Makropoulos*,[1] P. Rafferty*,[1] J. Quinn*,[2] R. Achuthanandam*,[3] M. Kam*,[3] L. Hrebien*,[3] P. Bugelski*,[1] (Intr. by Emmanuel C. Besa) *[1]Experimental Pathology, Centocor, Malvern, PA, USA; [2]School of Biomedical Engineering, Science, and Health Systems, Drexel University, Philadelphia, PA, USA; [3]Dept of Electrical and Computer Engineering, Drexel University, Philadelphia, PA, USA.*

Rheumatoid arthritis is a chronic inflammatory disease commonly associated with anemia of chronic disease (ACD). Heterozygous Tg197 transgenic mice expressing human tumor necrosis factor-α (TNF- α) develop a chronic progressive polyarthritis and weight loss and have been used as a model of rheumatoid arthritis (Shealy et al *Arth Res* 4:R7, 2002). As TNF-α is believed to play an important role in the pathogenesis of ACD, we used 4-color flow cytometry and routine hematology to evaluate the potential of Tg197 mice to serve as a model of ACD. Groups of 8 heterozygous Tg197 were euthanized at 5 or 10 weeks of age after 24 hr pulse labeling with BrdU. Age matched CBF1 mice served as controls. Hind limbs were collected and fixed in 10% formalin for paraffin sections. Blood and femoral bone marrow were collected for hematology and flow cytometry, respectively. Antibody to Sca-1 was used to evaluate hematopoietic stems cells, Ter-119/CD71 erythroid progenitors, Gr-1/CD11b granulocyte and monocyte progenitors and IL-7R-α, lymphoid progenitors. Annexin V binding, BrdU and 7-AAD were used to evaluate apoptosis and cell cycle in these populations. Histologic evaluation revealed a progressive arthritis and hematology a mild progressive, microcytic hypochromic anemia with reticulocytosis and monocytosis. Preliminary analysis of the flow cytometric data revealed increased numbers of dead and apoptotic erythroid precursors. The results of in-depth analysis will be presented. Taken together, our data suggest that Tg197 mice may be a useful model of ACD.

Abstract# 1857 **Poster Board #-Session: 28-II**

Relationship between Serum Erythropoietin and Hemoglobin Concentration in Community-Dwelling Older Women: The Women's Health and Aging Studies I and II. Paulo H. M. Chaves*, Qian-Li Xue*, Linda P. Fried*. (Intr. by Richard C. Woodman) *Johns Hopkins Center on Aging and Health, Baltimore, MD, USA.*

BACKGROUND. Anemia in older women is typically defined as Hb<12 g/dL. The current criteria used to define anemia were mainly derived from statistical distribution considerations. There is, however, limited physiologic data supporting or refuting these criteria. The objective of this study was to investigate the relationship between the continuous distributions of serum erythropoietin (EPO) – a surrogate marker for tissue oxygenation – and hemoglobin concentration (Hb) in older adults, focusing on the characterization of potential threshold effects. Specifically, we tested the assumption that within the Hb range defined as normal for older women by the World Health Organization (WHO); i.e., 12–16 g/dL, serum EPO is constant and lowest.

METHODS. This cross-sectional analysis used data from the Women's Health and Aging Studies (WHAS) I and II, two complementary population-based studies investigating the epidemiology of disability progression in 1002 women ≥ 65 yrs (WHAS-I, 1992-95), and disability onset in 436 high functioning women 70-80 yrs (WHAS-II, 1994-96). For this analysis, baseline WHAS I and WHAS II data from women 70-80 yrs were pooled (n=865). EPO was measured in sera samples stored at -70C by enzyme immunoassay. Within- and between-essays coefficients of variability for EPO were 13.3% and 11.6%, indicating acceptable reproducibility. Hb was measured by spectrophometry using the traditional cyanmethemoglobin method. Baseline EPO and Hb were available for 662 subjects. To minimize the impact of outliers, we restricted this analysis to subjects with Hb between 10–16 g/dL, and excluded 3 subjects with EPO values above the 99th percentile level (≥63 mU/mL). This study final sample size was 641. A generalized linear model with a log-link and gamma distribution was used to estimate the mean serum EPO per Hb concentrations, and EPO differences between different Hb concentrations with corresponding 95% confidence intervals (CI).

RESULTS. Median EPO was 7.1 mU/mL (25th – 75th percentiles: 3.1 – 13.6). Mean Hb was 13.2 ± 1.1 g/dL. Mean age of study group was 74.3 ± 2.9. The observed relationship between Hb and EPO was best described by a curvilinear, quadratic curve. EPO was highest at the lowest Hb, and monotonically decreased with increasing with Hb up to 14.2 g/dL (95% CI: 13.1, 15.3), the tangent to the curve. As compared to EPO at a Hb of 12 g/dL (11.3 mU/mL), mean EPO linked to a Hb of 10 g/dL (23.7 mU/mL) and 11 g/dL (15.4 mU/mL) were significantly higher; i.e., EPO was 2.1 (95%CI 1.4, 3.1) and 1.4 (95%CI 1.2, 1.6) times higher, respectively. Even within the normal Hb range, there was a substantial EPO gradient. For example, EPO at a Hb of 14.2 g/dL was .75 (95%CI .65, .88) times lower than at the low-normal Hb of 12 g/dL. Adjustment for age, race, and calculated creatinine clearance did not change these estimates.

CONCLUSION. We found a meaningful relationship between decreasing EPO and increasing Hb up to levels currently perceived as mid-normal Hb. These results support the hypothesis that mid-normal Hb concentrations might offer a clinical advantage over mild anemia (10-12g/dL) and possibly low-normal Hb (12-13 g/L). In addition, our findings are in accordance with recently published epidemiologic studies suggesting that a Hb of 12 g/dL might be a suboptimal criterion to define anemia in older women.

Abstract# 1858 **Poster Board #-Session: 29-II**

Eculizumab, a C5 Complement-Blocking Antibody, Controls Hemolysis in Paroxysmal Nocturnal Hemoglobinuria (PNH) with Responses Maintained over a Prolonged Period of Therapy. Peter Hillmen,[1] Claire Hall*,[1] Judith Marsh*,[2] Dupe Elebute*,[2] Stephen Richards,[1] Scott A. Rollins*,[3] Christopher F. Mojcik*,[3] Russell P. Rother*,[3] *[1]H.M.D.S., Leeds Teaching Hospitals NHS Trust, Leeds, United Kingdom; [2]Haematology, St. George's Hospital Medical School, London, United Kingdom; [3]Alexion Pharmaceuticals, Inc., Cheshire, CT, USA.*

Paroxysmal nocturnal hemoglobinuria (PNH) is characterized by intravascular hemolysis and venous thrombosis. Deficiency of the terminal complement inhibitor CD59 from PNH red cells is the principle cause of the complement-mediated hemolysis. Eculizumab is a humanized monoclonal antibody that binds to C5 inhibiting terminal complement activation. 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. To date all patients remain on eculizumab, completing between 11 to 15 months of therapy. Results presented below represent cumulative data through 6 months. Eculizumab continues to be well tolerated with no dose limiting toxicity. The adverse events in the extension study are similar to those in the pilot study. The dramatic improvement in the biochemical parameters of hemolysis occurring in all patients within a week of starting therapy persists. Mean LDH decreased from 3111 +/- 598 U/L over the 12 months prior to treatment to 670 +/- 69 U/L over 6 months following treatment (p=0.003). Hemoglobinuria (daily urine color scale) decreased from 3.0 to 0.2 paroxysms/patient-month since starting eculizumab therapy with a significant improvement in quality of life measures related to hemolysis. In addition, PNH type III red cells progressively increased during the treatment period from a mean of 37% to 57% at 6 months (p=0.009). There was also a continued reduction in transfusion requirement from a median 1.8 units/patient/month in the 12 months prior to treatment to 0.4 units/patient/month over the last 6 months (p=0.001), and 5 of the 11 patients remain completely transfusion independent after a year of therapy. Patients with purely hemolytic disease (macroscopic hemoglobinuria with a normal platelet count; n=6) had the best response

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with cessation of hemolysis, an increase in type III red cells from 39% to 67% and a decrease in transfusions from a median of 2.0 units/patient-month to 0 units/patient-month. Four patients who did not become transfusion independent have been treated with subcutaneous erythropoietin in addition to eculizumab with some improvement in hemoglobin and transfusion requirement. Interestingly, in most patients LDH levels remain just above the upper limit, the bilirubin remains slightly to moderately elevated (under 50 umol/l), the haptoglobins remain undetectable and the reticulocytosis persists indicating a low level of well-compensated, presumably extravascular, hemolysis. 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. These data support the role of terminal complement activation in hemolysis of PNH red cells and the mechanism of action of eculizumab in the control of hemolysis and symptoms in PNH. In summary, this study demonstrates that eculizumab is well tolerated, dramatically decreases hemolysis and transfusion requirement as well as improving the symptoms of PNH. The effectiveness of eculizumab has been maintained for a median of 12 months of therapy and is ongoing in all patients.

Abstract# 1859 **Poster Board #-Session: 30-II**

A Novel GATA-Specific Inhibitor (GSI) Rescues Anemia of Chronic Disease by Oral Administration. Shigehiko Imagawa,[1] Yoko Nakano*,[1] Naoshi Obara*,[1] Norio Suzuki*,[2] Toshiro Nagasawa*,[1] Masayuki Yamamoto.[2] *[1]Division of Hematology, Institution of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan; [2]TARA center, University of Tsukuba, Tsukuba, Ibaraki, Japan.*

The disorders associated with anemia of chronic disease (ACD) are characterized by the production of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), which increase the binding activity of GATA-2 and NF-κB and inhibit production of erythropoietin (Epo). Because Epo promoter activity is negatively regulated by GATA binding, GATA-2 may be responsible for impaired Epo synthesis in inflammatory disease *in vivo*. On the other hand, we found that N^{G}-monomethyl L-arginine (L-NMMA), which is increased in patients with chronic renal failure, inhibits NO and cGMP production, increases the binding activity of GATA and mRNA expression, and inhibits Epo promoter activity. Therefore, one common pathogenesis of ACD and anemia of renal disease appears to be *via* the stimulation of GATA binding activity. We have shown that intraperitoneal injection of K-7174, a GATA-specific inhibitor, improves Epo production which had been inhibited by IL-1β and TNF-α. In this study, a novel GSI, which has a 100-fold higher affinity than K-7174, was examined to further improve Epo production that had been inhibited by IL-1β and TNF-α. The addition of 100 nM GSI rescued the inhibition of Epo protein production and promoter activity induced by IL-1β and TNF-α, respectively. Electrophoretic mobility shift assays revealed that the addition of GSI decreased GATA binding activity, which was increased with the addition of IL-1β and TNF-α. Furthermore, intraperitoneal injection of mice with IL-1β or TNF-α decreased Epo levels, reticulocyte counts, hematocrits (Hts), hemoglobins (Hbs), the numbers of CFU-E from spleen and bone marrow, and the number of CD71+/Ter119+ cells from the spleen. However, oral administration of GSI reversed these effetcs. These results raise the possibility of using GSI as a novel drug for treating ACD by oral administration.

Effects of GSI on Hbs, Hts, reticulocyte counts, Epo levels, the number of CFU-E and CD71+/Ter119+cells from ICR mice induced by IL-1β or TNF-α

	Control	+IL-1β	+TNF-α	+IL-1β + 3 mg/kg GSI	+TNF-α + 3 mg/kg GSI
Hb (g/dL)	14.8 ± 0.8	13.5 ± 0.5	13.4 ± 0.6	14.0 ± 0.8	13.8 ± 0.5
	(15)	(15) *	(11) *	(14) ***	(12) *****
Ht (%)	46.4 ± 2.8	41.6 ± 2.2	42.7 ± 2.0	43.4 ± 2.7	43.2 ± 2.1
	(16)	(13) *	(12) *	(14) ***	(12) *****
Reticulocyte (‰)	163 ± 29	122 ± 19	92 ± 32	204 ± 38	253 ± 55
	(10)	(8)*	(12) *	(8) ***	(12) *****
Epo (mU/mL)	24.8 ± 31.9	3.0 ± 4.6	3.3 ± 5.6	17.1 ± 24.1	49.4 ± 45.8
	(9)	(7) *	(8) *	(7) ***	(9) ******
CFU-E (spleen)	10.0 ± 8.9	4.7 ± 3.6	7.5 ± 4.7	30.4 ± 19.8	67.8 ± 7.9
	(8)	(6) *	(8) **	(8) ***	(8) *****
CD71+/Ter119+	19.6 ± 6.9	16.8 ± 6.9	ND	41.8 ± 11.6	54.3 ± 1.2
	(4)	(3)		(3) ****	(2)#

(experimental numbers) * Significance compared with control, P<0.005; **Significance compared with control, P<0.025; Significance compared with +IL-1β, P<0.005; ***Significance compared with +IL-1β, P<0.025; ****Significance compared with +TNF-α, P<0.005, #+TNF-α + 1 mg/kg GSI, ND:not done

Abstract# 1860 **Poster Board #-Session: 31-II**

Stress Erythropoiesis Is Not Efficiently Supported by a Minimal Epo Receptor Phosphotyrosine-Null Allele. Don M. Wojchowski,[PSU] Vinit G. Karur*,[PSU] Madhu P. Menon*,[PSU] Ke Li,[PSU] Jamie Crater*,[PSU] *[1]Department of Veterinary Sciences, The Pennsylvania State University, State College, PA, USA; [2]Pathobiology program, Department of Veterinary Sciences, The Pennsylvania State University, State College, PA, USA; [3]Molecular Medicine, Huck Institute of Life Sciences, The Pennsylvania State University, State College, PA, USA; [4]Department of Veterinary Sciences, The Pennsylvania State University, State College, PA, USA; [5]Department of Veterinary Sciences, The Pennsylvania State University, State College, PA, USA.*

Anemia intensifies signals for red cell production and activates stress erythropoiesis mechanisms. This can involve accelerated medullary erythropoiesis, enhanced cytokine action, stimulation of latent progenitor cell populations and extra-medullary erythropoiesis. To investigate ways in which subsets of Epo receptor linked factors might regulate this process, mice with a knocked-in truncated and phosphotyrosine-null EpoR allele (EpoR-HM) were challenged with phenylhydrazine. In these EpoR-HM mice, stress erythropoiesis was discovered to falter in several ways: 1) Hemoglobin levels, hematocrits and RBC numbers failed to efficiently rebound; 2) Interestingly, reticulocyte levels nonetheless approximated those in wt-EpoR controls; 3) In response to this hemolytic anemia, spleens of EpoR-HM mice essentially failed to enlarge; 4) Immunohistochemical analyses revealed decreased red pulp expansion in spleens of EpoR-HM mice; 5) Analyses of constituent erythroid progenitor cells revealed an apparent developmental deficiency in EpoR-HM mice at a late CD71-high, Ter119(+) stage; 6) In splenocyte preparations, proliferative responsiveness to Epo was several-fold lower for EpoR-HM mice, and markedly lower in the absence of serum; 7) Affymetrix profiling of purified Ter119(+) erythroid progenitor cells from spleen revealed elevated expression of several genes in EpoR-HM mice including an interesting set of GTP-regulated factors.These findings in general reveal that important roles are exerted by distal EpoR PY-encoding domains during stress erythropoiesis (as illustrated, for example, by defective red cell production and splenic enlargement). In addition, insight into stress erythropoietic mechanisms also is advanced. This includes first a sustained deficit in mature red cells vs an efficient production of reticulocytes observed in EpoR-HM mice: this suggests that EpoR PY signaling normally may impact on reticulocyte half-life and/or maturation. Second, Epo-dependent stress erythropoiesis also may be significantly assisted in EpoR-HM mice by an accessory serum growth factor. Finally, profiling outcomes suggest that erythroid progenitor cell-intrinsic mechanisms also are activated in response to deleted EpoR-PY coupled events, and in part this may involve a selective engagement of GTP-protein linked pathways. Such novel stress erythopoietic mechanisms may have significant clinical relevance.

Abstract# 1861 **Poster Board #-Session: 32-II**

Complement-Mediated Hemolysis of Erythrocytes Is Blocked by RNA Aptamers to Complement Components C5 and C8. Jun-ichi Nishimura,[1] Shahid M. Nimjee*,[2] Haixiang Jiang*,[3] George Pitoc*,[2] Thad A. Howard*,[3] Christopher P. Rusconi*,[2] Mitsuhiro Omine*,[4] Michael M. Frank*,[3] Bruce A. Sullenger*,[2] Russell E. Ware.[3] *[1]Medical Oncology and Transplantation, Duke University Medical Center, Durham, NC, USA; [2]Experimental Surgery, Duke University Medical Center, Durham, NC, USA; [3]Pediatrics, Duke University Medical Center, Durham, NC, USA; [4]Medicine, Showa University Fujigaoka Hospital, Kanagawa, Japan.*

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematopoietic stem cell disorder characterized by intravascular hemolysis, venous thrombosis, and bone marrow failure. The lack of GPI-anchored surface complement regulatory proteins, such as CD55 (DAF) but especially CD59, results in complement-mediated hemolysis and hemoglobinuria, which is a major clinical manifestation of PNH and an important cause of morbidity and mortality. Complement plays a critical role in the pathophysiology of hemolysis in PNH, and may also be important in the development of thrombosis. Accordingly, complement inhibition in PNH should control hemolysis and potentially reduce the risk of venous thrombosis. RNA aptamers that bind and functionally inhibit human complement protein C5 have been identified using a combinatorial RNA library and the systematic evolution of ligands by exponential enrichment (SELEX) in vitro selection method (Biesecker G. et al. : Immunopharmacology, 42:219, 1999). However, C5 is critical not only for complement activation, but also for proper regulation of inflammatory processes. Prolonged inhibition of C5 could, therefore, have undesired side effects that would limit its utility in patients with PNH. CD59 inhibits C9 from binding to the C5b8 complex, and is critically important in protecting erythrocytes from complement-mediated lysis. Individuals deficient in C9 are usually healthy, although inherited deficiencies in other complement proteins typically lead to various degrees of illness. Thus, complement proteins C8 or C9 may be the ideal targets to protect PNH erythrocytes from complement-mediated lysis without adverse side effects. We first synthesized one of the known C5-binding aptamers (UCUCAUGCGCCGAGUGUGAGUUUACCUUCGU) and confirmed inhibition of human serum hemolytic activity using an in vitro assay with sensitized sheep erythrocytes, C5-depleted serum, and purified C5. We have begun to identify RNA aptamers that inhibit complement components C8 or C9 by the SELEX procedure, to isolate high affinity, nuclease-resistant RNA ligands that bind specifically to C8 or C9. After only 4 rounds of the SELEX procedure, aptamers bound purified C8 with a Kd = 970 pM and also inhibited human serum hemolytic activity using an in vitro assay with sensitized sheep erythrocytes, C8-depleted serum, and purified C8. Complement-mediated lysis was inhibited at 50-100nM C8 aptamer concentrations, similar in potency to the published C5 aptamers. Additional rounds of the SELEX procedure should yield specific high-affinity C8-binding aptamers, and C9 aptamers are also being developed. Inhibition of complement activation is a new therapeutic goal for patients with PNH that could control hemolysis, reduce transfusion dependency, and potentially reduce the risk of thrombosis. The development of specific RNA aptamers by the SELEX procedure should provide new therapeutic options for patients with PNH in the near future.

Abstract# 1862 **Poster Board #-Session: 33-II**

Increased Apoptosis of Erythroid Cells during Anemia Recovery in Prion Protein Deficient (Prnp-/-) Mice. Jan H. Zivny*,[1,2] Karel Holada*,[2] Jan Simak,[1] Jaroslav G. Vostal.[1] *[1]Laboratory of Cellular Hematology, Center for Biologics Evaluation and Research, FDA, Bethesda, MD, USA; [2]1st School of Medicine, Charles University, Prague, Czech Republic.*

The cellular isoform of prion protein (PrPc) is involved in the pathogenesis of prion diseases in both humans and animals but its normal function is not known. It has been proposed that PrPc may protect cells against oxidative stress damage, promote survival and differentiation of neuronal cells and mediate copper transport. However, prion protein gene knock-out mice (Prnp-/-) did not develop major phenotypic changes. PrPc is expressed on many cell types including hematopoietic cells and red cells. We tested the role of PrPc in erythropoiesis using a mouse erythroleukemia cell line (MEL) induced to erythroid differentiation by N,N'-hexamethylene bisacetamide (5mM) and a mouse model of accelerated erythropoiesis induced by acute anemia. Erythroid differentiation of MEL cells led to the induction of prion protein gene (Prnp) mRNA expression and up-regulation of cell surface

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INHIBITION OF COMPLEMENT ACTIVITY BY HUMANIZED ANTI-C5 ANTIBODY AND SINGLE-CHAIN Fv.

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Abstract—Activation of the complement system contributes significantly to the pathogenesis of numerous acute and chronic diseases. Recently, a monoclonal antibody (5G1.1) that recognizes the human complement protein C5, has been shown to effectively block C5 cleavage, thereby preventing the generation of the pro-inflammatory complement components C5a and C5b-9. Humanized 5G1.1 antibody, Fab and scFv molecules have been produced by grafting the complementarity determining regions of 5G1.1 on to human framework regions. Competitive ELISA analysis indicated that no framework changes were required in the humanized variable regions for retention of high affinity binding to C5, even at framework positions predicted by computer modeling to influence CDR canonical structure. The humanized Fab and scFv molecules blocked complement-mediated lysis of chicken erythrocytes and porcine aortic endothelial cells in a dose-dependent fashion, with complete complement inhibition occurring at a three-fold molar excess, relative to the human C5 concentration. In contrast to a previously characterized anti-C5 scFv molecule, the humanized h5G1.1 scFv also effectively blocked C5a generation. Finally, an intact humanized h5G1.1 antibody blocked human complement lytic activity at concentrations identical to the original murine monoclonal antibody. These results demonstrate that humanized h5G1.1 and its recombinant derivatives retain both the affinity and blocking functions of the murine 5G1.1 antibody, and suggest that these molecules may serve as potent inhibitors of complement-mediated pathology in human inflammatory diseases.
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Key words: complement, C5, humanized antibody, Fab, scFv.

INTRODUCTION

Activated components of the complement system are believed to contribute to many of the pathophysiological mechanisms underlying numerous acute and chronic inflammatory processes (reviewed in Morgan (1994)). For example, cleavage of C3 generates both C3a, a relatively weak chemoattractant for neutrophils and C3b, a potent opsonin. The subsequent cleavage of C5 generates C5a, a potent chemo-attractant and activator of neutrophils, as well as C5b, which initiates deposition of the membrane attack complex C5b-9 on the cell surface. The recruitment of neutrophils contributes significantly to inflammatory tissue injury in numerous experimental systems (reviewed in Korthius and Granger (1994)), while C5b-9 deposition can result in cell activation due to calcium influx (Morgan, 1989), upregulation of cell surface adhesion molecules (Hattori *et al.*, 1989) or in cell lysis.

The complement cascade is regulated by endogenous inhibitors which act at numerous points including C1, C4, C3 convertase activity, C5 convertase activity and the formation of C5b-9 (Lachmann, 1991). Development of a soluble form of complement receptor type 1 (sCR1), an inhibitor of C3 convertase activity, has provided a means to examine the potential of complement inhibition for the amelioration of several disease processes including xenotransplant rejection (Pruitt *et al.*, 1994), lung and dermal immune complex-mediated injury (Mulligan *et al.*, 1992), experimental allergic encephalomyelitis (Piddlesden *et al.*, 1994), cardiopulmonary bypass (Moat *et al.*, 1992; Gillinov *et al.*, 1993) and reperfusion injury (Weisman *et al.*, 1990; Smith *et al.*, 1993; Pemberton *et al.*, 1993). However, inhibition of the complement cascade by sCR1 at the level of C3 convertase activity may be predicted to have significant clinical side effects. Generation of C3b is essential for the normal phagocytosis of bacterial and fungal pathogens as well as the clearance of circulating immune complexes (Liszewski and Atkinson, 1993). In fact, humans genetically deficient in C3 are subject to recurrent life-threatening infections and also suffer from a greatly increased incidence of autoimmune diseases such as systemic lupus erythrematosus and glomerulonephritis (Ross and Densen, 1984).

To avoid these complications, we have focused on the

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inhibition of the complement cascade at the level of C5, thereby blocking formation of the proinflammatory complement activation products C5a and C5b-9, while preserving the generation of C3b. Patients with a genetic deficiency of C5 suffer only from an increased incidence of Neisserial infections, which are in fact milder than infections in patients having normal levels of C5 (Ross and Densen, 1984). As there are no endogenous inhibitors specific for the C5 convertases, monoclonal antibodies were developed, which bind human C5 and prevent its cleavage by C5 convertase (Würzner *et al.*, 1991; Khroshus *et al.*, 1995). These antibodies inhibit complement-mediated acute cardiac tissue injury occurring in perfused heart models of xenotransplantation (Kroshus *et al.*, 1995) and block leukocyte and platelet activation in an *ex vivo* recirculation model of cardiopulmonary bypass (Rinder *et al.*, 1995). Additionally, in pigs, a monoclonal antibody against C5a reduces myocardial infarct size by 35% (Amsterdam *et al.*, 1995). Finally, a monoclonal antibody against mouse C5 (Frei *et al.*, 1987) blocks progression of established arthritis in a murine collagen-induced arthritis model (Wang *et al.*, 1995) and prevents development of glomerulonephritis in a murine model of systemic lupus erythromatosis (Wang *et al.*, 1995).

The desired pharmacological properties of complement inhibitors are dramatically different for acute versus chronic settings. In acute settings, a complement inhibitor should have a relatively short serum half-life, coupled with a rapid rate of tissue penetration, since complement activation occurs within the tissue (Schäfer *et al.*, 1986; Hugo *et al.*, 1990). Large molecules such as monoclonal antibodies have very slow rates of tissue penetration as compared to smaller molecules such as Fab fragments (Covell *et al.*, 1986) or single chain Fv (scFv) molecules (Yokota *et al.*, 1992). A complement inhibitor used in chronic settings should have a prolonged serum half-life and not provoke an immune response. In humans, murine monoclonal antibodies have a half-life of approximately 1 day (LoBuglio *et al.*, 1993) and uniformly provoke an immune response (Khazaeli *et al.*, 1994). These problems have been addressed by the development of humanized monoclonal antibodies which have serum half-lives of several days (LoBuglio *et al.*, 1989; Khazaeli *et al.*, 1991; Saleh *et al.*, 1992; Schüpbach *et al.*, 1993) as well as greatly diminished immunogenicity (LoBuglio *et al.*, 1993)

In this study, variants of the 5G1.1 monoclonal antibody have been engineered, which can be used in either acute or chronic settings. Firstly, the 5G1.1 variable regions were humanized using the CDR-grafting technique (Riechmann *et al.*, 1988). The humanized variable regions were then used to construct humanized antibody, Fab and scFv molecules which all maintained a high affinity for human C5 and blocked the generation of both C5a and C5b-9.

MATERIALS AND METHODS

Cloning of 5G1.1 variable region genes

For N-terminal amino acid sequencing, 75 μg 5G1.1 mAb was subjected to SDS–PAGE under reducing conditions and transferred to ProBlott membrane (Applied Biosystems, Foster City, CA, U.S.A.) as previously described (Evans *et al.*, 1995). Protein bands were localized by staining with Ponceau S, excised and subjected to amino acid sequence analysis using Edman chemistry, performed on a pulsed liquid protein sequencer (ABI model 477A) with the PTH amino acids analysed using an on-line microbore HPLC system (ABI model 120A).

To deblock the amino terminus of the 5G1.1 heavy chain, 10 mg 5G1.1 monoclonal antibody was exchanged into reducing buffer (5 M guanidine–HCl, 50 mM Tris–HCl, 10 mM dithiothreitol, pH 8.5), using a PD-10 column (Pharmacia, Piscataway, NJ, U.S.A.). After incubation for 1 hr at room temperature, 50 mM iodoacetamide was added and the incubation allowed to continue for 30 min. The carbamidomethylated light and heavy chains were then separated by size exclusion chromatography on a Superose 12 (Pharmacia) column equilibrated with 5 M guanidine–HCl, 50 mM Tris–HCl (pH 8.5). The purified light chain was exchanged into 50 mM sodium phosphate (pH 7.0), using a PD-10 column, digested using 0.5 mU pyroglutamate aminopeptidase (PanVera, Madison, WI, U.S.A.) per nmol of heavy chain protein and sequenced as previously described. For determination of internal amino acid sequence, the isolated light chain was exchanged into 2 M urea, 25 mM Tris–HCl, 1 mM EDTA (pH 8.0) and incubated at 37°C overnight with endoproteinase Lys-C (Promega, Madison, WI, U.S.A.) at a protease:protein ratio of 1:40. The digested material was run on a C18 reversed phase HPLC column (Beckman Instruments, Fullerton, CA, U.S.A.) and eluted using a linear acetonitrile gradient (0–50%) in 0.1% trifluoroacetic acid. Peaks were subjected to amino acid sequence analysis as previously described.

Cloning of the 5G1.1 heavy chain variable region (VH) and light chain variable region (VL) was initially performed using a set of commercially available primers (Mouse Ig-Primer Set, catalogue number 69831-1; Novagen, Madison, WI, U.S.A.) essentially as previously described (Evans *et al.*, 1995). Comparison to the obtained peptide sequences confirmed that the correct VH had been cloned, whereas neither of the two light chain amino acid sequences were present in the cloned VL. To isolate the 5G1.1 VL, the UWGCG program TFASTA was used to search the GenBank rodent subdirectory with the amino acid sequence obtained from the purified light chain (IQMTQSPASLSASVGETVT). An exact match to this sequence was located in the murine germline gene encoding the v-kappa k2 variable region (Seidman *et al.*, 1978). The DNA sequence of this germline gene was used to design an oligonucleotide for use as a variable region 5′-primer for isolation of the correct VL gene.

Humanization of 5G1.1 variable regions

Two humanized variants of the 5G1.1 VL and VH regions were constructed by CDR grafting (Reichmann *et al.*, 1988). For CDR grafting, the 5G1.1 heavy and light complementarity determining regions were intro-

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duced into the human heavy variable region H20C3H (Weng *et al.*, 1992) to yield h5G1.1VHC (Fig. 1A) or the human light variable region L23 (Klein *et al.*, 1993) to yield h5G1.1VLC (Fig. 1B), respectively. Construction of each CDR-grafted variable region was performed by PCR amplification, using two synthetic overlapping oligonucleotides of approximately 200 nucleotides in length. The cloned PCR products were sequenced on both strands in their entirety to ensure the absence of PCR-introduced errors. Murine amino acids were introduced into the human framework regions at position 71 in the light variable region and positions 71 and 78 in the heavy variable region by PCR mutagenesis.

Expression and purification of Fab

Expression of chimeric and humanized 5G1.1 Fab molecules was achieved using the mammalian 293-EBNA expression system essentially as previously described (Evans *et al.*, 1995*b*). Briefly, a plasmid capable of producing a chimeric Fab was constructed by fusion of the murine VL to the human kappa (*Inv*3 allele) constant region and fusion of the murine VH to the human IgG1 (*G1m*(17) allele) Fd in the expression plasmid pAPEX-3P. Since the cloning strategy for the 5G1.1 VL did not obtain the leader sequence, the leader sequence of the human CD59 protein was cloned in frame to allow secretion of the chimeric light chain cDNA. Similarly, a plasmid capable of expressing the CDR grafted Fab, h5G1.1 Fab (CDR), was constructed by cloning the variable regions h5G1.1VHC and h5G1.1VLC into pAPEX-3P. Finally, a plasmid capable of expressing the CDR grafted Fab containing framework changes, h5G1.1 Fab (CDR + FW), was constructed by cloning the variable regions h5G1.1VHC+F and h5G1.1VLC+F into pAPEX-3P. 293-EBNA cells (Invitrogen, San Diego, CA, U.S.A.) were transfected with the pAPEX-3P expression plasmids and selected using 1 μg/ml puromycin. Secreted Fabs were purified using protein G Sepharose chromatography as described (Evans *et al.*, 1995*b*), dialysed into PBS, and stored at 4°C. Protein concentrations were determined by the Bradford assay (Bio-Rad, Melville, NY, U.S.A.).

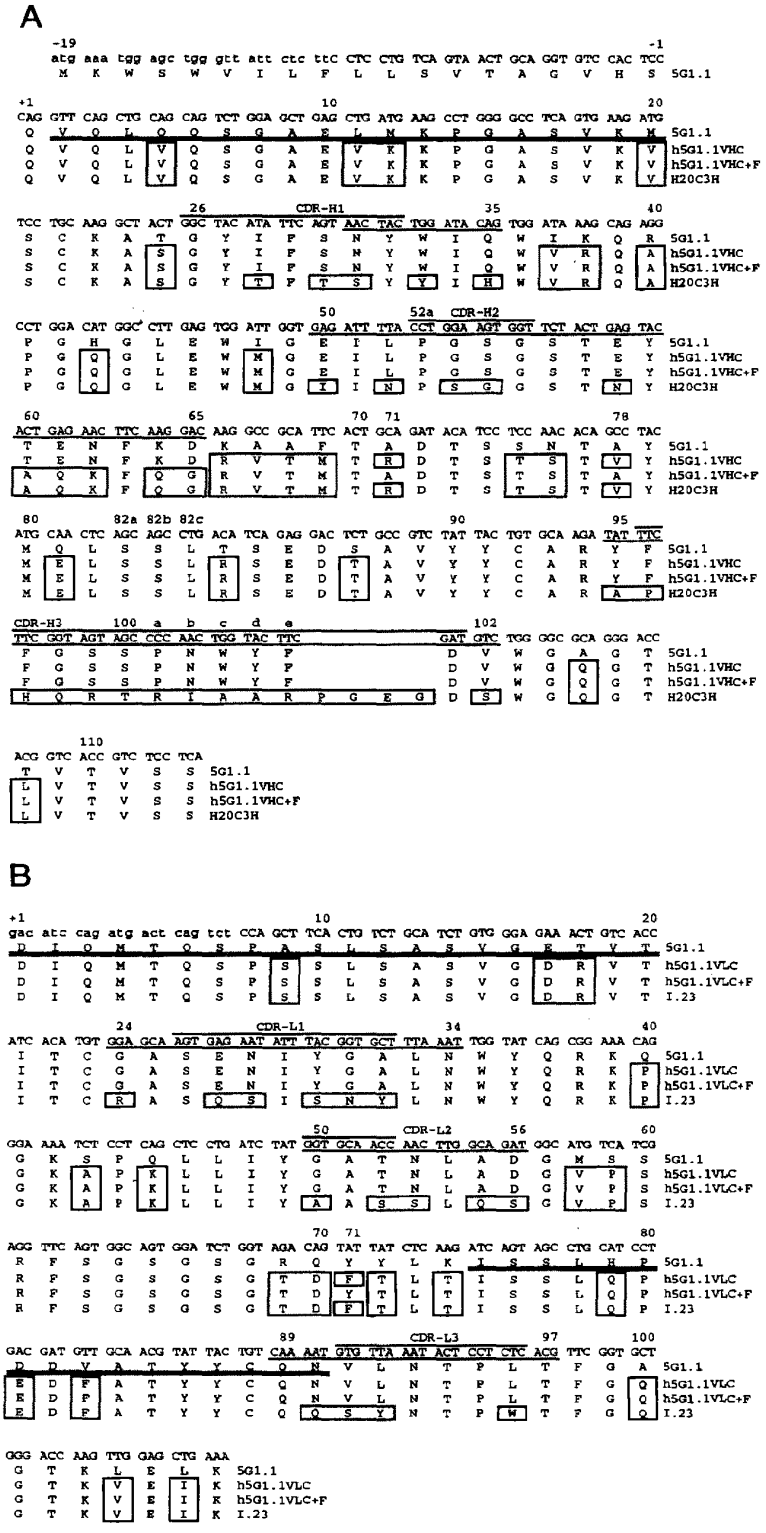
Construction of a humanized scFv

Two scFv proteins were constructed by the overlapping PCR technique. The h5G1.1 scFv (CDR) contained the h5G1.1VLC variable region, linked to the h5G1.1VHC variable region; while the h5G1.1 scFv (CDR + FW) contained the h5G1.1VLC+F variable region, linked to the h5G1.1VHC+F variable region. These scFv cDNAs were cloned into a plasmid in which expression is driven by the trc promoter. *Escerichia coli* strain ME1 was transformed with the resulting expression plasmids. Transformants were grown at 37°C in 2 l Applikon glass vessel fermentors containing Terrific Broth (1.2% (w/v) bactotryptone, 2.4% (w/v) bacto-yeast extract, 0.4% (v/v) glycerol, 90 mM potassium phosphate, pH 7.0), supplemented with 100 μg/ml ampicillin. The production of recombinant scFv was induced by the addition of 1 mM isopropylthio-β-D-galactoside when the OD_{550} of the culture reached 10. After an additional 3 hr incubation at 37°C, the cells were harvested by centrifugation and the cell pellets stored at −20°C. Cells were resuspended in 1 mM EDTA (pH 5.0), at 10 ml/g weight and lysed by a single pass through a microfluidizer M110-T (Microfluidics Corp., Newton, MA, U.S.A.). After centrifugation at 17 500**g** for 15 min, the resulting inclusion body pellet was resuspended in 20 mM Tris–HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 0.15% (w/v) deoxycholate at 10 ml/g inclusion body, using a Tekmar polytron. The inclusion bodies were again pelleted by centrifugation at 17 500***g*** for 15 min and resuspended in 20 mM Tris–HCl (pH 9.0) and 8 M urea at 10 ml/g. After stirring for 1 hr, the sample was centrifuged at 14 000***g*** for 30 min to pellet remaining insoluble material. The resulting supernatant was diluted 10-fold with 20 mM Tris–HCl (pH 9.0), 7 M urea and 50 μM cupric sulfate, and allowed to stir for at least 16 hr at 4 C to refold the scFv. Biocryl BPA-1000 (TosoHaas, Montgomeryville, PA, U.S.A.) was then added as a flocculation agent at 3 μl/ml (or at 10 μl/ml for the anti-mouse C5 scFv). After stirring for 5 min, the sample was centrifuged at 15 000***g*** for 10 min to pellet insoluble material. The supernatant was exchanged into 20 mM Tris (pH 9.0), 1 mM EDTA by diafiltration and concentrated by ultrafiltration using a stirred cell, fitted with a YM10 membrane (Amicon, Beverly, MA, U.S.A.). Properly refolded scFv was separated from aggregated material and contaminating proteins by anion exchange chromatography using Q Sepharose Fast Flow (Pharmacia). Bound scFv was eluted with 20 mM Tris–HCL (pH 9.0) and 1 mM EDTA containing a linear NaCl gradient (0–0.5 M). The fractions containing the scFv were combined, concentrated by ultrafiltration using a stirred cell fitted with a YM10 membrane and applied to a Sephacryl S200 HR 26/100 column (Pharmacia) equilibrated in 20 mM Tris–HCl, pH 9.0, 1 mM EDTA and 150 mM NaCl. Fractions containing the scFv were combined, exchanged into PBS by diafiltration, concentrated by ultrafiltration, filtered through a 0.22 mm Millex-GV filter (Millipore, Bedford, MA, U.S.A.) and stored at 4°C. Protein concentration was determined by the Bradford assay.

ELISA binding assays

For detection of recombinant Fab binding to human C5, F96 PolySorp microtiter plates (Nunc, Naperville, IL, U.S.A.) were coated overnight at 4°C with 0.1 mg/well human C5 (Quidel, San Diego, CA, U.S.A.) at a concentration of 2 mg/ml in 0.1 M Na_2CO_3, pH 9.6. The plates were then washed three times with 100 μl/well wash buffer (PBS containing with 0.5% (v/v) Tween 20) and blocked with 100 μl/well blocking buffer (PBS supplemented with 1% (w/v) bovine serum albumin, fraction V and 0.5% (v/v) Tween 20) at 37°C for 1 hr. The plates were again washed three times with wash buffer and incubated with 50 μl/well blocking buffer, containing 30 ng/ml murine 5G1.1 mAb, plus the indicated concentrations of inhibitor, at 37°C for 2 hr. The plates were

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again washed three times with wash buffer, and incubated with 50 μl/well blocking buffer, containing peroxidase-conjugated goat anti-mouse IgG Fc antibody, at a 2000-fold dilution (Sigma, St. Louis, MO, U.S.A.), at 37°C for 1 hr. After three final washes, the plate was developed with 50 μl/well substrate buffer (0.05 M phosphate–citrate buffer, pH 5.0, containing 0.3 mg/ml sodium perborate and 0.4 mg/ml *o*-phenylenediamine dihydrochloride). Reactions were stopped by the addition of 50 μl/well 1 M sulfuric acid and quantified using a Bio-Rad model 3550 plate reader set at 490 nm.

Hemolytic and C5a generation assays

The antibody, Fab, or scFv was pre-incubated with the indicated percent (v/v) human serum in 0.1 ml veronal buffered saline containing 1% gelatin ($GVBS^{++}$) for 30 min at room temperature. Chicken erythrocytes (Lampire Biological, Pipersville, PA, U.S.A.) were washed four times with $GVBS^{++}$ and resuspended at 5×10^7 cells/ml in $GVBS^{++}$, containing 1 μg/ml anti-chicken erythrocyte IgG (Inter-cell Technologies, Hopewell, NJ, U.S.A.). After incubation at 4°C for 15 min, the cells were washed twice with $GVBS^{++}$ and 3×10^6 cells were added to the pre-incubated human serum. After further incubation for 30 min at 37°C, the cells were pelleted by centrifugation at 10 000*g* for 2 min and the supernatant was assayed for released hemoglobin by measurement of the absorbance at 415 nm, and for C5a content by sandwich ELISA as described (Würzner *et al.*, 1991).

Porcine aortic endothelial cell (PAEC) lysis assays

PAEC were obtained from Cell Systems (Kirkland, WA, U.S.A.) at passage 1 and maintained in M199 medium, supplemented with 20% fetal calf serum, 20 mM HEPES, 10 μg/ml heparin (Sigma) and 5 μg/ml ECGF (Biomedical Technologies, Stoughton, MA, U.S.A.). PAECs at passage 3 were plated at 10 000 cells/well in a 96-well Falcon flat-bottomed microtiter plate. The following day, cells were washed twice with Hank's balanced salt solution, containing 1% (w/v) bovine serum albumin (HBSS/BSA) and incubated with 10 μM calcein AM (Molecular Probes, Eugene, OR, U.S.A.) in HBSS/BSA for 30 min at 37°C. Wells were washed twice with HBSS/BSA and incubated with 0.85 mg/ml rabbit anti-PAEC antibody (Kennedy *et al.*, 1994) for 30 min at 37°C. Wells were again washed twice with HBSS/BSA and incubated with HBSS containing 20% (v/v) human serum plus various concentrations of inhibitors for 30 min at 37°C. The supernatant was then transferred to a new 96-well Falcon flat-bottom microtiter plate and the remaining cells were solubilized with 1% SDS. The fluorescence of the supernatants and solubilized cells was determined using a Cytofluor 2350 (Millipore) with an excitation wavelength of 485 nm and an emmision wavelength of 530 nm. Percentage dye release was defined as the ratio of supernatant fluorescence to total (supernatant plus cell) fluorescence. Supernatant fluorescence in the absence of human serum was subtracted as background release.

RESULTS

Humanization of 5G1.1

The variable regions of the anti-C5 monoclonal antibody 5G1.1 were cloned by PCR amplification using 5′ primers, based on amino acid sequencing (for the light variable region) or degenerate 5′ primers (for the heavy variable region), coupled with 3′ primers specific for murine constant region sequences. Amino acid sequence analysis confirmed that the appropriate heavy and light variable regions had been cloned (Fig. 1). The 5G1.1 heavy variable region was most similar to V_H genes belonging to the V_H1/J558 family (Kofler *et al.*, 1992) and utilized the J_H1 gene. The most similar cloned murine V_H region was from the IgM secreting hybridoma 19.1.2 (Akolkar *et al.*, 1987). The 5G1.1 V_H differed from this clone at positions 20, 37 and 68 in framework regions, as well as at positions 28, 31 and 35 in CDR-H1, positions 58 and 65 in CDR-H2 and positions 95, 96 and 97 in CDR-H3. The 5G1.1 light variable region was most similar to the NYC V_κ gene (Jack *et al.*, 1992) and utilized the mouse $J_\kappa5$ gene (Hieter *et al.*, 1980), with amino acid differences present at framework position 72 in framework region 3 and at position 93 in CDR-L3.

For humanization, the 5G1.1 heavy variable regions CDRs were transposed into the human variable region

Fig. 1. Sequence of the 5G1.1 heavy (A) and light (B) variable regions. The DNA sequence and the translated amino acid sequence of the cloned 5G1.1 variable regions are shown. Amino acid position is numbered according to Kabat *et al.* (1992), with the complementarity determining regions according to the hypervariable sequence definition (Kabat *et al.*, 1992) or the structural variability definition (Chothia and Lesk, 1987) underlined and overlined, respectively. Lower case letters indicate nucleotide sequences derived from primers used for cloning. Amino acid sequences obtained from protein sequencing are indicated by double underlines. The protein sequences of the human variable regions H20C3H and I.23 are shown below the appropriate 5G1.1 variable regions. h5G1.1VHC and h5G1.1VLC denote humanized heavy and light variable regions constructed by grafting the CDRs from 5G1.1 on to the H20C3H and I.23 human framework regions. The variable regions h5G1.1 VHC+F and h5G1.1VLC+F contain murine amino acids at framework positions 71 and 78 in the heavy variable region and position 71 in the light variable region in addition to the murine CDR sequences. Amino acids in the human variable regions and the humanized 5G1.1 variable regions which differ from the murine 5G1.1 sequences are boxed.

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H20C3H (Weng *et al.*, 1992). This human V_H was derived from the human genomic V_H gene HG3 (Rechavi *et al.*, 1983), belonging to the V_H1 family and the human genomic J_H5 gene, and contains no changes in the framework regions from these genomic genes. The 5G1.1 light variable region CDRs were grafted into the human light variable region I.23 (Klein *et al.*, 1993). This human V_L region was derived from the human $V_\kappa I$ family gene O12 (Klein *et al.*, 1993) and the genomic $J_\kappa 1$ gene, with the introduction of an Arg residue in framework region 2 at position 38 in the I.23 cDNA as compared to the Gln residue encoded in the O12 genomic gene. Initial humanized 5G1.1 variable heavy and light regions were constructed by introducing the 5G1.1 CDRs into these human frameworks and are designated as h5G1.1 VHC and 5G1.1 VLC (Fig. 1).

Computer modeling of antibody variable regions has indicated that amino acid residues within the framework regions of a variable region can influence the structure of the CDRs. Comparison of the murine 5G1.1 variable region with the human acceptor variable regions H20C3H and I.23 suggested that the murine and human framework regions differed at three positions important for CDR structure. In the light chain, the presence of either Phe or Tyr at framework position 71 determines the canonical structure of CDR-L1 (Chothia and Lesk, 1987; Foote and Winter, 1992). Since I.23 contains a Phe residueat position 71, whereas 5G1.1 contains a Tyr residue, the Phe residue in h5G1.1 VLC was changed to a Tyr residue to create the variable region h5G1.1 VLC+F (Fig. 1). Similarly, the human H20C3H and murine 5G1.1 V_H regions differ at framework positions 71 and 78, which have been suggested to be important for maintaining the function of humanized antibodies (Carter *et al.*, 1992; Chothia *et al.*, 1989; Tramontano *et al.*, 1990; Foote and Winter, 1992). The murine amino acids were, therefore, introduced at these positions to create the variable region h5G1.1 VHC+F (Fig. 1).

Since choice of human antibody isotype can influence antibody avidity whereas isotype choice does not influence Fab affinity (Morelock *et al.*, 1994), initial analysis of the humanization procedure was performed using humanized Fab fragments. The murine 5G1.1 V_L and its humanized variants h5G1.1VLC and h5G1.1VLC+F were fused to the human kappa constant region (Inv3 allele), to create an intact light chain. The 5G1.1 V_H and its humanized variants h5G1.1VHC and h5G1.1VHC+F were fused to constant region 1 of human IgG1 (*G1m*(17) allele) to create an intact Fd. Three humanized Fabs were produced in 293-EBNA cells

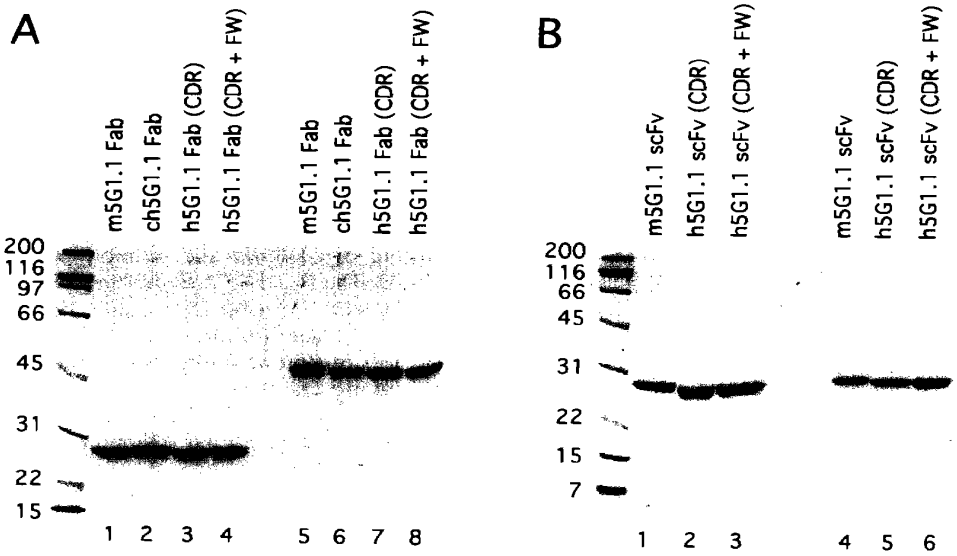


Fig. 2. SDS–PAGE analysis of purified recombinant proteins. (A) Fab fragments from the murine 5G1.1 mAb (m5G1.1 Fab) were produced by papain digestion of the m5G1.1 mAb followed by protein A chromatography to remove undigested antibody and Fc fragments. Recombinant chimeric Fab (ch5G1.1 Fab), CDR-grafted Fab (h5G1.1 Fab (CDR)), or CDR-grafted Fab containing murine framework amino acids (h5G1.1 Fab (CDR + FW)) were produced in 293 cells and purified from conditioned, serum-free medium using protein G-sepharose chromatography. Protein samples (10 mg) were subjected to SDS–PAGE under reducing (lanes 1–4) or non-reducing conditions (5–8) and stained with Coomassie R-250. (B) scFv molecules containing the murine variable regions (m5G1.1 scFv), the CDR-grafted variable regions (h5G1.1 scFv (CDR)), or the CDR-grafted variable regions containing murine framework amino acids (h5G1.1 scFv (CDR + FW)) were produced in *E. coli* and purified, as described in Materials and Methods. Ten micrograms of each purified scFv was subjected to SDS–PAGE under reducing (lanes 1–3) or non-reducing (lanes 4–6) conditions and stained with Coomassie R-250.

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by co-expression of light chain and Fd: (1) ch5G1.1 Fab, a recombinant chimeric Fab containing the murine 5G1.1 variable regions; (2) h5G1.1 Fab (CDR), a recombinant humanized Fab containing the h5G1.1VHC and h5G1.1VLC variable regions; and (3) h5G1.1 Fab (CDR + FW), a recombinant humanized Fab containing the h5G1.1VHC+F and h5G1.1VLC+F variable regions. SDS–PAGE analysis of the purified recombinant Fabs revealed a single band, which comigrated with Fab produced from 5G1.1 under non-reducing conditions and resolved into stoichiometric amounts of Fd and light chain bands, under reducing conditions (Fig. 2).

Binding of recombinant Fab and scFv to human C5

The affinities of Fab produced by papain digestion of the 5G1.1 mAb and the recombinant humanized Fabs were initially compared in a competitive binding ELISA, in which increasing concentrations of test Fab were used to displace murine 5G1.1 mAb binding to human C5 (Fig. 3). Biochemically produced Fab (m5G1.1 Fab) inhibited binding with an IC_{50} of approximately 2 nM. Similarly, the recombinant ch5G1.1 Fab, h5G1.1 Fab (CDR) and h5G1.1 (CDR+ FW) all inhibited binding by 50% at approximately 2 nM.

Although the humanized Fabs retained biological activity, neither the h5G1.1 Fab (CDR) nor the h5G1.1 Fab (CDR + FW) could be produced in significant quantities in *E. coli* (data not shown). Three scFv molecules were, therefore, constructed (Fig. 4); (1) m5G1.1 scFv, containing the murine variable light region and murine variable heavy region joined by a linker; (2) h5G1.1 scFv (CDR), containing the h5G1.1VHC and h5G1.1VLC variable regions; and (3) h5G1.1 scFv (CDR + FW), containing the h5G1.1VHC+F and h5G1.1VLC+F variable regions. These scFv molecules were produced as inclusion bodies in *E. coli*, refolded, and purified by anion exchange and sizing chromatography (Fig. 2). Gel filtration chromatography coupled with light scattering analysis demonstrated that at a concentration of 2.0 mg/ml (75 μM) the purified h5G1.1 scFv (CDR) was predominantly in the form of dimers. However, dilution of the h5G1.1 scFv resulted in a rapid shift of the equilibrium towards the monomer state (Thomas *et al.*, 1996). In the competition ELISA analysis, the m5G1.1 scFv inhibited binding of 5G1.1 mAb to human C5 with an IC_{50} value of 2 nM (Fig. 5), identical to the value obtained for the chimeric Fab (Fig. 3). Similarly, both the h5G1.1 scFv (CDR) and h5G1.1 scFv (CDR + FW) inhibited 5G1.1 mAb binding with IC_{50} values of 1.5 and 2 nM, respectively. In contrast, an scFv specific for mouse C5 (anti-mC5, Fig. 5) did not inhibit 5G1.1 binding, even at the highest concentrations assayed. Together these results indicated that the refolded scFv molecules bound to human C5 with an affinity equivalent to the Fab molecules produced in mammalian cells. Furthermore, no effect of the framework alterations could be discerned, based on the binding affinity of either the Fab or scFv molecules.

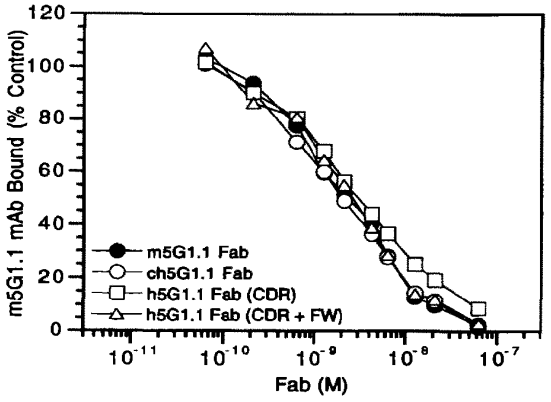


Fig. 3. Inhibition of 5G1.1 mAb binding to human C5 by recombinant Fab. 5G1.1 (0.2 nM) mAb plus the indicated concentrations of recombinant Fab were incubated together, for 2 hr at 37°C in microtiter plates coated with human C5. After washing, bound 5G1.1 mAb was detected using peroxidase-conjugated goat anti-mouse IgG (Fc specific) with quantitation performed by monitoring hydrolysis of *o*-phenylenediamine dihydrochloride at 490 nm. One hundred percent binding was defined in each experiment as the signal produced by binding of 5G1.1 mAb in the absence of competitor Fab. Data shown are mean values obtained from four to eight independent assays, with each datum point assayed in duplicate in each experiment.

Inhibition of C5b-9 formation by humanized Fab and scFv

The ability of the humanized Fab and scFv proteins to inhibit the formation of the C5b-9 membrane attack complex was evaluated using a hemolytic assay, in which the deposition of membrane attack complex on antibody-sensitized chicken erythrocytes is assessed by measuring hemoglobin release. Hemolysis was inhibited by greater than 90% when 0.25 μM of biochemically prepared murine 5G1.1 Fab, or each of the recombinant Fab molecules were added to the assay (Fig. 6A). This concentration corresponds to an approximate three-fold molar excess of Fab, as compared to the predicted human C5 concentration (0.08 μM) present in the assay. As with the Fab molecules, the scFv molecules also inhibited chicken erythrocyte hemolysis when present at a three-fold molar excess (Fig. 6B). The inhibition curves obtained with the scFv molecules were identical to those obtained with the Fab (Fig. 6B). The ability of the scFv to protect mammalian cells from complement-mediated damage was assessed by measuring their ability to block the lysis of antibody-coated porcine aortic endothelial cells (PAEC) by 20% human serum. The m5G1.1 scFv, h5G1.1 scFv (CDR) and h5G1.1 scFv (CDR + FW) all inhibited lysis of the PAEC when present at a three-fold molar excess, while the scFv specific for mouse C5 did not inhibit lysis, even at the highest concentration tested (Fig. 7A). The specificity of the 5G1.1 mAb for human C5 was maintained by the scFv, as neither the 5G1.1

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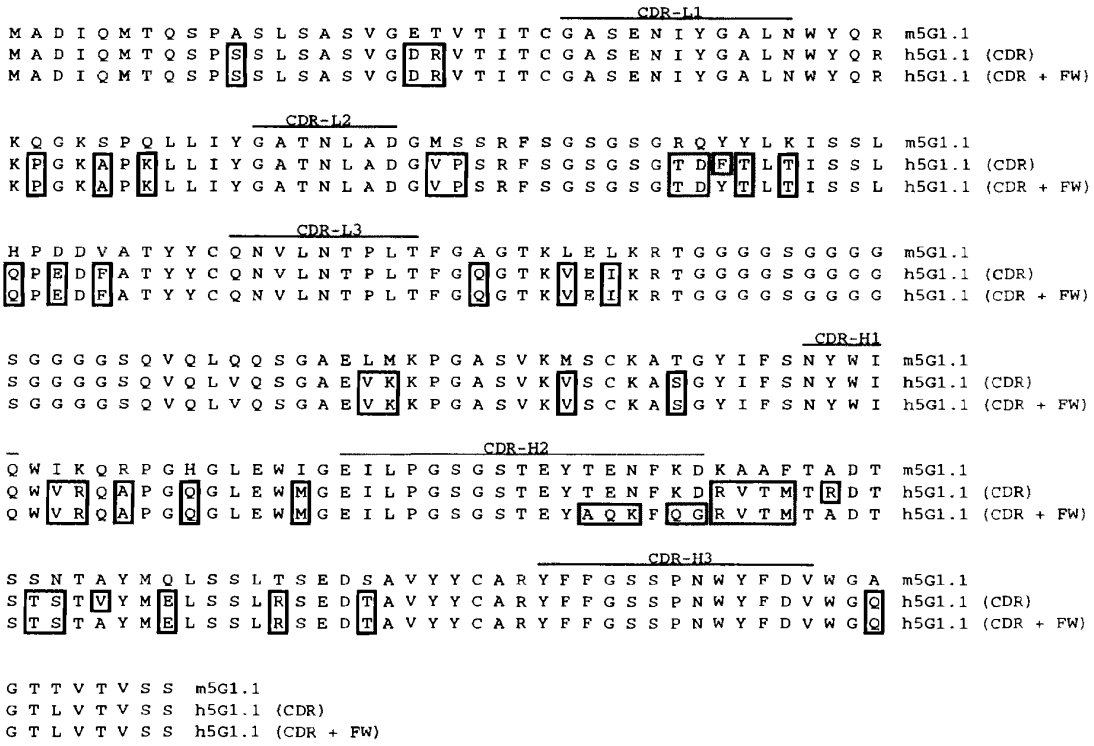


Fig. 4. Sequence of scFv molecules constructed using the 5G1.1 murine variable regions (m5G1.1) or humanized variable regions. The h5G1.1 (CDR) scFv contains the variable regions h5G1.1 VLC and h5G1.1 VHC, while the h5G1.1 scFv (CDR + FW) contains the variable regions h5G1.1 VLC+F and h5G1.1 VHC+F. The positions of the CDRs, as defined by Kabat *et al.* (1992), are indicated. Amino acid sequence analysis indicated removal of the initiator methionine, with the alanine residue at position two being the amino terminal residue of the bacterially produced scFv molecules. Amino acids in the humanized 5G1.1 variable regions which differ from the murine 5G1.1 sequences are boxed.

scFv, the h5G1.1 scFv (CDR) nor the h5G1.1 scFv (CDR + FW) could inhibit the lysis of PAEC by rat serum (Fig. 7B).

A critical parameter for use of an scFv as a C5 inhibitor is retention of the ability to block C5a generation. Previously, it has been found that a chimeric Fab, constructed from the monoclonal antibody N19-8 retained the ability to block both the formation of C5b-9 and generation of C5a, whereas an scFv constructed using the N19-8 variable regions retained the ability to block the formation of C5b-9, but only weakly inhibited the generation of C5a (Evans *et al.*, 1995). In contrast, analysis of supernatants obtained from the chicken erythrocyte hemolytic assays for C5a content (Fig. 8) indicated that equivalent concentrations of the humanized 5G1.1 Fab or scFv molecules blocked the generation of C5a. The concentration of inhibitor required for inhibition of C5a generation (Fig. 8) was comparable to the concentration of inhibitor required for inhibition of lysis (Fig. 6B).

Constuction of a humanized h5G1.1 antibody

Having demonstrated the effective humanization of the 5G1.1 variable regions, an intact humanized antibody (IgG4 isotype) was constructed and produced in 293-EBNA cells. The avidity of this humanized antibody (h5G1.1 HuG4) for human C5, was compared to the murine 5G1.1 mAb by determining the ability of each to compete binding of biotinylated 5G1.1 mAb to C5 (Fig. 9). The humanized h5G1.1 mAb had a two-fold lower avidity than the murine antibody. However, the humanized h5G1.1 HuG4 antibody was equipotent with the murine antibody at protecting PAEC from lysis by human serum, with a 0.5-fold molar ratio of antibody to C5 (1:1 ratio of antibody binding sites to C5) completely inhibiting lysis of the PAEC (Fig. 10).

DISCUSSION

This article describes the successful humanization of the anti-human C5 specific monoclonal antibody 5G1.1, utilizing the CDR-grafting technique. Although the original description of the CDR-grafting technique described the maintenance of antibody specificity by the transfer of the CDRs alone (Riechmann *et al.*, 1988), in most cases the transfer of a number of murine framework amino acids in addition to the CDRs has been found to be

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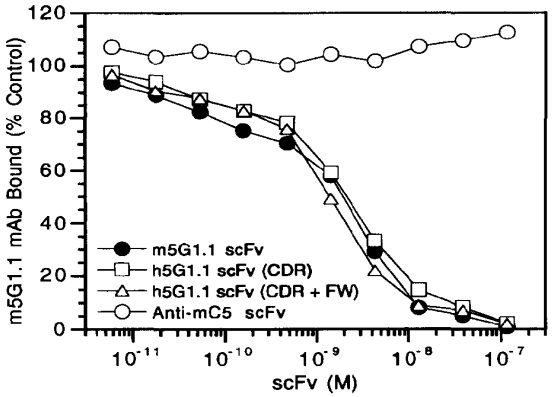


Fig. 5. Inhibition of 5G1.1 mAb binding to human C5 by recombinant scFv. 5G1.1 mAb (0.2 nM) plus the indicated concentrations of recombinant scFv were incubated together for 2 hr at 37°C, in microtiter plates coated directly with human C5. After washing, bound 5G1.1 mAb was detected using peroxidase-conjugated goat anti-mouse IgG (Fc specific) with quantitation performed by monitoring hydrolysis of *o*-phenylenediamine dihydrochloride at 490 nm. One hundred percent binding was defined in each experiment as the signal produced by binding of 5G1.1 mAb in the absence of competitor scFv. Data shown are mean values obtained from four to 10 independent assays, with each datum point assayed in duplicate in each experiment.

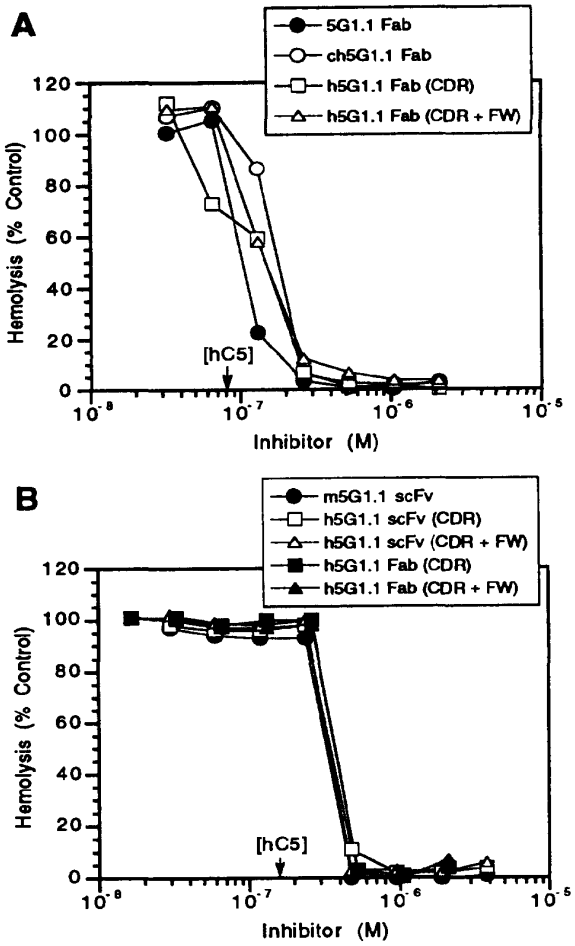


Fig. 6. Inhibition of chicken erythrocyte lysis by recombinant Fab and scFv. Twenty percent (A), or 40% (B), human serum in $GVBS^{++}$ was pre-incubated for 30 min at room temperature, with the indicated concentrations of competitors. Chicken erythrocytes, precoated with rabbit anti-chicken erythrocyte antibody, were added and the incubation was continued for 30 min at 37°C. The erythrocytes were then pelleted and the supernatants assayed for released hemoglobin by spectrophotometry (A_{415}). The A_{415} absorbance value obtained in the absence of inhibitor was defined as 100% hemolysis. Data shown are the average results of two to four independent determinations. The predicted hC5 concentrations in 20% and 40% serum are indicated by arrows.

necessary for retention of high affinity binding to the antigen (Winter and Milstein, 1991). These framework amino acids are believed to influence the structure of the CDRs. In particular, heavy variable region framework amino acid 71 determines the canonical structure of CDR-H2 (Tramontano *et al.*, 1990), while light variable region framework amino acid 71 determines the canonical structure of CDR-L1 (Chothia and Lesk, 1987; Foote and Winter, 1992). The human variable regions chosen for grafting of the 5G1.1 CDRs differed from the murine variable regions at heavy region framework position 71 and light region framework position 71. Therefore, humanized variable regions were constructed and designated h5G1.1 (CDR + FW), which maintained the murine amino acids at both of these positions.

The importance of these framework amino acids on the humanization process was initially assessed using recombinant Fab produced in 293 cells. Both the Fab containing the framework changes, h5G1.1 Fab (CDR + FW) and the Fab with unmodified human framework regions, h5G1.1 Fab (CDR), had the same binding affinity as murine Fab, when assayed by competition ELISA (Fig. 3). Similarly, no difference could be discerned between h5G1.1 Fab (CDR), h5G1.1 Fab (CDR + FW) and the murine Fab in their ability to inhibit human complement-mediated lysis of chicken erythrocytes (Fig. 6A). The h5G1.1 Fab (CDR) and h5G1.1 Fab (CDR + FW) were also equivalent in their ability to block the generation of C5a (Fig. 8). Similarly, bacterial-produced, refolded scFv, containing either human (h5G1.1 scFv (CDR)) or murine (h5G1.1 scFv (CDR + FW)) amino acids at these framework positions, had identical affinities as measured by ELISA (Fig. 5) and inhibited chicken erythrocyte lysis (Fig. 6), porcine aortic endothelial cell lysis (Fig. 7) and C5a generation (Fig. 8) with equivalent potencies. Therefore, although computer modeling suggests the importance of these framework amino acids in CDR canonical structure, amino acid changes at these positions had no effect on the functional activity of the humanized 5G1.1 molecules.

A clinically useful C5 inhibitor must block generation of both C5a and C5b-9, as both of these molecules have

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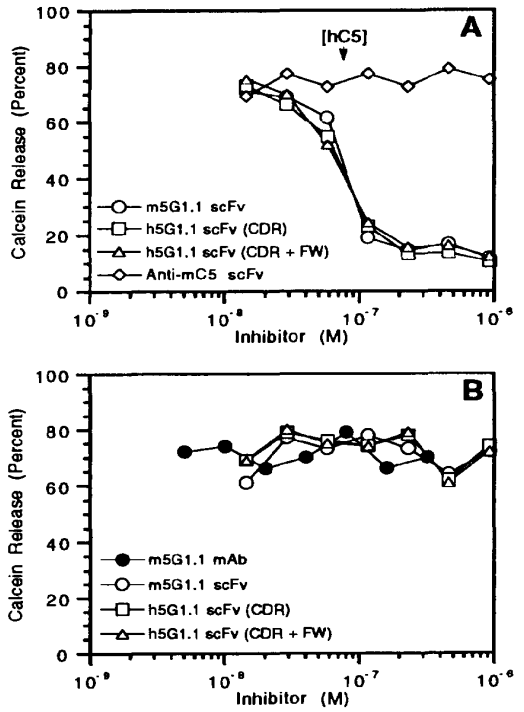


Fig. 7. Protection of porcine aortic endothelial cells (PAEC) from human serum complement by scFv. Calcein-labeled PAEC were incubated with HBSS containing 1% BSA and 0.85 mg/ml purified rabbit anti-PAEC polyclonal antibody for 30 min at 37°C. The cells were washed and incubated with HBSS containing 1% BSA plus 20% human serum (A), or 20% rat serum (B), and the indicated concentrations of inhibitor for 30 min at 37°C. Calcein dye release and retention were determined by measurement of the fluorescence of supernatants and SDS-solubilized cells, respectively, with dye release in the absence of human serum subtracted from all values. Results shown are the average of four to five independent determinations. The arrow denotes the predicted concentration of hC5 in 20% serum.

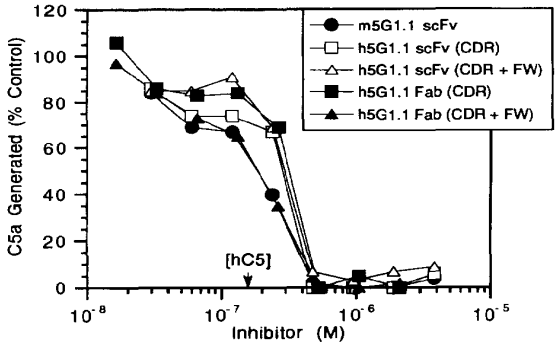


Fig. 8. Inhibition of C5a generation by recombinant Fab and scFv. Supernatants from chicken erythrocyte hemolytic assays performed using 40% human serum, as described in Fig. 5, were assayed for C5a content by ELISA. The C5a concentration obtained in the absence of inhibitor was defined as 100%. Data shown are from a representative experiment of several performed. The predicted hC5 concentration in 40% serum is indicated by the arrow.

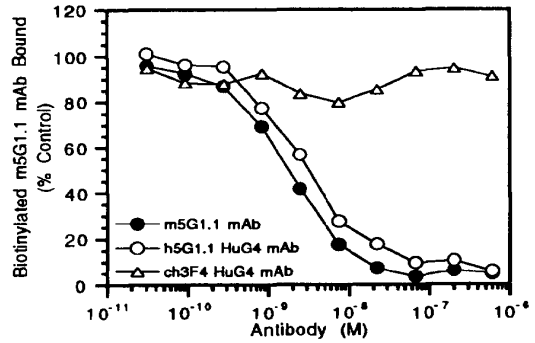


Fig. 9. Inhibition of 5G1.1 mAb binding to human C5 by recombinant humanized h5G1.1 HuG4 mAb. Biotinylated 5G1.1 mAb (0.2 nM), plus the indicated concentrations of unlabeled murine 5G1.1 mAb, recombinant h5G1.1 HuG4 mAb or a negative control recombinant HuG4 mAb (ch3F4 HuG4 mAb), were incubated together for 2 hr at 37°C in microtiter plates coated with human C5. After washing, bound biotinylated 5G1.1 mAb was detected using peroxidase-conjugated streptavidin, with quantitation performed by monitoring hydrolysis of *o*-phenylenediamine dihydrochloride at 490 nm. Absorbance values obtained in the presence of recombinant inhibitor were normalized in each individual experiment, to the value obtained in the absence of competitor. Results shown are mean values of four determinations.

potent pro-inflammatory effects. An scFv has been previously constructed from the variable regions of the monoclonal antibody N19-8 (Würzner *et al.*, 1991). The N19-8 antibody binds to human C5 with an avidity comparable to 5G1.1 (data not shown) and blocks generation of both C5b-9 and C5a. Although the N19-8 scFv has a high binding affinity for human C5 and inhibits the ability of human serum to lyse chicken erythrocytes, it only weakly inhibits C5a generation (Evans *et al.*, 1995). These findings suggest a model in which binding of the N19-8 mAb sterically blocks interaction of C5 with the C5 convertases. The N19-8 scFv may be too small to block cleavage by the C5 convertases, but remains bound to C5b and blocks formation of the C5b-9 complex. In contrast, the h5G1.1 scFv completely blocked generation of C5a when present at only a three-fold molar excess, relative to human C5 (Fig. 8). The 5G1.1 epitope may, therefore, be more directly involved in the interaction of C5 with the C5 convertases, than is the N19-8 epitope. Additionally, the h5G1.1 scFv maintains the ability to block the induction of both C5a- and C5b-dependent pro-inflammatory events in a closed loop model of cardiopulmonary bypass (S. A. Rollins, unpublished results).

Three important attributes of the humanized h5G1.1 scFv suggests its value as a therapeutic. Firstly, it main-

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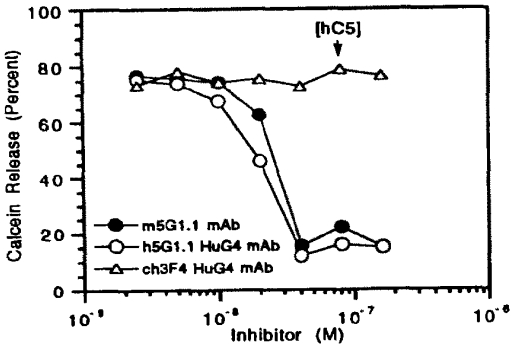


Fig. 10. Protection of porcine aortic endothelial cells from human serum complement by h5G1.1 HuG4 mAb. Calcein-labeled PAEC were incubated with HBSS containing 1% BSA and 0.85 mg/ml purified rabbit anti-PAEC polyclonal antibody for 30 min at 37°C. The cells were washed and incubated with HBSS containing 1% BSA plus 20% human serum and the indicated concentrations of inhibitor for 30 min at 37°C. Calcein dye release and retention were determined by measurement of the fluorescence of supernatants and SDS-solubilized cells, respectively, with dye release in the absence of human serum subtracted from all values. Results shown are the mean of three independent determinations. The arrow denotes the predicted concentration of hC5 in 20% serum.

tains the essential ability to block cleavage of C5 while preserving the immunoprotective properties associated with C3b generation. Secondly, it can be readily produced in large quantities in *E. coli*. Finally, the pharmacokinetics of scFv molecules are well matched to the time frame necessary for complement inhibition in acute settings where complement activation contributes significantly to the inflammatory process, such as cardiopulmonary bypass or myocardial infarction. Clearance of scFv molecules from the blood has previously been found to be extremely rapid with half lives of only a few minutes (Colcher *et al.*, 1990; Milenic *et al.*, 1991). At least two features of scFv molecules are likely to result in a rapid clearance rate, as compared to intact antibodies. Firstly, the prolonged plasma half-life of IgG is mediated through binding of the Fc region to specific "protection receptors", which have recently been demonstrated to be identical to the neonatal intestinal transport receptor (Ghetie *et al.*, 1996; Junghans and Anderson, 1996). Secondly, the small size of the scFv is well below the M_r 60 000 cut off of the glomerular basement membrane, resulting in the loss of the scFv into the urine. However, the binding of the scFv to C5 in the blood results in its incorporation into a high molecular weight scFv–C5 complex of nearly 220 000. Loss of bound scFv through glomerular filtration is, thus, dependent on the dissociation rate of the scFv from C5. Calculations based on the competitive ELISA data (Fig. 5), as well as direct measurements of h5G1.1 scFv (CDR) binding to human C5 by surface plasmon resonance (data not shown), indicated a very high affinity of the scFv for C5 ($K_d = 100$ pM) and a slow dissociation rate ($k_{off} = 1.0 \times 10^{-4}$/sec). This dissociation rate corresponds to an scFv–C5 complex half-life of approximately 2 hr. The 5G1.1 monoclonal antibody recognizes only human C5, precluding a direct assessment of h5G1.1 scFv pharmacokinetics in primates. In contrast, the N19-8 monoclonal antibody, which binds to human C5 with an avidity equivalent to 5G1.1, does bind to rhesus monkey C5. A single 100 mg bolus injection of N19-8 scFv into rhesus monkeys inhibits serum hemolytic activity for approximately 2 hr (Evans *et al.*, 1995b), further suggesting that the h5G1.1 scFv will have *in vivo* kinetic properties sufficient for use in acute clinical settings.

Complement activation has been suggested to be involved in the pathogenesis of several chronic human diseases including allograft rejection (reviewed by Baldwin *et al.* (1995)), systemic lupus erythematosus (reviewed by Mills (1994)), myasthenia gravis (reviewed by Drachman (1994)) and rheumatoid arthritis (reviewed by Morgan (1990)). Recently, it has been shown that prolonged treatment with an antibody to mouse C5 both blocks onset and disease progression in a collagen-induced arthritis model (Wang *et al.*, 1995), as well as inhibits development of glomerulonephritis in NZB/W mice (Wang *et al.*, 1996). As the half-life of the h5G1.1 scFv is likely to be too short to allow for chronic use in humans, a full length humanized antibody was also constructed. The human IgG4 isotype was chosen, as this isotype does not activate human complement (Tao *et al.*, 1993) and there is only one known allotype of IgG4 (Ghanem *et al.*, 1988), precluding the potential development of allo-antibodies in patients. The humanized h5G1.1 (CDR) HuG4 antibody bound to human C5 with a similar avidity as the murine antibody when assayed by ELISA (Fig. 9) and inhibited lysis of porcine aortic endothelial cells as effectively as the murine antibody, with a 1:1 molar ratio of antibody binding sites to human C5 being sufficient for inhibition (Fig. 10). Little information is available on the immunogenicity of CDR-grafted antibodies in humans. Repeated administration of chimeric antibodies containing intact murine variable regions, has induced an immune response directed against the murine variable regions in nearly all trials (reviewed in Khazaeli *et al.* (1994)). In this regard, it is significant that introduction of murine amino acids in the framework regions was not essential for maintenance of high affinity binding to C5. The h5G1.1 antibody is, therefore, likely to be minimally immunogenic in patients.

The recent successes of complement inhibitors in modulating numerous *in vivo* models of inflammation, suggest a great potential for complement inhibitors in the treatment of human inflammatory disease. The development of distinct C5-specific complement inhibitors with pharmacokinetic parameters, tailored for use in either acute or chronic settings, should facilitate the clinical assessment of the role of complement in such diseases.

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BESCHWERDEKAMMERN DES EUROPÄISCHEN PATENTAMTS

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Datasheet for the decision of 20 September 2011

Case Number: T 1979/09 - 3.3.09

Application Number: 03000403.0

Publication Number: 1300084

IPC: A23L 1/30

Language of the proceedings: EN

Title of invention:
Composition for the treatment of chronic venous insufficiencies comprising an extract of red vine leaves

Patentee:
Boehringer Ingelheim International GmbH

Opponent:
Frutarom Schweiz AG

Headword:
-

Relevant legal provisions:
EPC Art. 83, 54, 56

Relevant legal provisions (EPC 1973):
-

Keyword:
"Inventive step (no - main and auxiliary requests)"

Decisions cited:
T 1416/07

Catchword:
-

Case Number: T 1979/09 - 3.3.09

D E C I S I O N
of the Technical Board of Appeal 3.3.09
of 20 September 2011

Appellant: (Opponent)	Frutarom Schweiz AG Rütiwisstrasse CH-8820 Wädenswil (CH)
Representative:	Zink-Wild, Markus Peter Patentanwaltsbüro Zink Birchlistrasse 11 CH-8173 Riedt-Neerach (Zürich) (CH)
Respondent: (Patent Proprietor)	Boehringer Ingelheim International GmbH D-55216 Ingelheim am Rhein (DE)
Representative:	von Kreisler Selting Werner Deichmannhaus am Dom Bahnhofsvorplatz 1 D-50667 Köln (DE)
Decision under appeal:	**Interlocutory decision of the Opposition Division of the European Patent Office posted 14 July 2009 concerning maintenance of European patent No. 1300084 in amended form.**

Composition of the Board:

Chairman: W. Sieber
Members: J. Jardón Álvarez
F. Blumer

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Summary of Facts and Submissions

I. The mention of the grant of European patent No. 1 300 084, in respect of European patent application No. 03000403.0, in the name of Boehringer Ingelheim International GmbH, filed on 19 October 2000 as a divisional application of the earlier European patent application no. 00974420.2, was published on 19 April 2006 (Bulletin 2006/16). The granted patent contained 10 claims, whereby claim 1 read as follows:

"1. A composition in a form suitable for oral administration which consists of an active principle being capable of preventing or treating the discomfort associated with mild-to-moderate chronic venous insufficiency of the lower extremities and a pharmaceutically, cosmetically or dietetically acceptable carrier, the improvement wherein is that said active principle consists of an aqueous extract of red vine leaves containing 2 to 20% flavonoids, wherein said aqueous extract of red vine leaves is obtainable by a method comprising the steps of:

(a) collecting red vine leaves at a point of time when the content in flavonoids has reached an optimum;
(b) drying and crushing the leaves;
(c) cutting the leaves to pieces;
(d) extracting the leaves with water at temperatures from 60 to 80°C for 6 to 10 hours in an exhaustive percolation;
(e) optionally concentrating the obtained extract;

characterized in that said composition contains an amount of said aqueous extract of red vine leaves,

which corresponds to a daily dosage of 300-800 mg of the extract."

Claims 2 to 10 were dependent claims.

II. A notice of opposition was filed by Frutarom Schweiz AG on 29 December 2006 requesting the revocation of the patent in its entirety on the grounds of Articles 100(a) (lack of novelty and lack of inventive step), (b) and (c) EPC.

During the opposition proceedings, *inter alia*, the following documents were cited:

D1: Ministère des Affaires Sociales et de la Solidarité, Bulletin Officiel, No 90/22 bis, "Médicaments à base de plantes", 1990, pages 24, 25, 36 and 37;

D4: Monographies de la 10e édition de la Pharmacopée Française et du Formulaire National (FN) en vigueur à la date du 1er janvier 1996, Extrait de vigne rouge (sec) (altogether 4 pages);

D5: Rote Liste 1998, Venentherapeutika, 83 048 and 83 084; "Antistax®";

D6: H. Beck, "Rotes Weinlaub von A bis Z", PTA heute, Nr. 8, August 1997, pages 792-796; and

D28: Frutarom Switzerland LTD, Versuchsprotokoll VENT, dated 11 July 2005 (6 pages).

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III. By its interlocutory decision announced orally on 17 June 2009 and issued in writing on 14 July 2009, the opposition division held that the grounds for opposition did not prejudice the maintenance of the patent in amended form on the basis of claims 1 to 9 according to auxiliary request 1.

Claim 1 of auxiliary request 1 was based on granted claim 1 (see point I above) wherein the expression "A composition" had been amended to read "A dietary supplement composition" based on granted claim 8.

In view of the experimental results filed by the opponent (cf. D28), the opposition division was convinced beyond reasonable doubt that the method of D4 provided an extract which was indistinguishable from the extract obtained by carrying out steps (a)-(d) of claim 1, even if D4 did not specify the extraction temperature and the flavonoid content of the extract. Notwithstanding the above, the opposition division acknowledged novelty because D4 neither disclosed a material being suitable for use as dietary supplement nor a carrier that was pharmaceutically, cosmetically or dietetically acceptable.

The opposition division considered D5 or D6 to represent the closest state of the art, because the Antistax® products disclosed in these documents were intended for the same purpose as the claimed product. The problem underlying the patent in suit was to provide an alternative product for oral administration being suitable for treating chronic venal insufficiency. The claimed solution using the extract according to claim 1 was seen as involving an inventive step because

the skilled person would have regarded the presence of esculin in the Antistax® products of D5 and D6 as essential so that he would not have contemplated the exclusion of said component from the known products.

IV. On 14 September 2009 the opponent (appellant) lodged an appeal against the interlocutory decision of the opposition division. The appeal fee was paid on the same day.

The statement of grounds of appeal was filed on 12 November 2009 together with the following further documents:

D30: P.H. List and P.C. Schmidt, "Technologie pflanzlicher Arzneizubereitungen", Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, 1984, altogether 29 pages;

D31: R.O.B. Wijesekera, Ed., "The Medicinal Plant Industry", CRC Press, Inc, 1991, Chapter 7, pages 85-88, 93-96 and 111;

D32: Les médicaments à base de plantes (Septembre 1997), Agence du Médicament, Paris, 1998; pages 3-9, 54-57, 60 and 61; and

D33: Copy of the minutes of the oral proceedings of 22 September 2009 in appeal case T 1416/07-3309.

The appellant requested that the decision under appeal be set aside and the patent be revoked in its entirety because the claimed subject-matter lacked novelty and inventive step, and was insufficiently disclosed. The

appellant further requested that the point of law, which had been the subject in G 3/06, be again referred to the Enlarged Board of Appeal, because the referral proceedings in G 3/06 had been terminated following the withdrawal of the remaining appeals in the referring decision.

V. With its reply dated 28 May 2010 the patent proprietor (respondent) disputed all the arguments submitted by the appellant and requested that the appeal be dismissed.

It also filed the following fresh evidence:

D34: E. Schneider, "Gutachterliche Darstellung zur Aussagekraft von Leitsubstanzen als alleiniger Parameter zur Charakterisierung und zum Vergleich von Pflanzenextrakten unter Betrachtung der Phytoäquivalenz", dated 18 February 2008, 22 pages;

D35: M. Veit, "Stellungnahme zur Vergleichbarkeit pflanzlicher Extrakte *sui generis* und Gutachten zur Einordnung und Vergleichbarkeit von unterschiedlichen Weinlaubextrakten", dated 18 February 2008, 14 pages; and

D36: Pharmaton GmbH, Fachinformationen relating to Antistax® Kapseln, Januar 1996.

VI. On 1 April 2011 the board dispatched a summons to attend oral proceedings. In the attached communication the board drew the attention of the parties to the points to be discussed during the oral proceedings.

VII. On 19 August 2011 both the appellant and the respondent filed further arguments in support of their requests.

The respondent also filed a set of seven claims for an auxiliary request marked "Auxiliary Request I- Appeal" and the following two further documents:

D37: PLANTA-SUBTIL Arzneimittel GmbH, "Zusammenfassung der pharmakologischen, toxikologischen und klinischen Untersuchungen zur Wirksamkeit von ANTISTAX Liquidum bei Venenerkrankungen (Chronisch Venöser Insuffizienz)"; a non-dated internal document; and

D38: C. Diehm, "Comparison of leg compression stocking and oral horse-chestnut seed extract therapy in patients with chronic venous insufficiency" THE LANCET, vol. 347, February 1996, pages 292-294.

VIII. Oral proceedings were held before the board on 20 September 2011 during which the appellant withdrew its request for a referral to the Enlarged Board of Appeal (see above point IV). The appellant requested that documents D37 and D38 not be admitted into the proceedings because they were *prima facie* not relevant and/or not published before the filing date of the patent in suit. During the oral proceedings the appellant's main and auxiliary requests were discussed.

The claims of the main request are the claims of auxiliary request 1 found allowable by the opposition division (see point III above).

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The claims of the auxiliary request are the claims filed on 19 August 2011 as "Auxiliary Request I - Appeal". Claim 1 of the auxiliary request reads as follows:

"1. A dietary supplement composition for use in the prevention or alleviation of the discomfort associated with mild-to-moderate chronic venous insufficiency of the lower extremities in a form suitable for oral administration which consists of an active principle being capable of preventing or treating the discomfort associated with mild-to-moderate chronic venous insufficiency of the lower extremities and a pharmaceutically, cosmetically or dietetically acceptable carrier, the improvement wherein is that said active principle consists of an aqueous extract of red vine leaves containing 2 to 20% flavonoids, wherein said aqueous extract of red vine leaves is obtainable by a method comprising the steps of

(a) collecting red vine leaves at a point of time when the content in flavonoids has reached an optimum;
(b) drying and crushing the leaves;
(c) cutting the leaves to pieces;
(d) extracting the leaves with water at temperatures from 60 to 80°C for 6 to 10 hours in an exhaustive percolation;
(e) optionally concentrating the obtained extract;

characterized in that said composition contains an amount of said aqueous extract of red vine leaves, which corresponds to a daily dosage of 300-800 mg of the extract, and the composition is administered in a

daily dose of 300-800 mg of the extract in 1 to 3 capsules or tablets taken once daily."

IX. The arguments presented by the appellant in its written submissions and at the oral proceedings insofar as they are relevant for the present decision may be summarized as follows:

- The requirements of Article 83 EPC were not fulfilled, essentially because the specification did not describe a method for analyzing the complete group of flavonoids.

- The claimed subject-matter lacked novelty in view of D4. D4 disclosed the use of an auxiliary substance which could not be distinguished from a carrier.

- Concerning inventive step of both requests, the appellant pointed out that the situation in the present appeal case was the same situation as in the parent patent, which had been revoked for lack of inventive step (T 1416/07). The reasons given in that decision equally applied to the now claimed subject-matter.

X. The arguments of the respondent may be summarized as follows:

- The subject-matter of claim 1 was novel over the disclosure of D4 at least because there was no disclosure of a carrier as claimed. Additionally, the process of D4 did not result in the same extract as the one obtained when carrying out steps (a) to (e) of claim 1.

– Regarding inventive step the respondent saw the disclosure of Antistax®, D5, as the closest prior art document. The subject-matter of claim 1 differed from this prior art in that the composition did not include esculin; the red vine leaves extract was specified to contain 2 to 20% flavonoids; and in that the method of preparation of the extract was specified. It pointed out that the experiments of the appellant, D28, did not show any prior art extract and that it was the task of the appellant to prove that the extraction conditions mentioned in D4 would inevitably result in an extract being identical to the claimed extract. The respondent stressed that there was no suggestion in the prior art that the red vine leaves extract alone would be sufficiently active. The fact that this extract would have the same efficiency as the known product Antistax® was an unexpected result. To affirm the contrary could only be done with knowledge of the invention (*ex post facto*).

– The auxiliary request involved an inventive step because there was no hint in the available documents as to the claimed dosage regime. On the contrary, the prior art's constant teaching (D5, D36) was towards an intake two or three times daily, actually teaching away from the invention. In the respondent's opinion it was quite unexpected that good results in treating chronic venous insufficiency could be obtained by using a different red vine leaves extract, eliminating the esculin from the Antistax® composition and administering it only once daily. Moreover, a single daily

administration dosage regime was more likely to be complied with by the patients.

XI. The appellant (opponent) requested that the decision under appeal be set aside and that the European patent No. 1 300 084 be revoked.

The respondent (patent proprietor) requested that the appeal be dismissed (main request) or, subsidiarily, that the patent be maintained on the basis of "Auxiliary Request I - Appeal" as filed with letter dated 19 August 2011.

Reasons for the Decision

1. The appeal is admissible.

2. *Procedural matters*

2.1 The respondent filed documents D37 and D38 with letter dated 20 August 2010. The reason given for the late filing was that it had managed to get access to the unpublished study on Antistax® (D37) only during the preparation of its latest submission. D38 was necessary to properly understand the effects presented in D37.

2.2 The appellant requested not admitting these documents into the proceedings because they were *prima facie* not relevant and/or not published before the filing date of the patent in suit.

2.3 In the present appeal proceedings both parties filed new documents, namely D30 to D38. There is no evidence

that the filing of these new documents by the respective parties amounts to a tactical abuse of the proceedings. The submissions were made at an early stage of the appeal proceedings or at the moment the documents were made available to the party. Moreover, the documents were filed in support of previous arguments and/or to establish the skilled person's general knowledge. Finally, the other party had enough time to take them into consideration. As regards the fact that D37 was not pre-published, the board notes that this document was merely used as a witness document to provide evidence for the effect allegedly achieved by the claimed subject-matter.

2.4 Thus the board saw no reasons not to admit the newly filed documents, and in particular D37 and D38 into the appeal proceedings (Article 114(2) EPC).

MAIN REQUEST

3. *Interpretation of Claim 1*

3.1 Claim 1 is directed to a dietary supplement composition with the following features:

(α) a dietary supplement composition
(β) in a form suitable for oral administration which consists of
(γ) an active principle being capable of preventing or treating the discomfort associated with chronic venous insufficiency
(γ1) the active principle consists of an aqueous extract of red vine leaves obtainable by a method comprising the steps of collecting red vine leaves,

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drying and crushing the leaves, cutting them to pieces and extracting with water under certain conditions, and

(δ) a pharmaceutically, cosmetically or dietetically acceptable carrier wherein

(ε) said composition contains an amount of aqueous extract of red vine leaves, which corresponds to a daily dosage of 300-800 mg of the extract.

3.2 Concerning feature (ε), it is noted that the expression "daily dosage" is a feature relating to the use of the composition, namely the amount to be taken per day, not to the composition itself. This feature is therefore not a limiting feature of the claimed composition. It is interpreted as indicating that the composition must be suitable for administering a daily dosage of 300-800 mg of the aqueous extract.

3.3 This interpretation of the claim is in conformity with the respondent's own interpretation (page 2 of its letter dated 19 August 2011).

4. *Sufficiency of disclosure*

4.1 The appellant has maintained during the appeal proceedings the objection that the requirements of Article 83 EPC are not satisfied because of the absence of a method for analyzing the complete group of flavonoids in the specification of the patent in suit.

4.2 In fact, the same objection has been raised in the appeal proceedings dealing with the parent application/patent, namely T 1416/07. As set out in point 3.5 of the reasons of this decision, "… it is

well known which flavonoids are present in red vine leaves ... and that methods for analysing them, for instance high pressure liquid chromatography, are also well known and available to the skilled person without undue burden.... In these circumstances, the fact that it may be difficult and time consuming to identify and quantify all of the flavonoid species contained in the red vine leaves extract is not a matter to be criticised under the aspect of sufficiency." These reasons apply *mutatis mutandis* to the present case, in particular because the appellant has not provided any evidence whatsoever which could challenge the finding in T 1416/07.

4.3 Consequently the board is satisfied that the patent discloses the invention in a manner sufficiently clear and complete for it to be carried out by a person skilled in the art.

5. *Novelty (Article 54 EPC)*

5.1 The novelty of Claim 1 was contested by the appellant having regard to the disclosure of D4.

5.1.1 Document D4 discloses a method of preparation of a dry extract of red vine leaves (see under "Extrait de vigne rouge (sec), lines 1-10). According to D4, appropriate auxiliary substances can be incorporated, if necessary, before the drying step (lines 5-6).

5.1.2 However, the generic disclosure of "auxiliary substances" in D4 cannot take away the novelty of the specific carriers (pharmaceutically, cosmetically or dietetically acceptable carriers) now required in

claim 1, in particular because D4 does not disclose any use of the extract for a pharmaceutical, cosmetic or dietetic application.

5.1.3 For this reason alone the subject-matter of claim 1 of the main request is novel.

6. *Inventive step (Article 56 EPC)*

6.1 The present invention relates to a dietary supplement composition having as active principle an extract of red vine leaves, for preventing or alleviating the discomfort associated with mild-to-moderate chronic venous insufficiency of the lower extremities.

6.2 Closest prior art

6.2.1 The board considers, in agreement with the decision under appeal and the parties, that the closest prior art is represented by the orally administered Antistax®, a commercially available product disclosed in D5, D6 and D36. Antistax® contains as active principle a mixture of an extract of red vine leaves (98,4%) and esculin (1,6%) and is also used for the treatment of venous insufficiency of the lower extremities (cf. D5, product 83 048; D6, abstract and pages 795-796, last section of the article; and D36 whole document).

Document D5 is silent about the composition of the red vine leaves extract used. In D6 and D36 it is stated that the main components of the extract are flavonoids (D6, page 793, right column, first full paragraph and D36 middle column under "1. Weinlaub-Extrakt") and that the properties of the flavonoids are significant for

its use in the treatment of venous insufficiency (D6, page 794 section "*Venenleiden und Bioflavonoide*", in particular last paragraph and D36 middle column under "1. Weinlaub-Extrakt").

6.2.2 However, none of these documents disclose how the red vine leaves extract used in Antistax® is prepared. Thus, the composition of claim 1 differs from Antistax® as disclosed in these documents in that:

- it does not include esculin; and
- it is specified how the red vine leaves extract with a content of 2 to 20% flavonoids is prepared.

6.3 The problem to be solved and its solution

6.3.1 The compositions of claim 1 have the same use as the product Antistax®, namely the treatment of chronic venous insufficiency of the lower extremities. There is no evidence on file of any unexpected effect of the claimed compositions when compared with Antistax®. In fact, even the respondent saw the technical problem to be solved by the patent in suit in the provision of alternative compositions which are also useful for the treatment of venous insufficiency of the lower extremities.

6.3.2 This problem is solved by using the compositions defined in claim 1, wherein the active principle consists of an aqueous extract of red vine leaves containing 2 to 20% flavonoids and the extract is obtainable under specific extraction conditions.

In the light of the results of the study described in the patent specification the board is satisfied that

the above-defined problem has been credibly solved. This finding was not challenged by the appellant.

6.4 Obviousness

6.4.1 It remains to be decided whether the suggested solution is obvious from the prior art. In this context it has to be analysed as to whether or not (i) the specific method of preparing the extract and (ii) the use of the red vine leaves extract alone can contribute to inventive step.

6.4.2 The respondent has maintained during the proceedings that the method of preparation of the red vine leaves extract results in an extract having very specific properties. It pointed out that plant extracts are complex mixtures defined by their method of preparation. The claimed extracts being characterized by a content of 2 to 20% flavonoids, mandatorily included 98 to 80% of other ingredients which were defined by the method of extraction. The respondent supported this affirmation by the two expert opinions, D34 and D35, indicating that two extracts were phytoequivalent, if their pharmaceutical equivalence and their therapeutical equivalence were demonstrated. As this evidence had not been provided by the appellant, it should be recognized that a different extract was used in the claims.

6.4.3 It is true that the method of preparation of the red vine leaves extract is not specified in the prior art, and thus a comparison of the claimed extracts with the extract used in Antistax® is not possible. In the absence of this information it has to be assumed, as

pointed out by the appellant, that the prior art extract has been prepared according to the general knowledge of the skilled person using standard methods like the one disclosed in D4.

In D4 the extract is obtained by warm lixiviation of suitable, cut red vine leaves with water to complete exhaustion. The method used in D4 is described in a general way without indicating the specific conditions used in every process step.

Compared to this known process, the process of claim 1 requires:

(a) collecting red vine leaves at a point of time when the content in flavonoids has reached an optimum;
(b) drying and crushing the leaves;
(c) cutting the leaves to pieces; and
(d) extracting the leaves with water at temperatures from 60 to 80 °C for 6 to 10 hours in an exhaustive percolation.

Steps (a), (b) and (c) define in a very broad way how the leaves are handled before the extraction step. The leaves are to be collected when the content in flavonoids reaches an optimum and they are dried, crushed and cut into pieces. None of these steps are further concretized in the claim. In the absence of such further information it must be assumed that the skilled person would carry out these process steps according to the known standard technologies for the processing of medicinal plants (cf. D30 and D31) with the aim of optimizing the extraction of flavonoids, the known active components in red vine leaves. Steps (a),

(b) and (c) thus cannot contribute to essentially distinguishing the claimed extract from the prior art extracts.

6.4.4 Concerning step (d), the actual extraction step, this step is carried out at a temperature in the range of 60° to 80°C for 6 to 10 hours in an exhaustive percolation to achieve a high content in flavonoids during the extraction as indicated in paragraph [0018] of the specification. This extraction step by percolation is similar to the lixiviation to complete exhaustion disclosed in D4 as both processes aim to extract the drug until exhaustion of the soluble substances.

In this context, the appellant provided further experimental evidence (D28) that the extraction conditions had little influence on the flavonoid content of a particular sample of red vine leaves. Thus, regardless of whether the temperature was inside or outside the range required in claim 1, whether percolation or maceration or even a mixture of water and ethanol was used, the content of flavonoids extracted from said sample, that is to say the known main active ingredients of the extract used in Antistax®, is always within the range of 2 to 20%.

Insofar as the respondent relied on the presence of compounds in the extract other than the main active ingredients, the board notes the following:

- The extract used in the patent in suit contains as main active ingredients quercetin-3-O-ß-D-glucuronide and isoquercitrin ([0009]). These are

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exactly the same main components present in the red vine leaves extract used in Antistax® according to D6 (page 793, right column, lines 25 - 29) and D36. The skilled person when preparing a red vine leaves extract for treating venous insufficiency would ensure that the components which are said to be the main active components are present in the extract in good yield.

– There is no information on file showing that the activity of the compositions could be due to other unspecified components of the extract which would not be extracted when carrying out the process of D4.

6.4.5 Thus, it is evident from the above that the extract obtained by steps (a) to (d) of claim 1 is very similar, if not identical, to the extract used in Antistax®. But even if there is no identity, the slight differences in the preparation leading to a slight difference in the extract, it has not been shown that slight variations in the preparation contribute to inventive step. On the contrary, all the process steps identified in claim 1 are common in this field.

The preparation of the red vine leaves extract can for these reasons not contribute to justifying an inventive step.

6.4.6 Concerning the use of the extract of red vine leaves alone, that is to say without esculin (question (ii) above), it is noted that D6 emphasizes the importance of the flavonoids present in the red vine leaves extract as the main active principle of the Antistax® product and being essentially responsible for its

activity in the treatment of venous insufficiency (cf. D6, page 792, last sentence of the abstract and page 794, second and third columns). It would therefore be evident to the skilled person from studying these passages of D6 that a composition containing only the red vine leaves extract and being free of esculin would also show the desired activity.

This finding is also confirmed by further documents on file indicating that the red vine leaves extract was already known for the treating of venous insufficiency (for instance, D1, page 25) and that the effect on venous insufficiency is due to the presence of flavonoids (D36, middle column, "1. Weinlaub-Extrakt"). Furthermore, it is stated in D36, right hand column that esculin and the extract of wine leaves act in the same way ("gleichsinnig"). Nothing is said of the two components working in a synergistic way.

Consequently, the exclusion of esculin from the Antistax® product cannot justify the presence of an inventive step.

6.4.7 The respondent has filed document D37 during the appeal proceedings in order to show an unexpected result of the claimed compositions. In its opinion it was an unexpected result that the activity of the claimed composition would be maintained even if one active component of Antistax®, namely esculin, were removed. This was demonstrated when comparing the results of D37 with those of the patent in suit.

However no direct comparison of a treatment with the compositions of claim 1 with a treatment with Antistax®

has been made. As discussed in detail during the oral proceedings it is rather difficult, if not impossible, to compare the results presented in the patent with the results of D37. The study in D37 was conducted with patients with only one leg suffering chronic venous insufficiency and its results cannot be compared with those of the patent conducted with patients suffering from chronic venous insufficiency in both legs. The assumption made by the respondent that the results in D37 were likely to be overestimated by a factor of 2 appears rather speculative. Consequently, no conclusion can be achieved by comparing both experimental results.

6.5 For these reasons the subject-matter of claim 1 of the main request lacks inventive step.

AUXILIARY REQUEST

7. *Inventive step (Article 56 EPC)*

7.1 Claim 1 of the auxiliary request is drafted in the form of a first medical use claim ("A dietary supplement composition for use in the prevention or alleviation of the discomfort associated with mild-to-moderate chronic venous insufficiency of the lower extremities …") which contains in addition to the features of claim 1 of the main request a dosage regime, namely that "the composition is administered in a daily dose of 300-800 mg of the extract in 1 to 3 capsules of tablets taken once daily" (point VIII above).

7.2 Since claim 1 of the main request already refers to "an active principle being capable of preventing or treating the discomfort associated with mild-to-

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moderate chronic venous insufficiency of the lower extremities", the mere reformulation of claim 1 to a first medical use claim cannot contribute to overcoming the inventive step objection against claim 1 of the main request.

Thus, the actual difference of the subject-matter of claim 1 of the auxiliary request over claim 1 of the main request is the dosage regime.

7.3 The recommended dose of Antistax® in D5 and D36 varies from 360 mg to 540 mg taken in two or three capsules twice a day. The now claimed dosage regime uses an amount of extract covering the known amount but it requires that it is taken only once a day.

7.3.1 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.

7.3.2 In the present case the amount of active ingredient remains the same as the amount used in D5 and there is no evidence on file of any unexpected technical effect caused by administering the red vine leave extract once daily. 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.

7.3.3 In this respect the board cannot accept the argument of the appellant that it was unexpected that the same activity would be achieved by a change of dosage regime. In fact, a comparison of the claimed dosage regime with the dosage regime of D5/D36 has not been made. The only comparison which has been made compares the now required dosage regime with the use of compression stockings and/other oedema-reducing agents (last paragraph of the patent specification). The information given in the patent is that the red vine leave extract in a daily dose of 300-800 mg taken once daily is useful for treating chronic venous insufficiency. It has never been shown that taking a dose of 300-800 mg once daily has the same effect as taking the same dose twice daily.

Finally, insofar as the respondent relied on the fact that by changing the dosage regime from two times a day to only once a better adherence of the regime by patients is achieved, this advantage is in itself known and its exploitation in the present case cannot justify an inventive step.

7.4 Consequently, the subject-matter of claim 1 of the auxiliary request also lacks inventive step.

8. In summary, none of the requests relate to patentable subject-matter.

Order

For these reasons it is decided that:

1. The decision under appeal is set aside.

2. The patent is revoked.

The Registrar The Chairman

D. Magliano W. Sieber

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Acknowledgement of receipt

We hereby acknowledge receipt of the Notice of Opposition:

Submission number	2041017
Application number	EP05712607.0
Patent number	EP1720571
Date of receipt	12 March 2013
Your reference	SR-01675
Opponent	Pfizer Inc.
Title	METHOD OF TREATING HEMOLYTIC DISEASE

Documents submitted

package-data.xml	ep-opposition-data.xml
ep-oppo.pdf (6 p.)	OTHER-1.pdf\SR-01675-Notice of oppo.pdf (1 p.)
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	Published-Evidence-12.pdf\D12 - Hillmen 2004 BJH.pdf (1 p.)	Other-evidence-1.pdf\T-1979-09.pdf (26 p.)

Submitted by	CN=Isabelle Campion 12135
Method of submission	Online
Date and time receipt generated	12 March 2013, 16:57 (CET)
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Correction by the EPO of errors in debit instructions filed by eOLF
Errors in debit instructions filed by eOLF that are caused by the editing of Form 1038E entries or the continued use of outdated software (all forms) may be corrected automatically by the EPO, leaving the payment date unchanged (see decision T 152/82, OJ EPO 1984, 301 and point 6.3 ff ADA, Supplement to OJ EPO 10/2007).

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Date
18-03-2013

Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571

Applicant/Proprietor
ALEXION PHARMACEUTICALS, INC.

Communication of a notice of opposition

You are hereby informed of a notice of opposition to the European patent specified above (see attached copy in the enclosure). The documents specified as patent documents in the notice of opposition are available for inspection via the European Patent Register at **www.epo.org/register**. Should you wish to receive these documents in paper format, they will be supplied to you free of charge if specifically requested on receipt of this communication (see OJ EPO 2009, 434).

If oral proceedings are to take place, parties are advised to check the electronic file via European Patent Register in advance of the hearing to ensure they are in possession of all relevant documents.

An invitation to file observations and to file amendments, where appropriate, to the description, claims and drawings (R. 79(1) EPC) will be issued separately.

The period within which such observations may be filed will not be fixed until the following conditions are met:

(a) the opposition period has expired;

(b) the notice of opposition has been examined for certain formal requirements (R. 77 EPC).

For the Opposition Division

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Enclosure: Notice of opposition 01 with non-patent literature

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Date
19-04-2013

Reference		Application No./Patent No.
SR-01675	OPPO 01	05712607.0 - 1412 / 1720571
Applicant/Proprietor		
ALEXION PHARMACEUTICALS, INC.		

Communication of further notices of opposition pursuant to Rule 79(2) EPC

No further opposition has been filed.

If oral proceedings are to take place, parties are advised to check the electronic file via the European Patent Register at **www.epo.org/register** in advance of the hearing to ensure that they are in possession of all relevant documents. (OJ EPO 2009, 434)

A copy of the communication pursuant to Rule 79(1) EPC sent to the proprietor of the patent is enclosed for your information.

The patent proprietor's (proprietors') observations on the notice of opposition to the above-mentioned patent will be communicated to the opponent without delay. A time limit for reply will be fixed if the Opposition Division considers this expedient.

If no reply to a communication is received within the time limit set, the proceedings will be resumed forthwith. Your attention is drawn to Article 114(2) EPC.

For the Opposition Division

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Enclosures: copy of the communication pursuant to Rule 79(1) EPC (Form 2317A)

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Date
19-04-2013

Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571
Applicant/Proprietor	
ALEXION PHARMACEUTICALS, INC.	

Communication of notices of opposition (R. 79(1) EPC)

Notice of opposition has been filed within the opposition period by:

01. Pfizer Inc./235 East 42nd Street/New York, NY 10017/ETATS-UNIS D'AMERIQUE//

The notice of opposition indicated above has already been communicated to you.

You are requested to file your observations within a period of **four months** from notification of this communication.

You may also file amendments, where appropriate, to the description, claims and drawings within the period specified. One set of these documents is to be filed.

If you introduced documents which have not yet been mentioned during the proceedings, your attention is drawn to Rule 83 EPC.

Enclosures:

For the Opposition Division

Europäisches Patentamt • European Patent Office • Office européen des brevets

Registered letter
EPO Form 2317A 12.07 (11/04/13)

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Page 3436

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Patentanwälte Rechtsanwälte

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European Patent Office

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EPO - Munich
27
19. Aug. 2013

Opposition against EP-Patent 1720571
(Application No.: 05 71 2607.0)
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

München, August 19, 2013
KRD/FLG/OOV

Reference is made to the Communication dated April 19, 2013 in connection with the above-referenced case.

It is respectfully requested that the four-month period specified therein for filing observations be extended by two months, i.e. until

October 29, 2013.

As a precautionary measure oral proceedings are requested.

D. Krämer

Dana Krämer, Ph.D.
European Patent Attorney

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EUROPEAN PATENT ATTORNEYS
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Samsung Bioepis Exhibit No. 1002
Page 3437

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Date
26-08-2013

Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571
Applicant/Proprietor	
ALEXION PHARMACEUTICALS, INC.	

Extension of time limit pursuant to Rule 132 EPC

Opposition procedure

With reference to your request, the time limit for replying to the communication dated 19.04.2013 has been extended

by 2 months

to a total of 6 months

from the date of notification of the above-mentioned communication.

Please note: To the extent that your request exceeded the above extension, your request has been refused.

Note:
The granting of extensions to time limits is governed by the Implementing Regulations to the EPC and the Guidelines for Examination in the EPO, part E-VII, 1.6. A request for extension, which would result in the total period set exceeding six months, must be reasoned and supported by evidence.

If no reply to the communication is received in due time, the procedure will be continued. Attention is drawn to Article 114(2) EPC.

For the Opposition Division

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Date: 26-08-2013

Reference	Application No./Patent No.
SR-01675 OPPO 01	05712607.0 - 1412 / 1720571
Applicant/Proprietor: ALEXION PHARMACEUTICALS, INC.	

BRIEF COMMUNICATION

Subject:
- ☐ Your letter of
- ☐ Our telephone conversation of
- ☐ Communication of
- ☐

Enclosure(s):
- ☒ Letter from the proprietor of the patent of 19.08.2013
- ☐ Letter from the opponent of
- ☒ Copy (copies) EPO Form 2944C

Communication:

☐ A letter from the opponent /proprietor was received on ..... . The documents specified as patent documents in this letter are available via the European Patent Register at **www.epo.org/register.** Should you wish to receive these documents in paper format, they will be supplied to you free of charge if specifically requested on receipt of this communication (OJ EPO 2009, 434).

☐

For the Opposition Division

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☐ **Registered letter**

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European Patent Department

VIA EPOLINE

EUROPEAN PATENT OFFICE
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07 October, 2013

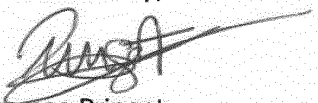
OUR REF.: SR-01675
THP/IC

Re: European Patent No 1 720 571 B1 (ALEXION PHARMACEUTICALS, INC.)
Application No. 05712607.0
Opponent: PFIZER INC.

Dear Sirs,

The opponent, Pfizer, Inc., hereby formally and irrevocably withdraws its opposition to EP1720571B1 in the name of Alexion Pharmaceuticals, Inc. entitled "Method of treating hemolytic disease".

Yours faithfully,



Thomas Pringot
European Patent Attorney
Association Pfizer - No. 462
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Submission in opposition proceedings

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- representing the opponent(s):	Pfizer Inc.
Opponent/representative's reference	SR-01675

The information given below is pertaining to the following patent in opposition proceedings:

Patent No.	EP1720571
Application No.	EP05712607.0
Date of mention of the grant in the European Patent Bulletin (Art. 97(3), Art. 99(1) EPC)	13 June 2012
Title of the invention	METHOD OF TREATING HEMOLYTIC DISEASE
Proprietor of the patent	ALEXION PHARMACEUTICALS, Inc.

Requests:

- Opposition is herewith withdrawn.

Documents attached:

	Description of document	Original file name	Assigned file name
1	Any annexes (other than citation) to an opposition letter - Withdrawal of opposition	SR-01675-Withdrawal.pdf	OTHER-1.pdf

Signatures

Place:	**Paris**
Date:	**07 October 2013**
Signed by:	**/Thomas PRINGOT/**
Association:	**462**
Capacity:	**(Representative)**

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European Patent Office
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Acknowledgement of receipt

We hereby acknowledge receipt of the following submission by the opponent:

Submission number	2336621	
Application number	EP05712607.0	
Patent number	EP1720571	
Date of receipt	07 October 2013	
Your reference	SR-01675	
Opponent	Pfizer Inc.	
Title	METHOD OF TREATING HEMOLYTIC DISEASE	
Documents submitted	package-data.xml ep-oppo.pdf (2 p.)	ep-opposition-data.xml OTHER-1.pdf\SR-01675-Withdrawal.pdf (1 p.)
Submitted by	CN=Isabelle Campion 12135	
Method of submission	Online	
Date and time receipt generated	07 October 2013, 16:17 (CEST)	
Message Digest	62:F8:CB:C6:AD:87:27:49:C0:A7:F1:61:7A:40:34:8F:17:4E:97:6C	

Correction by the EPO of errors in debit instructions filed by eOLF
Errors in debit instructions filed by eOLF that are caused by the editing of Form 1038E entries or the continued use of outdated software (all forms) may be corrected automatically by the EPO, leaving the payment date unchanged (see decision T 152/82, OJ EPO 1984, 301 and point 6.3 ff ADA, Supplement to OJ EPO 10/2007).

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VOSSIUS & PARTNER

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EPO - Munich
81
09. Okt. 2013

05 71 2607.0 / 1720571
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

München, October 9, 2013
WA/FLG

This is in reply to the Communication of notices of opposition (Rule 79(1) EPC) issued in the above-referenced opposition proceedings on April 19, 2013.

The Opponent, Pfizer Inc., formally and irrevocably has withdrawn its opposition on October 7, 2013. It is therefore respectfully requested that the patent is maintained as granted and the opposition proceedings are promptly terminated.

Dr. Joachim Wachenfeld
European Patent Attorney

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Date: 09-10-2013

Reference	Application No./Patent No.
SR-01675 OPPO 01	05712607.0 - 1412 / 1720571
Applicant/Proprietor: ALEXION PHARMACEUTICALS, INC.	

BRIEF COMMUNICATION

Subject:
- ☒ Your letter of 07.10.13
- ☐ Our telephone conversation of
- ☐ Communication of
- ☐

Enclosure(s):
- ☐ Letter from the proprietor of the patent of
- ☐ Letter from the opponent of
- ☐ Copy (copies)

Communication: Your above notice of withdrawal of opposition is hereby acknowledged. You are no more a party in this proceedings.

☐ A letter from the opponent /proprietor was received on ..... . The documents specified as patent documents in this letter are available via the European Patent Register at **www.epo.org/register.** Should you wish to receive these documents in paper format, they will be supplied to you free of charge if specifically requested on receipt of this communication (OJ EPO 2009, 434).

☐

For the Opposition Division

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☐ **Registered letter**

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Date: 09-10-2013

Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571

Applicant/Proprietor: ALEXION PHARMACEUTICALS, INC.

BRIEF COMMUNICATION

Subject:
- ☐ Your letter of
- ☐ Our telephone conversation of
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Enclosure(s):
- ☐ Letter from the proprietor of the patent of
- ☒ Letter from the opponent of 07.10.13
- ☒ Copy (copies) of brief comm. of 09.10.13

Communication:

☐ A letter from the opponent /proprietor was received on ..... . The documents specified as patent documents in this letter are available via the European Patent Register at **www.epo.org/register.** Should you wish to receive these documents in paper format, they will be supplied to you free of charge if specifically requested on receipt of this communication (OJ EPO 2009, 434).

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For the Opposition Division

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CC20871

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44

29. Okt. 2013

Opposition against EP 1720571
(Application No.: 05 71 2607.0)
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

München, October 29, 2013
WA/ARO/BUK

Reference is made to the Communication dated April 19, 2013 as well as our petition of October 9, 2013 in the above-mentioned case.

In view of the recent withdrawal of the opposition by Pfizer Inc. and the fact that so far we have not heard back from the Opposition Division, we would respectfully ask for an exceptional two-month extension of time, i.e. until

December 29, 2013.

Only in case the Opposition Division would be inclined to continue the proceedings and our above request for extension would not be granted, we request that nevertheless we get a further opportunity to file observations in written form.

As a precautionary measure oral proceedings are requested.

Dr. Joachim Wachenfeld
European Patent Attorney

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Date
06-11-2013

Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571
Applicant/Proprietor	
ALEXION PHARMACEUTICALS, INC.	

Extension of time limit pursuant to Rule 132 EPC

Opposition procedure

With reference to your request, the time limit for replying to the communication dated 19.04.2013 has been extended

by 2 months

to a total of 8 months

from the date of notification of the above-mentioned communication.

Please note: To the extent that your request exceeded the above extension, your request has been refused.

Note:
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Application No.: 05 712 607.0

Patent No.: EP-B-1 720 571

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)

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2.2 Parties' submissions in preparation for the oral proceedings, if any, should be made no later than

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3. ☐ Arrange for the following special equipment to be provided in the conference room:

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- dispatch the dossier to the primary examiner and
- dispatch Form 2041 with copies for the other members of the examining division.

19.12.2013
Date

Cilensek, Zoran	Weikl, Martina	Chapman, Rob	
Chairman	2nd examiner	1st examiner	Legal member

Enclosure(s):

Opposition against EP 1720571
Patentee: Alexion Pharmaceuticals, Inc.
Our Ref.: M2367 OPP(EP)

1017

D13

Eculizumab Alexion

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Eculizumab (5G1.1), a humanized monoclonal antibody that prevents the cleavage of human complement component C5 into its pro-inflammatory components, is under development by Alexion as a potential treatment for several chronic inflammatory diseases, including rheumatoid arthritis (RA) and nephritis [190673], [292328]. In January 2002, a phase IIb trial was initiated for RA [437814]. This trial was ongoing in April 2002, at which time, eculizumab was also in phase II trials for the treatment of membranous nephritis and lupus nephritis, and in earlier stage clinical trials for dermatomyositis and pemphigoid [446377]. The company is also developing a single-chain version of this antibody, pexelizumab, for use in acute cardiovascular indications [188760].

In October 2000, eculizumab was granted Orphan Drug status by the FDA for the treatment of dermatomyositis [385057]. In February 2002, the product received Orphan Drug designation for its use in patients with membranous nephritis [440583].

In September 2001, analysts at US Bancorp Piper Jaffray predicted eculizumab's launch for dermatomyositis and pemphigus in 2003, RA and nephritis in 2004, and chronic heart failure (CHF) after 2006 [426537]. In March 2002, analysts at US Bancorp Piper Jaffray predicted that the product would have peak worldwide sales in RA of US $175 million and US $400 million for nephritis. Sales for the RA indication are predicted to reach US $35 million in 2006, rising to US $110 million in 2008, and US $10 million in 2006, rising to US $50 million in 2008, in the US and the rest of the world, respectively. For the nephritis/other indication(s), sales are pegged at US $50 million in 2006, rising to US $200 million in 2008, and US $15 million in 2006, rising to US $100 million in 2008, again in the US and the rest of the world, respectively [446992].

Originator Alexion Pharmaceuticals Inc

Status Phase II Clinical

Indication Dermatological disease, Glomerulonephritis, Nephritis, Pemphigus, Psoriasis, Rheumatoid arthritis, Systemic lupus erythematosus

Action Complement cascade inhibitor

Biotechnology Monoclonal antibody, humanized

Synonyms 5G1.1, h5G1.1, C5 complement inhibitor (Alexion), h5G1.1scFv

CAS 5G1.1
Registry No: 339087-76-2

Introduction

The complement system comprises more than 30 human proteins in plasma and on cell surfaces that interact in a cascade sequence to mediate a variety of inflammatory events. These events include the opsonization of particles for phagocytosis, leukocyte activation and assembly of the membrane attack complex (MAC) [435653], [435654], [435718], [445228]. The system is responsible for killing and removing organisms, cells and cell components from the circulation which are recognized as foreign. In addition to host defense against bacterial agents, complement is involved in the disposal of immune complexes and the products of inflammatory injury, and serves as an interface between innate and adaptive immunity [435653].

Several complement proteins are cleaved during activation of the complement system and three pathways of activation have been described; the classical, the alternative and the lectin pathways [435653]. The pathways leading to the cleavage of C3 are triggered enzyme cascades. Downstream in these pathways, C3 is cleaved into C3a and C3b. C3a is released while C3b forms C5 convertases, which in turn cleave C5 to C5a and C5b. Following complement activation, pro-inflammatory peptides, such as the anaphylotoxins C3a and C5a, are generated and C5b-9 (MAC) is formed. Assembly of the MAC from the terminal components (C5 to C9) of the cascade leads to membrane damage. In addition, complement activation products, especially the anaphylotoxins, elicit a number of biological effects, including chemotaxis of leukocytes, degranulation of phagocytic cells, mast cells and basophils, smooth muscle contraction and the increase of vascular permeability [435653], [445228], [445736].

Under normal circumstances, the regulatory mechanisms of complement are balanced, so that the activation of complement occurs on the surface of invading microorganisms and the deposition of complement on normal cells and tissues is usually limited [435653], [445228]. If the mechanisms that regulate this balance are abnormal, the complement system might then cause tissue injury [435656]. Excessive or inappropriate activation of the complement system can lead to harmful and life-threatening consequences which are secondary to severe inflammatory tissue destruction. These consequences can manifest clinically in a variety of ways, including multiple organ failure, hyperacute graft rejection and septic shock [435656]. Inappropriate complement activation and its deposition on host cells can also lead to direct complement-mediated cell lysis of target structures, or indirectly to tissue destruction due to the generation of powerful mediators of inflammation [435653], [445228].

Complement plays an important role in the pathogenesis of many autoimmune and inflammatory diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) [435694], [445236] and dermatomyositis [445242]. It is also responsible for disease states associated

with bio-incompatibility, including transplant rejection [445249]. In addition, complement activation plays an important role in the pathogenesis of cardiac and intestinal ischemia/reperfusion injury [445251]. Other conditions in which complement activation has been proposed to play a role include stroke [445254], multiple sclerosis [445384], pancreatitis [445255], psoriasis [445272], Alzheimer's disease [445278] and prion-induced diseases [445286].

Therapeutic inhibition of complement is, therefore, potentially important in protecting against the development of certain diseases and it has been shown to be useful in animal models of sepsis, intestinal ischemia/reperfusion injury, myocardial reperfusion injury, nephritis, arthritis and graft rejection.

Several complement inhibitors are currently under investigation [212463], [225318], [281110], [435656], [435722], [435723], [435725], [436916]. Eculizumab (5G1.1), under development by Alexion Pharmaceuticals Inc, is a humanized C5 inhibitory monoclonal antibody (mAb) that prevents the cleavage of human complement component C5 into its pro-inflammatory components and targets the complement components, C5a and C5b-9 [445382]. As mentioned, the early steps of the complement cascade play an important role in the removal of infectious agents and several normal functions of the immune system. The rationale of specifically blocking C5 is that the normal upstream disease-preventing functions of complement remain intact, while the production of the abnormal downstream disease-causing actions of complement are blocked. This antibody, therefore, potentially avoids the disruption of antibacterial-protective mechanisms, which are believed to be mediated primarily through C3b [438165].

Eculizumab is undergoing phase II clinical trials for a variety of chronic inflammatory conditions, and is being developed as a potential treatment for RA, SLE and nephritis [190673], [292328], [412090]. The drug is currently in phase II trials for nephritis and has completed phase I/II trials in SLE and phase II trials in RA [328001], [335241], [335995], [396672]. Eculizumab is also currently undergoing phase I trials for psoriasis and pemphigus [384948], and has been granted Orphan Drug status by the FDA for the treatment of dermatomyositis [385057], and membranous nephritis [440583].

Alexion has also developed a short-acting C5 complement inhibitor that is a humanized single chain mAb fragment. This compound is known as pexelizumab (formerly 5G1.1-SC) and it is being tested for the treatment of acute cardiovascular diseases associated with complement activation, including acute myocardial infarction (MI), unstable angina and revascularization/reperfusion therapies. This drug was designed to have the advantage of enhanced tissue penetration, desirable for effective treatment of these acute life-threatening conditions [188760]. Pexelizumab and eculizumab are currently in at least eight clinical development programs [435963]. A phase III clinical study with pexelizumab in cardiopulmonary bypass patients has been initiated. In addition, two large phase II studies with pexelizumab in acute myocardial infarction (MI) patients are under way. Since pexelizumab has recently been evaluated, this review will focus primarily on eculizumab.

Synthesis and SAR

Humanized 5G1.1, Fab and scFv molecules were produced by grafting the complementary determining regions of 5G1.1 on to human framework regions [258884]. Competitive ELISA analysis indicated that no framework changes were required in the humanized variable regions for retention of high affinity binding to C5, even at framework positions predicted by computer modeling to influence CDR canonical structure. The humanized Fab and scFv molecules blocked complement-mediated lysis of chicken erythrocytes and porcine aortic endothelial cells in a dose-dependent fashion. Complete complement inhibition occurred at 3-fold molar excess, relative to the human C5 concentration. Humanized 5G1.1 scFv also effectively blocked C5a generation. In addition, intact humanized 5G1.1 antibody blocked human complement lytic activity at concentrations identical to the original murine monoclonal antibody, indicating that humanized 5G1.1 and its derivatives retained both the affinity and blocking functions of murine 5G1.1 antibody [258883], [258884]. 5G1.1 is supplied as a sterile, non-pyrogenic solution (2 mg/ml) for iv injection [212467], [258884] and it has picomolar affinity for a sequence within human C5. 5G1.1 can be administered either intramuscularly or subcutaneously, allowing self-administration [438165].

Pharmacology

Heart disease

There is a growing body of evidence pointing to a substantial disease-promoting role of complement components in MI and unstable angina [445289]. Activation of the complement cascade in MI patients is shown by elevated levels of activated complement byproducts in the blood of patients during acute MI and by the deposition of activated complement components in damaged heart tissue [445382]. Interestingly, terminal complement components play a key role in the apoptotic process in heart tissue during MI [241313], and blockade of the complement cascade at C5 has been shown to substantially reduce myocardial apoptosis and tissue damage in rats [241313]. Complement activation was also found in patients undergoing cardiopulmonary bypass (CBP) [445311]. This finding was associated with increased patient morbidity, including MI, stroke, cognitive dysfunction and blood loss [445311].

Studies in primates demonstrated that inhibition of complement activation reduces heart damage associated with coronary ischemia and reperfusion in preclinical models. Furthermore, treatment of hypoxic/reoxygenated human umbilical vein endothelial cells (HUVECs) with h5G1.1 scFv attenuated C5b-9 deposition, NFκB translocation and vascular cell adhesion molecule (VCAM)-1 expression [353137].

Intestinal and lung ischemia-reperfusion

A recent study reported that anti-C5a therapy significantly improved intestinal ischemia-reperfusion tissue injury as well as lung injury in rats [445313]. These results suggested that C5 inhibition could also limit the complaints in the broader general surgery population as well as in patients with ulcerative colitis [357827] Interestingly, C5 inhibition was associated with a significant reduction in tumor necrosis factor (TNF)α levels [357827].

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Inflammatory arthritis

Animals deficient in the complement component C5 are not susceptible to the onset of active arthritis [363495]. In the absence of C5 cleavage, the presence of TNFα is not sufficient to promote arthritis. A recent study reported that administration of anti-C5 mAb ameliorates the established collagen-induced arthritis in mice and rats [190673], [212456]. Systemic administration of the anti-C5 mAb effectively inhibited terminal complement activation *in vivo* and prevented the onset of arthritis in immunized animals. In addition, anti-C5 mAb treatment was also highly effective in ameliorating established disease. These results suggested a critical role for activated terminal complement components, not only in the induction, but also in the progression of collagen-induced arthritis [190673], [212456].

Renal disease and lupus

A C5 inhibitor was administered in both an acute and rapid progressive glomerulonephritis (RPGN) model that normally leads to crescentic changes, the most common histological lesion observed in lupus nephritis. The inhibitor was also administered to the NZB/NZW F1 lupus-prone mouse model. C5 inhibition prevented histological and clinical development of nephritis in the RPGN model and C5 inhibitor administration in the lupus-prone mouse model reduced histologic and biochemical evidence of kidney disease. Survival at 10 months was markedly improved from 5% in placebo-treated lupus-prone mice to 85% in C5 inhibitor-treated lupus-prone mice [190673], [232606], [280073].

Hematological diseases

C5 inhibition has also been shown to prevent platelet loss in a model of idiopathic thrombocytopenic purpura (ITP) [392090].

Metabolism

5G1.1 blocks complement activity for 1 to 2 weeks after a single dose [438165].

Toxicity

No toxicity has been reported in humans [228105], [392382] or rhesus monkeys [212462]. Interestingly, a recent study reported that C5 plays an important role in liver regeneration, strongly implicating the complement system as a crucial regulatory component of hepatic homeostasis. C5-deficient mice show severely defective liver regeneration and persistent parenchymal necrosis after exposure to CC_{l_4}. In addition, these mice show marked delays in the re-entry of hepatocytes into the cell cycle (S phase) and diminished mitotic activity. Reconstitution of C5-deficient mice with murine C5 or C5a significantly restores hepatocyte regeneration after toxic injury. Furthermore, blockade of the C5a receptor abrogates the ability of hepatocytes to proliferate in response to liver injury [445317]. The role of eculizumab on hepatic regeneration is unclear at this point.

C5 is also known to mediate chemotactic and activation events that are the basis for granulomatous responses during murine tuberculosis. Mycobacterium tuberculosis-infected mice with natural deficiency in C5 are unable to develop productive granulomatous responses, and are impaired in limiting organism growth within the lung [445321].

Clinical Development

Phase I

Rheumatoid arthritis

A phase I/II, multicenter, double-blind, placebo-controlled, dose-escalating trial tested the role of eculizumab in ameliorating RA. The trial was designed to gather clinical data on the safety profile and biological effects of a single administration of eculizumab in RA. The preliminary analysis demonstrated that the drug was well tolerated and was associated with a significant reduction in disease activity in these patients [445382]. The safety and biological activity of the antibody was examined in 40 patients with mild-to-moderate RA, each of whom received a single dose of eculizumab (0.1 to 8 mg/kg). The drug was safe and well tolerated, and showed no detectable immunogenicity in the population studied. Furthermore, a single dose of eculizumab potently and rapidly blocked complement activity in a dose-dependent fashion for up to 2 weeks [353141]. Complement inhibition after a single dose was associated with a beneficial clinical effect, with the highest dose (8 mg/kg; n = 6) resulting in a significant 30% reduction in C-reactive protein levels, compared to placebo-treated patients in which C-reactive protein increased by 24%. Eculizumab was also associated with a trend in reductions in other measurement of disease activity, including tender joint count, swollen joint count, patients' global assessment of disease and patients' global assessment of pain [291142], [305950], [322159], [335241], [347452], [335995].

Systemic lupus erythematosus

Alexion has completed preliminary analysis of the phase I study of eculizumab in SLE. The trial was designed to gather data on pharmacodynamics, safety and biological effects of a single administration of the drug in SLE patients. Eculizumab was well tolerated and its administration was associated with significant reduction in the incidence of proteinuria. The phase I, double-blind, placebo-controlled, dose-escalating trial examined the safety and biological activity of eculizumab in 24 patients with mild SLE, each of whom received a single dose of the antibody (0.1 to 8 mg/kg). There was no detectable immunogenicity in the patients studied and a single dose of antibody blocked complement activity in patients for up to 2 weeks. Administration of a single dose of 8 mg/kg was associated with significant decrease in proteinuria [292328], [328001].

Dermatomyositis

In 2001, Alexion completed a 2-month, phase I, pilot safety trial of eculizumab in 13 dermatomyositis patients with persistent dermatomyositis, undergoing concomitant treatment with moderate doses of methotrexate or steroids. The patients were evaluated in either a placebo (n = 3) or a single drug treatment arm (n = 10). Drug treatment consisted of eculizumab (8 mg/kg/week iv) for 5 weeks and then 8 mg/kg every 2 weeks for up to 2 months. The patients were evaluated after 2 months of treatment for safety and for trends in clinical improvement. In this placebo-controlled, multicenter trial, the drug was safe and well tolerated and associated with an improvement in skin rash. Exploratory clinical measurements included clinical and laboratory assessments of skin rash and muscle strength. There were consistent trends in improvements with drug administration in subjective and objective

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measurements of skin rash during the 2-month trial. While there was little baseline skin inflammation in the placebo group, a majority of drug-treated patients who completed the trial experienced an improvement of ≥ 50% in their skin rash score [354030], [434351], [435888].

Pemphigoid

Published observations have shown that clinical improvement of pemphigoid is associated with reduced levels of complement activation in the previously affected skin, further supporting the rationale for testing the C5 inhibitor in this disease. Phase I trials of eculizumab in pemphigoid syndrome are ongoing [361798], [426537], [435888], but no data are currently available.

Psoriasis

A single-center, double-blind, placebo-controlled study was designed to evaluate the safety profile and clinical effects of eculizumab in severe psoriasis [352110]. In June 2001, Alexion completed a phase I pilot safety trial involving 40 psoriasis patients, which showed that the drug was well tolerated. Drug administration did not influence the clinical outcome as measured by Psoriasis Area and Severity Index (PASI) score, although favorable trends in certain measures of disease activity were observed. Drug administration dose-dependently blocked hemolytic activity in the blood of treated patients and dose-dependently reduced deposition of activated terminal complement in psoriatic plaques [412091].

Phase II

Rheumatoid arthritis

In a phase II trial in 209 patients, eculizumab administration appeared to be safe and well tolerated and the adverse event profile was comparable to placebo. Patients were treated with placebo, eculizumab (8 mg/kg iv) once per week for 4 weeks and then once every month (induction/monthly group); eculizumab (8 mg/kg iv) once per week for 4 weeks followed by once every 2 weeks (induction/biweekly group); or, eculizumab (8 mg/kg iv) once every 2 weeks (biweekly group). The results after 3 months of treatment showed that the induction/monthly group met the primary endpoint of the trial (improvement in American College of Rheumatology response criteria score (ACR) 20 after 3 months of treatment), while the induction/biweekly and biweekly groups did not statistically meet the endpoint. The ACR20 response in the induction/monthly group was 44% as compared to 18% ACR20 in the placebo group, by per protocol analysis. Both induction/monthly and induction/biweekly groups also met the secondary endpoint of changes in C-reactive protein after 3 months of therapy [429348].

In patients with elevated C5b-9 levels over 200 ng/ml, the ACR20 at 3 months of treatment were dose-dependent. The ACR20 results obtained in patients with elevated baseline C5b-9 levels were: placebo (9%); biweekly (33%); induction/monthly (57%); and induction/biweekly (50%). Further data from this trial are to be released following unblinding [397163], [429348].

Alexion has also started a phase IIb trial in RA patients. The trial is designed to assess the safety and efficacy of eculizumab and to confirm the most efficacious dose regimen. The trial will consist of approximately 300 patients with mild-to-moderate disease who are being treated concomitantly with disease-modifying antirheumatic drugs such as methotrexate or leflunomide. The trial will consist of three treatment arms; patients will be treated with placebo; eculizumab (8 mg/kg/week iv) for 4 weeks and then once every month; or eculizumab (8 mg/kg/week) for 4 weeks and then bimonthly. The patients will be evaluated after a 6-month drug phase for safety and efficacy, and the primary efficacy endpoint will be the ACR20 score [437814].

Systemic lupus erythematosus and nephritis

In August 1999, Alexion initiated a phase II study with eculizumab in lupus patients suffering from membranous nephritis [335995]. The trial is expected to enroll approximately 40 lupus nephritis patients at four clinical sites in the US and has been designed to test the safety and biological efficacy of chronic administration of eculizumab for up to 6 months [412090]. In September 2001, Alexion expected phase II results to be available in mid-2002 [426537].

Side Effects and Contraindications

In clinical trials to date, the drug was well tolerated and showed no detectable immunogenicity in the patients studied. No major adverse reactions have been reported. In the dermatomyositis trial, the most common side effects were headache and rash, and it appeared that these side effects were comparable to the placebo group. In the psoriasis trial, the most common side effects were headaches and unspecified pain. The most common side effects in the RA trial were comparable to placebo and included diarrhea and headaches [429348], [434351].

Patent Commentary

In March 2002, Alexion was issued US-06355245 entitled 'C5-specific antibodies for the treatment of inflammatory diseases.' The patent covers the composition of Alexion's lead drug candidates, eculizumab and pexelizumab, and other C5-binding anti-inflammatory antibodies [443717]. WO-09529697, entitled 'methods and compositions for the treatment of glomerulonephritis and other inflammatory diseases' describes a method for the possible treatment of glomerular inflammation and enlargement, involving the administration of low dosages of anti-C5 antibodies. WO-09609043 describing a method for the treatment of established joint inflammation is claimed in which a C5 blocker is administered.

Current Opinion

C5 inhibitors appear to intervene at a point that allows preservation of the anti-inflammatory and antibacterial responses at the C3 level, while conceivably inhibiting the downstream disease-causing actions. Selective suppression of this immune response could certainly provide a significant therapeutic advantage compared to existing therapies. Eculizumab blocks the production of harmful complement components and appears to be promising for the treatment of various inflammatory diseases, including RA, SLE and a variety of conditions with skin involvement, including dermatomyositis, pemphigoid and psoriasis. There are other conditions in which C5a seems to play an important role, including asthma and HIV infection, and these conditions might potentially become therapeutic targets in which C5a inhibition could be useful [445327], [445336].

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Since complement activation has both positive and negative effects, the risk associated with complement inhibition is probably not negligible and long term studies will be necessary to assess possible risks. In particular, a theoretical concern is the role that C5 plays in hepatic regeneration and in combating granulomatous infections such as tuberculosis. Long term studies will determine whether C5 inhibition could have deleterious effects in these processes.

So far, limited animal and human studies have shown that eculizumab inhibits complement in a dose-dependent manner, is well tolerated and has no significant side effects. The results have been encouraging so far but more clinical trials are needed. There are still concerns that this drug does not block activation of the early stages of the complement system. This inhibitor allows the generation of C3a. While C3a is considered a less potent inflammatory molecule than C5a, the C3a receptor tissue expression appears to be much broader than originally expected [445349], [445352], and recent studies have shown that C3a can induce the production of inflammatory cytokines in a fashion similar to C5a [445354]. C3a could, in theory, play a significant role in inflammatory conditions and acute-phase response even after C5 inhibition. Nevertheless, if clinical trials prove successful, this drug as well as pexelizumab, could have a broad range of applications where complement-mediated inflammation contributes to disease pathology. There are currently no biological therapies on the market with the actions and specificity of these drugs and eculizumab could have a very strong therapeutic potential.

Development history

Developer	Country	Status	Indication	Date	Reference
Alexion Pharmaceuticals Inc	US	Phase II clinical	Rheumatoid arthritis	09-JUL-98	291142
Alexion Pharmaceuticals Inc	US	Phase II clinical	Nephritis	12-AUG-99	335995
Alexion Pharmaceuticals Inc	US	Phase I clinical	Psoriasis	06-OCT-00	384948
Alexion Pharmaceuticals Inc	US	Phase I clinical	Dermatological disease	06-OCT-00	384948
Alexion Pharmaceuticals Inc	US	Phase I clinical	Pemphigus	06-OCT-00	384948
Alexion Pharmaceuticals Inc	US	Preclinical	Glomerulonephritis	29-JUL-97	190673
Alexion Pharmaceuticals Inc	US	NDR	Systemic lupus erythematosus	11-APR-02	-

Literature classifications

Biology

Study Type	Effect Studied	Experimental Model	Result	Reference
In vivo	Anti-arthritic effect.	Collagen-induced arthritis in mice and rats.	An anti-C5 antibody both prevented the onset of disease and reduced established disease.	190673 212456
In vivo	Reduction in evidence of kidney disease.	NZB/NZW F1 lupus-prone mouse model.	An anti-C5 antibody reduced both the histological and biochemical signs of nephritis, and increased survival to 85% at 10 months.	232606

Metabolism

Effect Studied	Model Used	Result	Reference
Pharmacokinetics.	Complement activity.	A single dose of eculizumab blocks complement activity for 1 to 2 weeks.	438165

Clinical

Effect Studied	Model Used	Result	Reference
Safety and efficacy.	Phase I trial in 40 psoriasis patients.	Eculizumab was well tolerated and dose-dependently reduced complement activity in patients. Favorable trends were observed in PASI, although these were not significant.	412091
Safety and efficacy.	Phase I trial in 13 patients with persistent dermatomyositis.	Eculizumab (8 mg/kg/week iv) for 5 weeks, then every 2 weeks for up to 2 months, was safe and well tolerated. Drug treatment reduced skin rash score by ≥ 50%.	434351
Safety and efficacy.	Double-blind, placebo-controlled, phase I, dose-escalating trial in 24 patients with mild SLE.	Eculizumab (0.1 to 8 mg/kg) was safe and well tolerated, and no detectable immunogenicity was observed. The highest dose significantly reduced proteinuria.	328001
Safety and efficacy.	Phase II trial in 209 RA patients.	Eculizumab produced ACR20 in 44% of the patients treated. Adverse effects were similar to placebo, with nausea and diarrhea being the most common complaints.	429348
Safety and efficacy.	Phase II trial in RA patients in whom the C5b-9 levels were > 200 ng/ml.	A total of 57% of patients achieved ACR20 when treated with induction/monthly treatment of eculizumab.	429348

Associated patent

Title Method for reducing immune and hemostatic dysfunctions during extracorporeal circulation.

Assignee Alexion Pharm Inc

Publication WO-09525540 28-SEP-95

Priority US-00217391 23-MAR-94

Inventors Rollins SA, Smith BR, Squinto SP.

Associated references

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• *This press release documents the theoretical actions of eculizumab and its effectiveness in reducing histologic and clinical development of nephritis in a murine model of lupus nephritis.*

212456 **Anti-C5 monoclonal antibody therapy prevents collagen-induced arthritis and ameliorates established disease.** Wang Y, Rollins SA, Madri JA, Matis LA *PROC NATL ACAD SCI USA* 1995 92 19 8955-8959

212462 ***In vitro* and *in vivo* inhibition of complement activity by a single-chain Fv fragment recognizing human C5.** Evans MJ, Rollins SA, Wolff DW, Rother RP, Norin AJ, Therrien DM, Grijalva GA, Mueller JP, Nye SH, Squinto SP *et al MOL IMMUNOL* 1995 32 16 1183-1195
• *This paper documents the effectiveness of the anti-C5 monoclonal antibody (N19-8 scFv) at inhibiting complement-dependent myocardial injury in isolated mouse heart. In other studies, the ability of the antibody to inhibit complement hemolytic activity in rhesus monkeys while being well tolerated at doses up to 100 mg was documented.*

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212467 **Rapid expression of an anti-human C5 chimeric Fab utilizing a vector that replicates in COS and 293 cells.** Evans MJ, Hartman SL, Wolff DW, Rollins SA, Squinto SP *J IMMUNOL METHODS* 1995 184 1 123-138
• *This paper describes the generation and expression of the 5G1.1-SC antibody.*

225318 **Anti-C5 monoclonal antibody: a novel anti-inflammatory agent.** *EXP OPIN THER PAT* 1996 6 11 1229-1230

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• *This press release reports on the ability of 5G1.1-SC to block complement activation after intravenous administration into healthy male volunteers*

232606 **Amelioration of lupus-like autoimmune disease in NZB/WF1 mice after treatment with a blocking monoclonal antibody specific for complement component C5.** Wang Y, Hu Q, Madri JA, Rollins SA, Chodera A, Matis LA *PROC NATL ACAD SCI USA* 1996 93 16 8563-8568
• *This paper documents the effectiveness of an intact anti-C5 antibody in reducing glomerulonephritis and increasing survival in a murine model of systemic lupus using autoimmune mice.*

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RN 219685-50-4 REGISTRY REGISTRY COPYRIGHT 2009 ACS on STN
RN 219685-50-4 REGISTRY
ED Entered STN: 14 Feb 1999
CN Immunoglobulin, anti-(human complement C5 .alpha.-chain) (human-mouse monoclonal 5G1.1 heavy chain), disulfide with human-mouse monoclonal 5G1.1 light chain, dimer (9CI) (CA INDEX NAME)
OTHER NAMES:
CN Eculizumab
CN h5G1.1
CN h5G1.1VHC+h5G1.1VLC
CN Soliris
FS PROTEIN SEQUENCE
MF Unspecified
CI MAN
SR US Adopted Names Council (USAN)
LC STN Files: ADISINSIGHT, BIOSIS, CA, CAPLUS, CBNB, CHEMCATS, EMBASE, IMSDRUGNEWS, IMSPATENTS, IMSPRODUCT, IMSRESEARCH, IPA, MRCK*, PHAR, PROUSDDR, TOXCENTER, USAN, USPATFULL
(*File contains numerically searchable property data)

*** STRUCTURE DIAGRAM IS NOT AVAILABLE ***
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59 REFERENCES IN FILE CA (1907 TO DATE)
2 REFERENCES TO NON-SPECIFIC DERIVATIVES IN FILE CA
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Opposition against EP 1720571
(Application No.: 05 71 2607.0)
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

München, December 30, 2013
WA/ARO/FLG

This is in response to the Official Communication of April 19, 2013 issued by the European Patent Office in the above-referenced opposition proceedings.

It is requested that the patent EP-B1 1720571 is maintained on the basis of the claims as granted. As will be evident from our written submissions herein below, our request that the patent be maintained on the basis of the granted claims is fully justified. Since furthermore the Opponent, Pfizer Inc., formally and irrevocably has withdrawn its opposition on October 7, 2013, it is requested that the opposition proceedings are promptly terminated.

In case the Opposition Division would be inclined to proceed differently, we request oral proceedings in accordance with Article 116 EPC. In that case we request that we be allowed to submit one or more additional claim request(s).

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1. INADMISSIBLE BROADENING (Articles 100(c) and 123(2) EPC)

1.1. The then Opponent alleges that the subject-matter of independent claims 1 and 5 and the claims dependent thereon is not directly and unambiguously disclosed by the application a filed thereby contravening Article 123(2) EPC.

1.2. The second medical use claims 1 and 5 comprise identical features. Claim 1 is drafted as purpose-limited product claim in accordance with the EPC 2000 and claim 5 is drafted as a Swiss-type claim in accordance with the EPC 1973.

The Opponent has divided these claims into the following features:

(1) the use of eculizumab,
(2) the treatment of a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH),
(3) intravenous administration,
(4) administration of 600 mg each week for four weeks,
(5) administration of 900 mg 1 week later,
(6) administration of 900 mg on a biweekly basis,
(7) a breakthrough in serum hemolytic activity when treated under a dosing schedule according to (4) to (6), and
(8) administration of 900 mg every 12 days.

1.2.1. In more detail, the opponent is of the view that each of features (1) and (3) requires the selection of an item from a list of equally categorized items. In other terms, three selections from three lists are allegedly required in order to arrive at features (1) and (3) thereby violating Article 123(2) EPC.

1.2.2. The Opponent furthermore purports that feature (3) has no basis in the application as filed but only the more narrow feature "intravenous infusion by injection" (see page 20, lines 4-8 of the application as filed).

1.2.3. The Opponent considers that the general specification of the applications does not describe any specific dosage regimen. The Opponent concludes that the only basis for features (4) to (8) can be found in the Examples of the application as filed. However, the Opponent contends that features (4) to (8) are an undue

generalisation of the specific contents of the Examples and that in particular the following essential features of the Examples would have been omitted in claims 1 and 5:

(i) the PNH patients are transfusion-dependent and hemolytic

(ii) the meaning of transfusion dependency in the context of the application

(iii) the infusion time of approx. 30 min, and

(iv) the occurrence of the breakthrough between weeks 12 and 64.

1.2.4. With regard to feature (4) the Opponent further alleges that the term "600 mg each week for four weeks" only requires the administration of 600 mg once per week for four weeks, while the Examples would require that the 600 mg are administered at a time interval of exactly 7 days (i.e. weekly intervals) for four weeks.

1.2.5. Finally, the Opponent believes that it would not be clearly supported in the Examples whether the dosages according to features (5) and (6) were intravenously administered. The route of administration would not have been specified in the Examples.

1.3. <u>The new claims have a clear and unambiguous basis in the application as filed.</u>

1.3.1. Granted claim 1 reads:

> "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)."

As will be detailed herein below, granted claim 1 and consequently also granted claim 5 has a clear and unambiguous basis in the application as filed, because

(i) all features of these claims can be directly and unambiguously derived from the Examples of the application as filed, and

(ii) no technically essential feature of the examples has been omitted or changed.

In the following we will provide a clear and unambiguous basis for each of features (1) to (8) as classified by the Opponent.

1.3.2. *Feature (1) - use of eculizumab*

Eculizumab is the antibody which is used in the examples of the application as filed; see page 32, line 14 reading

> "The specific antibody used in the study was eculizumab."

Thus - in contrast to the allegations of the opponent - no selection from a list is required in order arrive at the anti-C5 antibody which is eculizumab. Hence, feature (1) is directly and unambiguously supported by the Examples of the application as filed.

1.3.3. *Feature (2) - treatment of a patient afflicted with PNH*

The Examples of the application as filed at page 32 start to read:

> "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." (emphasis added)

Hence - contrary to the assertions of the opponent - no selection from a list is required in order to define the patient to be treated as patient being afflicted with PNH.

The Opponent further considers that the PNH patient has to be further defined as (i) being hemolytic and (ii) having a history of four or more transfusions within twelve months whereas these features are not reflected by the claim.

(i) hemolytic anemia

At page 2, lines 9 to 15 of the application as filed it is described that PNH is characterized by hemolytic anemia. Hemolytic anemia is one of the characterizing symptoms of the disease PNH.

The Patentee has informed us that every individual to a very slight extent has some PNH cells, but they are present at an extremely low level and of course these individuals are not considered to be PNH patients. In a very small number of individuals these PNH clones of cells begin to expand and when they expand enough, hemolysis becomes apparent. These individuals will be diagnosed as being a PNH patient. Consequently, every individual given a clinical diagnosis of PNH will show hemolysis.

In summary, a PNH patient is inherently hemolytic. The omission of an inherent feature cannot violate Article 123(2) EPC. Compliance of Article 123(2) EPC has to be assessed from the perspective of a skilled person.

(ii) history of blood transfusions

As is evident from the above-citation from page 32 of the application as filed, the PNH patients used in the examples of the application had a history of four or more blood transfusions within twelve months. In order to judge whether this information on the PNH patients has to be considered a technical feature which cannot be omitted in claims 1 and 5 without violating Article 123(2) EPC we refer to the pertinent case law. In T 331/87 the Board developed the following general three criteria for the replacement or removal of a feature from a claim:

> "The replacement or removal of a feature from a claim may not violate Article 123(2) EPC provided the skilled person would directly and unambiguously recognise that
> (1) the feature was not explained as essential in the disclosure,
> (2) it is not, as such, indispensable for the function of the invention in the light of the technical problem it serves to solve, and
> (3) the replacement or removal requires no real modification of other features to compensate for the change (cf. point 6 of the Reasons; following Decision T 260/85, "Coaxial connector/AMP" OJ EPO 1989, 105)." (see headnote of T 331/87)

As regards requirement (1) of the above-described three-point test, we note that nothing in the application as filed conveys the impression that only PNH patients being transfusion-dependent can be treated by eculizumab.

In view of requirement (2) we note that administration of eculizumab prevents hemolytic events by blocking the complement cascade. Since all PNH patients suffer from hemolytic anemia, all PNH patients will benefit from the claimed use of eculizumab. It is of further note that the skilled person immediately understands from the Examples of the application as filed that all PNH patients showing a complement breakthrough should be treated by 900 mg of eculizumab every 12 days. A requirement of a certain transfusion history for the treatment to be successful is not apparent.

With regard to requirement (3) Patentee asserts that all PNH patients and not only transfusion dependent PNH patients may exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii). In more detail, the Patentee has indicated that, to the best of its knowledge, the ability to show breakthrough appears to be independent of the severity of the disease. Instead, breakthrough is related to how rapidly eculizumab is cleared from the circulation. Some patients seem to clear eculizumab at a higher rate than other patients. The severity of the disease - which may be expressed at least in part by the blood transfusion history of a PNH patient - has therefore no technical effect on the other features of granted claims 1 and 5.

It is thus our firm believe that the PNH patient in claims 1 and 5 neither has to be further defined as (i) being hemolytic nor as (ii) having a history of four or more transfusions within twelve months in order to render these claims compliant with Article 123(2) EPC.

1.3.4. *Feature (3) - administered intravenously*

In the Examples on page 32, lines 12-16 it is stated:

> "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." (emphasis added)

Hence, in the Examples eculizumab is administered intravenously. Again - contrary to the statements of the opponent - no selection from any list in the specification is required in order to define that eculizumab is administered intravenously.

The allegation of the Opponent that only the first dosage of weekly 600mg for four weeks has been administered intravenously makes no technical sense. As the Opposition Division knows, an application has to be read with a mind willing to understand; see, e.g., decision T 190/99. Also the prior art cited by the opponent itself shows that eculizumab is routinely administered intravenously. For example, it is stated in D4 that

> "Patients received 600mg eculizumab by intravenous infusion over 30 minutes weekly for 4 doses and then 900 mg every other week". (emphasis added)

Essentially corresponding statements can be found in D3 and D6.

Granted claims 1 and 5 refer to intravenous administration and not intravenous infusion. The skilled person knows, though, that an intravenous administration is always by a "push" - which is a one-time, rapid injection of the medication into the bloodstream – or by an "infusion" - which is a slow drip of medication into the vein over a period of time. The technical effect is in both cases that eculizumab is delivered in the blood stream. The only difference is the time frame in which the complete dose of eculizumab is administered. However, the time is not characterized as being essential in the application as filed. The indication of the time is also certainly not indispensable in order to work the invention, and varying the infusion time does not require a modification of the other features of the claim. Hence, in connection with the omittance of the intravenous time the criteria of the "three-point test" developed in T 331/87 are clearly met. Omittance of the infusion time of approx. 30 minutes does not constitute a violation of Article 123(2) EPC.

1.3.5. *Feature (7) - breakthrough in serum hemolytic activity*

The study described in the Examples of the application as filed has been initially carried out for 12 weeks and then has been extended to 64 weeks (see, for example, Table 1 or Figure 3 of the application as filed). The breakthrough in 2 out of 11 PNH patients has been observed between weeks 12 and 64 of the study (see Figure 4 of the application as filed).

It is not essential with respect to the claimed dosage regimen when a PNH patient has a complement breakthrough. The essential step is to increase the dosage from 900mg/14d to 900mg/12d once a complement breakthrough is observed. As it is described in the Examples at page 38, lines 15-17, the complement blockade was permanently restored by shortening the interval of administration - although both PNH patients likely did not show the complement breakthrough at the same time point during the 64-weeks study.

Hence, it is not technically necessary to define in granted clams 1 and 5 at which particular point in time a breakthrough is observed in a PNH patient to be treated.

1.3.6. *Features (4) to (6) and (8)*

The fact that also the dosages according to features (5) and (6) were administered intravenously has been discussed herein above in connection with feature (3).

The Opponent's interpretation of the wording of feature (4) is a mere play on words and should not attract any attention.

Beyond these points the Opponent apparently does not contest that features (4) to (6) and (8) as such can be clearly and unambiguously derived from the Examples of the application as filed. Having said that, the Opponent alleges that these features would only find support in connection with further features of the Examples. These further allegedly omitted features have been discussed herein above in connection with features (1) to (3) and (7).

As is evident from the above discussion in connection with features (1) to (3) and (7) these features do not violate Article 123(2) EPC.

1.3.7. The Opponent does not consider that the features in the dependent claims give raise to any further violation of Article 123(2) EPC. Hence, all of the granted claims comply with Article 123(2) EPC.

2. PRIORITY (Article 87 EPC)

2.1. For essentially the same reasons put forward by the Opponent in section 4 entitled "added subject-matter", the Opponent believes that the subject-matter of the claims cannot be directly and unambiguously derived from the priority application.

The Opponent concludes that the effective date of the granted claims is not the priority date of February 3, 2004 but the international filing date of February 3, 2005.

2.2. As will be shown in the following the relevant contents in the Examples of the application as filed - which form a clear and unambiguous basis for the granted claims - are also comprised in the priority document US 10/771,552; compare page 30, lines 17-18 of the priority document and page 32, lines 16-20 of the application as filed.

In more detail, page 30 of US 10/771,552 reads:

> 5 **EXAMPLES**
>
> **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 transfusion within twelve months. The**

and

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 bi-weekly basis. The first twelve weeks of the study constituted the pilot study. All patients participated in an extension study conducted to a total of 48 weeks.

These two passages give rise to priority for features (1) to (6).

Page 30 of US 10/771,552 reads

0.001). As seen in Figure 4, break-through of complement blockade resulted in hemoglobinuria, dysphagia, and increased LDH and AST. At the next dose, symptoms resolved (Fig 5) and reduction from 900mg every 14 days to 900mg every 12 days resulted in a regain of complement control which was maintained for over 9 months in both patients. This patient shows a 24 hour resolution of

and page 34 of US 10/771,552 reads

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).

These two passages give rise to priority for features (7) and (8).

Otherwise arguments in favour of clear and unambiguous support in the application as filed as detailed in section 1, *supra* apply *mutatis mutandis* for entitlement of priority.

Hence, the subject-matter of granted claims 1 and 5 has also a clear and unambiguous basis in the priority application. The priority claim is valid.

3. NOVELTY (Articles 100(a) and 54 EPC)

3.1. The Opponent considers that the claims lack novelty in view of D1.

3.2. D1 has been published on February 5, 2004 and hence 2 days after the priority date of the patent. D1 is not prior art because the claims are entitled to the priority date; see section 2, *supra*.

4. INVENTIVE STEP (Article 100(a) and 56 EPC)

4.1. The Opponent alleges that the granted claims lack inventive step in view of D5 considered alone or in combination with any one of D2, D3, D4 or D6. The Opponent also contends that the granted claims are no inventive in view of any one of D2, D3, D4 or D6.

4.2. D5 may be considered as closest prior art in the assessment of inventive step of the granted claims. D5 describes:

> "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. [...] Two patients experienced transient breakthroughs in complement activity associated with LDH, hemoglobinuria and mild symptoms; these resolves immediately upon next dose of eculizumab and further breakthroughs were prevented by a slight decrease in dosing interval." (emphasis added)

Details on the 3-months pilot study are, for example, described in D3, D4 and D6 (see page 53, left column). Hence, D5 (in combination with any one of D3, D4 and D6) could be seen as describing that 2 out of 11 PNH patients exhibited a complement breakthrough when treated under a dosage regimen similar, but not identical to that recited in the granted claims. D5 furthermore teaches that the complement breakthrough can be permanently prevented by a slight decrease in dosing interval.

4.3. Objective technical problem

Independent granted claims 1 and 5 are distinguished from D5 in that

(i) the dosing interval is the administration of 900 mg eculizumab every 12 days; and

(ii) feature (5) requiring "administration of 900 mg 1 week later" is absent from D5.

Accordingly, the objective technical problem underlying the granted claims in view of D5 may be seen as the determination of the specific dosing interval which prevents further breakthroughs in patients having the prior treatment history as defined in the claims.

The solution is the administration of 900 mg eculizumab every 12 days to patients having the prior treatment history as defined in the claims.

4.4. Non-obviousness

D5 neither teaches nor suggests the time interval of administration which is designated as a "slight decrease in dosing interval". Patentee submits that the skilled person would not have arrived at the exact dose of 900 mg eculizumab every 12 days without the need of inventive skills.

This is particularly evident when considering the prior art at the time of the invention in a comprehensive manner. Pursuant to the pertinent case law, e.g. T 24/81, second headnote, in the examination of inventive step all previously published embodiments must be taken into consideration which offered a suggestion to the skilled practitioner for solving the problem addressed, even where those embodiments were not particularly emphasized.

We enclose an article of Maria Kaplan in Current Opinion on Investigational Drugs 2002, 3(7):1472-4472. This article has been cited as document D10 during examination proceedings of the opposed patent and is introduced into the present opposition proceedings as document D13.

D13 describes at page 4, left column, fourth paragraph a Phase II trail on the treatment of RA patients with eculizumab:

> "Patients were treated with placebo, eculizumab (8 mg/kg iv) once per week for 4 weeks and then once every month (induction/monthly group); eculizumab (8mg/kg iv) one per week for 4 weeks followed by once every 2 weeks (induction/biweekly group); or eculizumab (8 mg/kg iv) once every 2 weeks (biweekly group). The results after 3 months of treatment showed that the induction/monthly group met the primary endpoint of the trial (improvement in American College of Rheumatology response criteria score (ACR) 20 after 3 months of treatment), while the induction/biweekly and biweekly groups did not statistically meet the endpoint."

Importantly, only patients receiving treatment every month, but not the patients receiving treatment every two weeks meet the endpoint of the trial – being an improvement of RA. In other words, an increase of the dose of eculizumab by increasing the dosing intervals did not have a beneficial effect. Hence, D13 shows that shortening of dosing intervals may not result in an effective treatment by eculizumab.

The inventors of the opposed patent have unexpectedly found that the 12-days period is sufficient to successfully treat PNH patients which suffer from breakthrough in serum hemolytic activity when treated under a dosing schedule according to the above-defined features (4) to (6), and at the same time is not too short such that an effective treatment by eculizumab is prevented, as observed in D13. This surprising key finding of the present invention is described at page 35, lines 5-23 of the application as filed:

> "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." (emphasis added)

Hence, the critical point in order to consistently block serum hemolytic activity is maintaining levels of eculizumab above 35 µg/ml. Thus is achieved in accordance with the invention by the reduction of the dosing interval from 900 mg every 14 days to 900 mg every 12 days. Arriving at the 12-days period is consequently not a matter of routine but required due consideration and mindful experimentation. Applicant therefore submits that the granted claims are inventive over D5. The deficiencies of D5 cannot be cured by any one of D2, D3, D4 or D6. No prior art document teaches or suggests a 12-days dosing interval, let alone the superior efficacy in preventing complement breakthrough hemolysis in PNH patients associated with this dosing interval.

5. SUFFICIENCY OF DISCLOSURE (Articles 100(b) and 83 EPC)

5.1. Eculizumab

5.1.1. The Opponent purports that the claims lack sufficiency of disclosure, because the complete sequence of eculizumab (or h5G1.1) would neither be disclosed by the application as filed nor any one of the prior art documents cited by the Opponent.

5.1.2. The Patentee asserts that the sequence for eculizumab was publicly available prior to the priority and filing date. A sequence for eculizumab was submitted to Chemical Abstracts Services (CAS) and entered into their STN database on 14 February 1999; please see the attached copy of the CAS entry No. 219685-560-4.

5.2. The term "breakthrough in serum hemolytic activity"

5.2.1. The Opponent considers that the term "breakthrough in serum hemolytic activity" is not sufficiently disclosed by the application as filed, because it is allegedly not

> "[...] clear whether one, two, three, four of these events [breakthrough of complement blockade] are necessary and/or sufficient to characterize a

breakthrough according to the claims." (see the Opponent's Appeal Brief at section 7.2, end of second paragraph)

The Opponent concludes that the skilled person would not be able to characterize and identify a "breakthrough in serum hemolytic activity".

5.2.2. The argument put forward by the Opponent reads on a lack of clarity rather than on a lack of sufficiency of disclosure. An alleged lack of clarity is not a valid ground for opposition.

Notwithstanding we wish to submit that Figure 4 of the application as filed shows that a complement breakthrough can be identified by blood in the morning urine. Breakthrough in serum hemolytic activity which is the result of the complement activity can also be diagnosed using a blood sample (see page 35, lines 5-14 of the application as filed). As such, and for the mere sake of arguments we submit that the term "breakthrough" is clear for the skilled person and means for its determination are at the skilled person's disposal.

6. CONCLUSION

In view of the above we submit that the claims as granted fulfill the requirements of the EPC. Our request that the patent be maintained on the basis of the granted claims is therefore fully justified.

Dr. Joachim Wachenfeld
European Patent Attorney

Enclosure:
CAS entry
D13

Samsung Bioepis Exhibit No. 1002
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14-01-2014

Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571
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.

The matters to be discussed are set out in the communication accompanying this summons (EPO Form 2906).

The oral proceedings, which will be public, will take place before the opposition division

on 15.05.14 at 09.15 hrs in Room 1662 at the EPO, Grasserstr. 2, PschorrHöfe, D-80335 München

No changes to the date of the oral proceedings can be made, except on serious grounds (see OJ EPO 1/2009, 68). If you do not appear as summoned, the oral proceedings may continue without you (R. 115(2) EPC).

Your attention is drawn to Rule 4 EPC, regarding the language of the oral proceedings, and to the Special edition No. 3 OJ EPO 2007, 128, 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 17.03.14.

You are requested to report in good time beforehand to the porter in the EPO foyer. Room 1601 and 1657 are available as waiting rooms.

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 the porters' lodge in the main foyer "Bayerstrasse", the parking ticket will be revoked.

1st Examiner:	2nd Examiner:	Chairman:
Chapman R	Weikl M	Cilensek Z

For the Opposition Division

Annexes:
Confirmation of receipt (Form 2936)
Rule 4 EPC (EPC Form 2043)
Communication (EPO Form 2906)

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Registered letter with advice of delivery

to EPO postal service: 10.01.14

1 Facts and Submissions

Reference is made to the following documents cited by the Opponent; the numbering will be adhered to for the rest of the proceedings.

D1 Hillmen et al., (2004) The New England Journal of Medecine, 350:552-9

D2 Jane Salodof MacNeil (2002) Medscape Medical News

D3 Hillmen et al., (2003) British Journal of Hematology 121 (Suppl. 1) 87-95, Abstract 257

D4 Hillmen et al., (2002) Blood. 100(11), Abstract 154

D5 Hillmen et al., (2003) Journal of The American Society of Hematology 102(11) Abstract 1858

D6 Rother et al., (2003) Molecular Immunology. 40, p 197, Abstract 54

D7 Rosse et al., (2004) American Society of Hematology, p 48-62

D8 Biotechnology News (2004) 24(30), p 10

D9 US 6,355,245

D10 Thomas et al., (1996) Molecular Immunology, Vol. 33, No. 17/18, p 1389-1401

D11 Drugs of the Future (2004) 29(7): 673-676

D12 Hillmen et al., British Journal of Haematology, 125 (Suppl. 1), 1-72, Abstract 5

1.1 European patent EP1720571 (hereinafter designated the patent) entitled "Method of treating hemolytic disease", was published in the official bulletin on 13.06.2012 and a corrigendum was issued on 24.10.2012. The Proprietor (hereafter designated 'P') is Alexion Pharmaceuticals, Inc., Cheshire, US.

Said patent is based on European application 05712607.0, filed on 03.02.2005 and claims the priority from US 10/771,552 filed on 03.02.2004. The International publication number is WO2005/074607.

1.2 Notice of opposition against the above-mentioned patent was received from the following party (hereinafter named the Opponent or 'O').

1. Pfizer Inc., 235 East 42nd Street, New York 10017, US (filed online with letter dated 12.03.2013, received on 12.03.2013).

1.3 The O requested the revocation of the patent as granted in its entirety on the basis of Articles 100(a)(b)(c) because the subject-matter granted was not novel, inventive, or disclosed in a manner sufficiently clear and complete to be

carried out by a skilled person (Articles 52(1),54,56 and 83 EPC), and comprised subject-matter that extended beyond the application as originally filed (Article 123(2) EPC). The O filed D1-D12 therewith.

1.4 In their letter dated 07.10.2013, the O withdrew their request of opposition against the above-mentioned patent.

1.5 With their letter dated 09.10.2013, the P requested that their patent be maintained as granted and that the opposition procedure be closed.

1.6 The P requested oral proceedings in case their respective request would not be granted.

2 Preliminary, non-binding opinion of the opposition division (OD)

The admissibility of the opposition has not been contested by the P. The opposition is thus considered admissible (Article 99(1) and Rule 76 EPC).

The O submitted documents and arguments that would appear to be *prima facie* prejudicial to the maintenance of the patent as granted. Since the O has withdrawn his opposition, the OD feels it pertinent to continue opposition *proceedings upon its own volition* (GL D, VII-5.2,5.3; Article 114(1) EPC).

Added subject-matter (Articles 100(c) and 123(2) EPC)

2.1 The O objected to claims 1 and 5 of the patent on grounds that they are not directly and unambiguously disclosed in the application, rather an intermediate generalisation of a specific example (p.32-33) combined with general teachings of the application.

2.2 The O considered the claims to contain the following features:

1. the use of eculizumab
2. the treatment of a patient affected with PNH
3. I.V. administration
4. administration of 600mg each week for 4 weeks
5. administration of 900mg a week later
6. administration of 900mg bi-weekly thereafter
7. a breakthrough in serum hemolytic activity when the patient is treated under a dosing schedule according to points 4 - 6
8. administration of 900mg every 12 days thereafter

2.3 Features 1 and 2 would appear to have a general basis on p.12, li.1-3 and p. 18, li.12-20, respectively. The Examples on p.33 also contain these features.

2.4 Feature 3 would appear to be broader than the basis provided on p.20, li.4-8, which relates merely to 'intravenous infusion by injection'. Feature 4 would also encompass both injection into a vein using a needle and the use of a catheter. The OD finds that since p.20, li.4-8 neither specifically states the use of a needle or a catheter, 'intravenous infusion by injection' could be considered to encompass both I.V. infusion via needle or catheter, i.e. intravenous administration; however, 'I.V. administration' does not necessarily imply administration of an infusion. 'Infusion' implies the use of a catheter, but this is not stated in the application. The OD is thus of the opinion that **Feature 3 has no basis** in the application as filed as it also encompasses administration of pharmaceutical formulations other than infusions.

2.5 Features 4 - 7 relate to a dosage schedule. The general teaching of the application on p.19-20 states that the dose will vary according to the compound used, the manner of administration, the symptoms, the size and the age of the patient. The only basis for the dosage regimen in the claim comes from the specific example on p.32-33, which describes steps 1 - 7 applied to a patient group. Two of the patients in that group exhibited breakthrough in serum hemolytic activity which was controlled by reducing the dosage interval to 12 days (p.35, li.8-17). The O is of the opinion that the combination of Features 1 - 8 cannot be derived from either the general part of the description or the examples.

2.6 The OD agrees that the only basis for claims 1 and 5 is in the specific examples.

2.7 However, the O argues that 'essential features' of the example are missing from the claim. The OD notes that clarity is not a grounds of opposition. There is no indication in the application to suggest that the specific patient sub-group to be treated must be transfusion-dependent and hemolytic (p.32, li.7-9), that an infusion time of 'approximately 30 mins' (p.30, li.12-14), or that the time point of breakthrough occurrence (between weeks 12-64) are essential to the application claimed. The OD finds that it is clear from the examples that any patient receiving the claimed dosage regimen for PNH and experiencing breakthrough could be treated by shortening the interval between doses, as claimed. The OD does not see that there is an intermediate generalisation of the examples in this respect.

2.8 The O further argues that there is no basis for the route of administration used following the initial four week dosing schedule, i.e. Feature 4, since the route thereafter is not specified. The OD finds that it would be obvious that further antibody treatments would be administered by the same route, however, the O is correct in that this is not clearly stated in the application. This point will require further discussion at oral proceedings.

Lack of novelty (Articles 100(a) and 54 EPC)

2.9 The O raised an objection on grounds of lack of novelty regarding claims 1 and 5 with regard to D1.

2.10 D1 published on 05.02.2004 discloses all the features of claims 1-8 (see Methods and section 'Biochemical indicators of hemolysis' on p.552-556 and Fig.3A) but is published after the date of priority of the patent.

2.11 The patent claims priority from US10/771552, filed on 03.02.2004, and appears to disclose identical subject-matter to the application filed on 03.02.2005 on which the patent is based.

2.12 The O considered the priority document not to disclose the subject-matter of the present claims for the same reasons they consider the patent's claims not to have a basis in the application as filed (Article 123(2) EPC).

2.13 Since doubt has been raised as to whether a basis for claims 1 and 5 exists in the application, the same doubts result for the disclosure of the priority document. Therefore, the subject-matter of D1 is considered relevant for the assessment of the novelty and inventive step of the claims.

2.14 The OD is of the provisional opinion that claims 1-8 of the contested patent lack novelty over D1.

Lack of inventive step

2.15 The O objected to the patent on grounds of a lack of inventive step, taking D2, D3,D4 or D6 as the closest prior art to the subject matter of claims 1 and 5.

2.16 Documents D3,D4 and D6 all disclose the following features:

1. the use of eculizumab
2. the treatment of a patient affected with PNH
3. I.V. administration
4. administration of 600mg each week for 4 weeks

5. administration of 900mg a week later
6. administration of 900mg bi-weekly thereafter

2.17 Document D2 does not disclose the Feature 3 and thus will not be considered further.

2.18 The difference between either D3,D4 or D6 and the present claims is considered to be the dosage.

2.19 The technical effect associated with this difference is the alleviation of symptoms associated with a breakthrough in hemolytic activity.

2.20 The problem to be solved can therefore be considered to be the provision of an improved dosage regimen for patients suffering a breakthrough in hemolytic activity.

2.21 The solution provided in the granted claims would appear obvious, since according to D5, transient breakthroughs in complement activity associated with increased LDL, hemoglobinuria and mild symptoms in an extension trial of patients receiving the dosage regimen described in D3,D4 or D6 were known to be preventable by a slight decrease in the dosing interval.

2.22 The skilled person with a knowledge of any of D3,D4 or D6, and observing a breakthrough in hemolytic activity in patients would therefore find it obvious to slightly reduce the dosing interval until no further hemoglobinuria event is observed (as shown in Fig.5 of the patent).

2.23 The establishment of optimal dosage regimens is considered to be routine according to the case law of the Technical Board of Appeal T1979/09, wherin under point 7.3.1 it is stated *'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.'*

2.24 and further under point 7.3.2, *'In the present case the amount of active ingredient remains the same as the amount used in D5 and there is no evidence on file of any unexpected technical effect caused by administering the red vine leave extract once daily. 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.'*

2.25 It is therefore the provisional opinion of the OD that at least the subject-matter of claims 1 and 5 does not involve an inventive step, in light of either D5 alone, or in combination with any one of D3,D4 or D6.

2.26 Dependent claims 2-4 and 6-8 do not appear to contain any additional features which, in combination with any other claim to which they refer, would meet the requirements of the EPC with respect to inventive step.

Insufficiency of disclosure (Articles 100(b) and 83 EPC)

2.27 The O raised the objection that the trade name 'eculizumab' did not sufficiently disclose such an antibody, since, at the time of filing, such an antibody was not completely described.

2.28 The application makes reference to D9 and D10, which do not make reference to deposits or sequences that disclose the humanised anti-C5 antibody to which it allegedly refers.

2.29 The OD is of the opinion, that in the absence of evidence that eculizumab *could be identified as a specific antibody, either by deposit or* ***full*** *sequence of* its light and heavy chains, the antibody and thus the subject-matter of claims 1-8 cannot be considered to be sufficiently disclosed.

2.30 The O made further objections to the clarity of the term 'breakthrough in serum hemolytic activity'. The OD will not pursue this objection since a lack of *clarity is not a ground for opposition*.

Conclusion

3 At present the OD is of the preliminary opinion that the patent contains added subject-matter (Articles 100(c) and 123(2) EPC), does not involve an inventive step (Articles 100(a) and 56 EPC), and lacks sufficient disclosure (Article 100 (b) and 83 EPC).

In view of the request of the P, oral proceedings are hereby appointed.

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Reference	Application No./Patent No.
M2367 EPOPPCEP)	05712607.0 - 1412 / 1720571
Applicant/Proprietor	
ALEXION PHARMACEUTICALS, INC.	

EPA/EPO/OEB Formblatt/Form/Formulaire : 2310

Empfangsbescheinigung über den Zugang des vorstehend bezeichneten Schriftstücks
Acknowledgement of receipt of the document specified above
Récépissé du document spécifié ci-dessus

Unter Bezugnahme auf die Mitteilung im ABl EPA 7/2010, 377 wird gebeten, die Empfangsbescheinigung mit Empfangsdatum und Unterschrift zu versehen und **umgehend** an das EPA zurückzusenden:

With reference to the Notice in OJ EPO 7/2010, 377, you are requested to date and sign the acknowledgement of receipt and return it to the EPO **immediately**:

Conformément au communiqué paru au JO OEB 7/2010, 377, vous êtes prié d'indiquer sur le récépissé la date de réception du document, de signer le récépissé et de le renvoyer **sans délai** à l' OEB:

- **über die Online-Dienste des EPA** (als Anlage zu EPA Form 1038) / **through EPO Online Services** (as annex to EPO Form 1038) / **par les services en ligne de l'OEB** (en tant que pièce jointe au formulaire OEB 1038),
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18-02-2014

Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571

Applicant/Proprietor
ALEXION PHARMACEUTICALS, INC.

BRIEF COMMUNICATION

Subject:
- ☒ Your letter of 30.12.13
- ☐ Our telephone conversation of
- ☒ Communication of 14.01.14
- ☐

Enclosure(s):
- ☐ Letter from the proprietor of the patent of
- ☐ Letter from the opponent of
- ☐ Copy (copies)

Communication:

☐ A letter from the opponent /proprietor was received on ..... . The documents specified as patent documents in this letter are available via the European Patent Register at **www.epo.org/register.** Should you wish to receive these documents in paper format, they will be supplied to you free of charge if specifically requested on receipt of this communication (OJ EPO 2009, 434).

☒ Please find an additional statement by the Opposition Division in the annex.

For the Opposition Division

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☐ **Registered letter**

The following opinion incorporates the P's response to the O's objections, that were not available to the OD at the time of issuance of the summons to oral proceedings.

The following non-binding preliminary opinion serves to augment those objections raised in the summons to Oral Proceedings.

The P further cited the following document:

D13 KAPLAN M: "ECULIZUMAB", CURRENT OPINION IN INVESTIGATIONAL DRUGS, PHARMAPRESS, US, vol. 3, no. 7, 1 July 2002 (2002-07-01), pages 1017-1023, XP009045356, ISSN: 1472-4472

Added subject-matter (Articles 100(c) and 123(2) EPC)

1.1 The O objected to claims 1 and 5 of the patent on grounds that they are not directly and unambiguously disclosed in the application, rather an intermediate generalisation of a specific example (p.32-33) combined with general teachings of the application.

1.2 The O considered the claims to contain the following features:

1. the use of eculizumab
2. the treatment of a patient affected with PNH
3. I.V. administration
4. administration of 600mg each week for 4 weeks
5. administration of 900mg a week later
6. administration of 900mg bi-weekly thereafter
7. a breakthrough in serum hemolytic activity when the patient is treated under a dosing schedule according to points 4 - 6
8. administration of 900mg every 12 days thereafter

1.3 Features 1 and 2 would appear to have a general basis on p.12, li.1-3 and p. 18, li.12-20, respectively. The Examples on p.33 also contain these features.

1.4 Feature 3 would appear to be broader than the basis provided on p.20, li.4-8, which relates merely to 'intravenous infusion by injection'. Feature 4 would also encompass both injection into a vein using a needle and the use of a catheter.

1.5 The P refers to the Examples on p.32, li.12-16 for a basis. This passage refers to 'intravenous infusion of anti-C5 antibody'. The P also states that 'intravenous administration', referred to in granted claims 1 and 5 is not the same as 'intravenous administration', the former referring to a one-time rapid injection of the medication into the blood stream and the latter to a slow drip of medication into the vein. The P argues, however, that the technical effect is the same, the only difference being the time frame in which the dose is administered.

1.6 The OD finds that since p.20, li.4-8 neither specifically states the use of a needle or a catheter, 'intravenous infusion by injection' could be considered to encompass both I.V. infusion via needle or catheter, i.e. intravenous administration; however, 'I.V. administration' does not necessarily imply administration of an infusion. 'Infusion' implies the use of a catheter, but this is not stated in the application.

1.7 The OD is thus of the opinion that **Feature 3 has no basis** in the application as filed, and as confirmed by the P, as it also encompasses administration of pharmaceutical formulations other than infusions.

1.8 Features 4 - 7 relate to a dosage schedule. The general teaching of the application on p.19-20 states that the dose will vary according to the compound used, the manner of administration, the symptoms, the size and the age of the patient. The only basis for the dosage regimen in the claim comes from the specific example on p.32-33, which describes steps 1 - 7 applied to a patient group. Two of the patients in that group exhibited breakthrough in serum hemolytic activity which was controlled by reducing the dosage interval to 12 days (p.35, li.8-17).

1.9 The O is of the opinion that the combination of Features 1 - 8 cannot be derived from either the general part of the description or the examples.

1.10 The P argues that the basis for claims 1 and 5 is in the specific examples.

1.11 The OD agrees with the view of the P, since all essential features of the examples would appear to be in said claims.

1.12 However, the O argues that 'essential features' of the example are missing from the claim. The OD notes that clarity is not a grounds of opposition. There is no indication in the application to suggest that the specific patient sub-group to be treated must be transfusion-dependent and hemolytic (p.32, li.7-9), that an infusion time of 'approximately 30 mins' (p.30, li.12-14), or that the time point of breakthrough occurrence (between weeks 12-64) are essential to the application claimed. The OD finds that it is clear from the examples that any

patient receiving the claimed dosage regimen for PNH and experiencing breakthrough could be treated by shortening the interval between doses, as claimed.

1.13 The OD does not see that there is an intermediate generalisation of the examples in this respect.

1.14 The O further argues that there is no basis for the route of administration used following the initial four week dosing schedule, i.e. Feature 4, since the route thereafter is not specified. The OD finds that it would be obvious that further antibody treatments would be administered by the same route, however, the O is correct in that this is not clearly stated in the application. This point will require further discussion at oral proceedings.

Lack of novelty (Articles 100(a) and 54 EPC)

1.15 The O raised an objection on grounds of lack of novelty regarding claims 1 and 5 with regard to D1.

1.16 D1 published on 05.02.2004 discloses all the features of claims 1-8 (see Methods and section 'Biochemical indicators of hemolysis' on p.552-556 and Fig.3A) but is published after the date of priority of the patent.

1.17 The patent claims priority from US10/771552, filed on 03.02.2004, and appears to disclose identical subject-matter to the application filed on 03.02.2005 on which the patent is based.

1.18 The O considered the priority document not to disclose the subject-matter of the present claims for the same reasons they consider the patent's claims not to have a basis in the application as filed (Article 123(2) EPC).

1.19 The P argues that the claims have a basis in both the application and priority document, and therefore the subject-matter is new.

1.20 Since the OD doubts that a basis for claims 1 and 5 exists in the application regarding feature 3, the same doubts result for the disclosure of the priority document. Therefore, the subject-matter of D1 is considered relevant for the assessment of the novelty and inventive step of the claims.

1.21 The OD is of the provisional opinion that claims 1-8 of the contested patent lack novelty over D1.

1.22 The P should understand that if a basis can be found, or amendments made to remove the objections regarding added subject-matter, the objections on grounds of lack of novelty will also be overcome.

Lack of inventive step

1.23 The O objected to the patent on grounds of a lack of inventive step, taking D2, D3,D4 or D6 as the closest prior art to the subject matter of claims 1 and 5.

1.24 Documents D3,D4 and D6 all disclose the following features:

1. the use of eculizumab
2. the treatment of a patient affected with PNH
3. I.V. administration
4. administration of 600mg each week for 4 weeks
5. administration of 900mg a week later
6. administration of 900mg bi-weekly thereafter

1.25 Document D2 does not disclose the Feature 3 and thus will not be considered further.

1.26 The difference between either D3,D4 or D6 and the present claims is considered to be the dosage.

1.27 The technical effect associated with this difference is the alleviation of symptoms associated with a breakthrough in hemolytic activity.

1.28 The problem to be solved can therefore be considered to be the provision of an improved dosage regimen for patients suffering a breakthrough in hemolytic activity.

1.29 The solution provided in the granted claims would appear obvious, since according to D5, transient breakthroughs in complement activity associated with increased LDL, hemoglobinuria and mild symptoms in an extension trial of patients receiving the dosage regimen described in D3,D4 or D6 were known to be preventable by a slight decrease in the dosing interval.

1.30 The skilled person with a knowledge of any of D3,D4 or D6, and observing a breakthrough in hemolytic activity in patients would therefore find it obvious to slightly reduce the dosing interval until no further hemoglobinuria event is observed (as shown in Fig.5 of the patent).

1.31 The P provides a similar problem-and-solution approach, but starting from D5 and combining with any of D3,D4 or D6. A similar problem is posed by the P, albeit that the problem they suggest is formulated as a 'determining a dosing interval' rather than the provision of a medical use.

1.32 The P states that the skilled person starting from D5 would not decrease the time interval to 12 days as claimed, since said D5 refers only to 'a slight decrease in dosing interval'. The P refers to D13 (cited in examination as D10), p.1020, col.1, §4, wherein a Phase II trial is described. The P notes that the only regimen to reach the primary endpoint was a weekly/monthly regimen, whereas weekly/bi-weekly and biweekly regimens did not. Although, the OD notes that the same passage states that both weekly/monthly and weekly/biweekly groups met the secondary endpoint after 3 months of therapy. The P concludes that the skilled person would conclude from D13 that shortening of dosing intervals may not result in an effective treatment by eculizumab, and that in accordance with T24/81 this prior art should be considered alongside all other published embodiments.

1.33 The OD considers that D13 is not directed to the same technical problem posed. The claim is clearly directed to patients suffering breakthrough who are already receiving a standard regimen. The standard regimen has been described in D2,D3,D4 and D6, and D5 clearly discloses how cases in which breakthrough is experienced can be resolved. D5, as with all these prior art documents was published after D13 and represent the actual stand of knowledge at the time of priority filing. The OD therefore considers that given the nature of D13 in reporting preliminary research results, and the subsequent publications D2-D6, the skilled person would not consider D13 to teach increasing dosing intervals as opposed to decreasing them (as in D5).

1.34 The OD further notes that the establishment of optimal dosage regimens was considered to be routine according to the case law of the Technical Board of Appeal T1979/09, wherein under point 7.3.1 it is stated *'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.'*

1.35 and further under point 7.3.2, *'In the present case the amount of active ingredient remains the same as the amount used in D5 and there is no evidence on file of any unexpected technical effect caused by administering the red vine leave extract once daily. 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.'*

1.36 It is therefore the provisional opinion of the OD that at least the subject-matter of claims 1 and 5 does not involve an inventive step, in light of either D5 alone, or in combination with any one of D3,D4 or D6.

1.37 Dependent claims 2-4 and 6-8 do not appear to contain any additional features which, in combination with any other claim to which they refer, would meet the requirements of the EPC with respect to inventive step.

Insufficiency of disclosure (Articles 100(b) and 83 EPC)

1.38 The O raised the objection that the trade name 'eculizumab' did not sufficiently disclose such an antibody, since, at the time of filing, such an antibody was not completely described.

1.39 The application makes reference to D9 and D10, which do not make reference to deposits or sequences that disclose the humanised anti-C5 antibody to which it allegedly refers.

1.40 The P states that eculizumab was publicly available prior to priority filing: its sequence was available through CAS on the STN database since 14.02.1999 (CAS 219685-560-4).

1.41 The OD is of the opinion, that the antibody and thus the subject-matter of claims 1-8 can be considered to be sufficiently disclosed as the antibody was available to the skilled person at the time of priority filing.

1.42 The O made further objections to the clarity of the term 'breakthrough in serum hemolytic activity'. The OD will not pursue this objection since a lack of clarity is not a ground for opposition.

Conclusion

2 At present the OD is of the preliminary opinion that the patent contains added subject-matter (Articles 100(c) and 123(2) EPC), and does not involve an inventive step (Articles 100(a) and 56 EPC).

The scheduled date for Oral Proceedings is thus maintained.

R. Chapman for the O.D.

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17. März 2014

European Patent Office

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EP 05 71 2607.0 / 1720571
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

München, March 17, 2014
WA/ARO/FLG

This is in response to the Summons to attend oral proceedings issued on January 14, 2014 and the Brief Communication issued on February 18, 2014 by the European Patent Office in the above-referenced opposition proceedings.

We enclose a new main request and a new auxiliary request which replace the claims as granted of the opposed patent EP-B1 1720571. As it will be evident from the following, both new main requests fulfill the requirements of the EPC. In case patentability of the main request cannot be accepted, consideration of the auxiliary request is requested.

1. AMENDMENTS TO THE CLAIMS (Rule 137(4) EPC)

1.1. Main Request

1.1.1. New claim 1 is based on claim 1 as granted. New claim 5 is based on claim 5 as granted. The term "intravenously" has been replaced by the phrase "by intravenous infusion". This amendment is supported at page 32, line 13 of the

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application as filed. Further support can be found at page 20, line 6 of the application as filed.

1.1.2. New claims 2-4 and 6-8 correspond to claims 2-4 and 6-8 as granted.

1.2. Auxiliary Request

1.2.1. New claim 1 is based on claim 1 of the main request. New claim 5 is based on claim 5 as granted. The phrase "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)" has been added. This amendment is supported by page 38, lines 11-17 and Figure 8 of the application as filed.

1.2.2. New claims 2-4 and 6-8 correspond to claims 2-4 and 6-8 of the main request.

2. INADMISSIBLE BROADENING (Articles 100(c) and 123(2) EPC)

2.1. The feature "Intravenous administration" of claim 1

2.1.1. The Opposition Division is of the preliminary view that the feature "intravenous administration" of claim 1 (i.e. feature 3 as characterized by the Opposition Division) extends beyond the contents of the application as filed, because it may also encompass administration by means other than infusions.

2.1.2. New claims 1 and 5 of the main request have been amended as described above. In more detail, the term "intravenously" has been replaced by the phrase "by intravenous infusion". The same amendment is present in claims 1 and 5 of the auxiliary request. The phrase "by intravenous infusion" has a clear and unambiguous basis at page 32, line 13 and page 20, line 6 of the application as filed.

We therefore submit that new claims 1 and 5 of the main request as well as auxiliary request comply with Article 123(2) EPC.

2.2. Mode of administration of the dosage schedule

2.2.1. The Opponent has argued that there is no basis in the application as filed that the dosages following the administration of 600mg eculizumab each week for 4 weeks are administered by the same route. The Opposition Division states that it is obvious that also the remainder of the administration steps are administered by the same route. However, since the Opponent's observation that no

administration route is expressly mentioned in connection with the further administration steps is correct, the Opposition Division wishes to further discuss this point at the oral proceedings.

2.2.2. In preparation of the discussion of this issue at the oral proceedings, we wish to provide the following comments in writing. We fully concur with the Opposition Division's statement that it is obvious that also the further administration steps are administered by the same route. The disputed passage at page 32 of the application reads:

> "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." (emphasis added)

Nothing in this passage conveys the impression that the mode of administration via intravenous infusion is changed after the initial dosing step of 600mg eculizumab per week for four weeks. The allegation of the Opponent that only the first dosage of weekly 600mg for four weeks has been administered by intravenous infusion makes no sense. In fact, if the mode of administration were to be changed, a corresponding statement would be expected in the corresponding passages. This is, however, not the case. The only mode referred to in the examples is intravenous infusion.

In fact, the attitude of the skilled person is to read the contents of a patent specification with a mind willing to understand the technical sense, not a mind desirous of misunderstanding (T 190/99; confirmed *inter alia* in T 1204/06 or T 1771/06). Although this case law on how a skilled person reads the contents of an application has been established in connection with Article 84 EPC, the Boards have established essentially the same standard under Article 123(2) EPC. In this regard reference is made to T 609/95, reasons for the decision 2.3:

> "The board takes the position that where a drafting defect or inconsistency in an application would be evident to a reader skilled in the art, the person to whom the application is addressed, it is reasonable to suppose that he would, in the light of the content of the application, attempt to formulate a notional amendment which would enable him to make sense of what he reads, and to the extent that the amendment might be said to leap to the mind of the reader, although perhaps only after close study of the document, it can be regarded as implicit in the application and would not contravene Article 123(2) EPC, if effected in practice." (emphasis added)

Such standard applies all the more to the present case, given that there is no drafting defect or inconsistency. The principle that the skilled person tries to sensibly interpret the contents of the application also with regard to claim amendments has been confirmed in T 887/97, reasons for the decision 2.2. Hence, also when it comes to the assessment of the contents of the application under Article 123(2) EPC, the skilled person aims at making sense of what he reads in an application.

It is furthermore emphasized that the application is addressed to the skilled person and hence the contents of the application as filed have to be assessed from the perspective of the skilled person. The skilled person unequivocally reads the contents of the above-quoted passage of the application as filed in the light of the available knowledge at the priority date of the opposed patent.

In view of the above-recited case law, the skilled person cannot understand the above-quoted passage from the application other than that eculizumab is administered by intravenous infusion throughout the entire study, especially in view of the fact that no different mode of administration is mentioned in the Examples.

2.3. In summary we submit that the new claims of the main and auxiliary request have a clear and unambiguous basis in the application as originally filed.

3. NOVELTY (Articles 100(a) and 54 EPC)

3.1. The Opposition Division is of the preliminary view that feature 3 of granted claim 1 and arguably also the mode of administration of the dosage schedule have no proper basis in the priority application. For this reason, the Opposition Division is of the view that the claims are no entitled to priority and consequently D1 becomes prior art.

The Opposition Division states that D1 anticipates claims 1 to 8.

3.2. The feature "Intravenous administration" of claim 1

The new claims have been amended as described above. In more detail, the term "intravenously" has been replaced by the phrase "by intravenous infusion". The skilled person can clearly and unambiguously derive the mode of "intravenous infusion" from page 30, line 14 and page 19, line 17 of the priority-establishing application.

3.3. Mode of administration of the dosage schedule

The passage at page 32, lines 12-15 of the application as filed corresponds to the contents of the priority-establishing application at page 30, lines 13-19.

In G 1/03, G 2/03 and G 2/10 the Enlarged Board held that the disclosure as the basis for the right to priority under Article 87(1) EPC, and as the basis for amendments in an application under Article 123(2) EPC have to be interpreted in the same way. Hence, for the same reasons set forth in section 2.2.2, *supra* it is immediately evident to the skilled person – in the sense that nothing else can be meant - that eculizumab is administered by intravenous infusion in the entire study as described in the priority-establishing application.

3.4. With regard to the auxiliary request it is to be added that the phrase "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)" can be clearly and unambiguously derived from page 34, lines 13-19 and Figure 8 of the priority document.

3.5. We therefore submit that the skilled person can immediately derive the subject-matter of the new main and auxiliary request from the priority-establishing application. The time rank of the new claims is consequently February 3, 2004; i.e. before D1 became available to the public. D1 is not prior art for the new set of claims.

4. INVENTIVE STEP (Articles 100(a) and 56 EPC)

4.1. The Opposition Division is of the preliminary view that at least claims 1 and 5 lack an inventive step in view of D5 optionally combined with D3, D4 or D6.

The Opposition Division defines the technical problem underlying the claimed subject-matter as the provision of an improved dosage regimen of eculizumab.

The solution of this technical problem - i.e. the administration of 900mg eculizumab every 12 days instead of every 14 days – is allegedly obvious, because D5 teaches that transient complement breakthrough can be prevented by a slight decrease in dosing interval.

The Opposition Division thus takes the position that a skilled person starting from D5 would have reduced the dosing interval until no complement

breakthrough is observed. By referring to decision T 1979/09, reasons for the decision sections 7.3.1 and 7.3.2, the Opposition Division emphasizes that finding the optimal dosage interval cannot justify the presence of an inventive step.

4.2. Our arguments indicating the presence of an inventive step submitted on December 31, 2013 are fully maintained and still applicable to the enclosed new main and auxiliary requests.

In order to underline our position that the skilled person starting from D5, optionally combined with D3, D4 or D6, would not have arrived at the exact dose of 900 mg eculizumab every 12 days without the need of inventive skills, the following further arguments are provided.

4.2.1. D5 teaches:

> "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." (emphasis added)

For a proper assessment of inventive step, we would like to elaborate on the following issues: What would the skilled person have observed upon slightly decreasing the interval, in particular from 14 days to 13 days? Would he/she have been encouraged to further decrease the interval?

4.2.2. The attention of the Opposition Division is respectfully drawn to Figure 4 of the application as filed. Figure 4 shows hemolysis tests on urine samples of the studied PNH patients. In more detail, in the urine samples the levels of lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) were measured. LDH and AST are often used in the art as a marker of hemolysis, as LDH and AST are abundant in red blood cells. Upon red blood cell lysis LDH and AST are released from the cell to the extracellular compartments of the body. Consequently, LDH and AST can be detected in a urine sample if red blood cell lysis occurs. In the absence of red blood cell lysis LDH and AST cannot be detected in a urine sample. It follows that the more LDH or AST is found in the urine sample, the more hemolysis occurs.

The pharmacokinetics (PK) and pharmacodynamics (PD) of eculizumab were also measured. PK is a measure of the concentration of eculizumab in the serum of a patient over time and PD is a measure of the level of complement activity in the patient's serum. The method used for the determination of PD levels is described at page 37, line 10 to page 38, line 10 of the application as filed. High PD levels indicate high levels of complement activity in the patient's serum, which in turn correlates with high levels of hemolysis.

The urine samples tested in Figure 4 have been obtained from PNH patients who received a dose of 900mg eculizumab every 14 days. The day on which 900mg eculizumab was administered is characterized as day "0" in Figure 4. Urine samples were obtained at day "0" and each day between day "9" and day "13" of the thirteen days between the mentioned fortnightly administrations of 900mg eculizumab.

At day 0, i.e. before the administration of eculizumab, LDH, AST and PD levels were consistently high, thereby indicating the occurrence of hemolysis in the tested PNH patients. LDH, AST and PD levels again were measured on days 9, 12 and 13 after administration of eculizumab. The measured LDH, AST, and PD consistently show that no hemolysis was found until day 12. However, on day 13, LDH, AST and PD levels surprisingly increased dramatically, thereby indicating an abrupt relapse of hemolytic activity in the PNH patients. In particular, the PD levels as shown in Figure 4 demonstrate the dramatic increase of serum hemolytic activity from day 12 to day 13:

	Day 12	Day 13	Day 14 = day 0[1]
PD	2	56	56

(excerpt of contents of Figure 4 of the application as filed)

Hence, the data in Figure 4 of the application as filed evidence that the administration of 900mg eculizumab every 12 days – as claimed – is the optimum dosage regimen for the administration of eculizumab. Assuming that 900mg eculizumab would be administered every 13 days, complement

[1] We note that day 14 is designated day 0 in Figure 14, given that the next dose is administered.

breakthrough may be still observed in PNH patients. If 900mg eculizumab were administered every 11 days, this would be an unnecessarily short interval which is of no further benefit for the PNH patients. Hence, Figure 4 evidences that the optimal time window for the administration of 900mg eculizumab is very narrow. In fact, the claimed 12 days dosing interval is ideal, while administration every 11 or 13 days cannot be viewed as ideal dosing intervals.

Importantly, the data in Figure 4 evidence that a decrease from 14 to 13 days is not encouraging. The breakthrough patients would remain in the realm of high LDH and unfavorable PD.

4.3. Since the Opposition Division relies on T 1979/09, reasons for the decision 7.3.1 and 7.3.2, for concluding that finding an optimal dosage interval may justify the presence of an inventive step, we will demonstrate in the following that the case decided in T 1979/09 has to be held distinct from the present situation.

4.3.1. *Dosage range vs. exact dosage*

It can be taken from reason for the decision 7.1 that the claims at stake in T 1979/09 did not refer to an exact dosage but to a broad dosage range of 300mg to 800mg of the extract in 1 to 3 capsules of tablets taken once daily. Thus, irrespective of whether 300mg of the extract or 800mg of the extract are administered as daily treatment, success is ensured.

On the other hand, claim 1 of the enclosed new main and auxiliary request refers to a distinct dose of 900mg eculizumab at a distinct time interval of every 12 days. As it has been detailed in section 4.2, *supra*, the precisely and narrowly defined dosing scheme according to step (iv) of claim 1 of the enclosed new main and auxiliary request – being the administration of 900mg eculizumab every 12 days – ensures successful treatment of PNH breakthrough patients. As such the facts of the case underlying in T 1979/09 on the one hand and the present case on the other hand are fundamentally different. It was by no means obvious from D5 optionally combined with D3, D4 or D6 for the skilled person to arrive at the mentioned narrow and specific dosing schedule.

4.3.2. *Closest prior art in T 1979/09 and in the present case*

We consider it also important to note that the prior art in T 1979/09 was considerably closer to the claimed subject-matter as in the present case. As it is evident from reason for the decision 7.3, the prior art taught to administer a total daily dose of 360mg to 540mg (which is within the claimed range of 300mg to 800mg) of the extract in 2 to 3 capsules (which is within the claimed range of 1 to 3 capsules) twice a day. It follows that in T 1979/09 the claimed total daily dose of the extract comprised that of the prior art. The only difference is that the dose is administered once a day instead of twice.

In the present case D5 may be seen as the closest prior art document. D5 merely mentions a "slight decrease in dosing interval" but fails to suggest any specific interval. Hence, the gap between D5 and the claimed subject-matter is indeed wider than the gap between the closest prior art and the claimed subject-matter in the case at issue in T 1979/09. The skilled person was not able to close the gap in the present situation without the need of inventive skills. D5 conveys the impression that a slight decrease of the dosing interval of 14 days would prevent complement breakthrough. However, as set forth in section 4.2 of this petition a decrease from 14 days to 13 days – which certainly is much closer to a "slight decrease" than from 14 to 12 days - fails to prevent further complement breakthroughs. As detailed herein above, administering 900mg eculizumab at an interval of every 12 days ensures optimal treatment.

This was by no means foreseeable for the skilled person on the basis of D5 optionally combined with D3, D4 or D6. None of these documents guides the skilled person to the interval of 12 days.

4.3.3. *Presence of an unexpected technical effect*

Section 7.3.2 of the Reasons for the decision of T 1979/09 reads:

> "In the present case the amount of active ingredient remains the same as the amount used in D5 [i.e. the closest prior art document in T 1979/09] and there is no evidence on file of any unexpected technical effect caused

> by administering the red vine leave extract once daily." (information in brackets and emphasis added)

Hence, in T 1979/09 no technical advantage associated with the distinguishing feature from the closest prior art could be demonstrated.

Moreover, section 7.3.3 of the Reasons for the decision of T 1979/09 teaches:

> "It has never been shown that taking a dose of 300-800 mg once daily has the same effect as taking the same dose twice daily."

As such, it even has not been shown in the case at issue in T 1979/09 that administering the extract once a day is as beneficial as twice a day.

On the other hand, in the present application it has been demonstrated that unexpected technical effects are associated with administering 900mg eculizumab every 12 days. The interval of 12 days is the optimum while shorter as well as longer intervals are associated with the discussed drawbacks. It is our firm belief that the advantageous technical effects being associated with an interval of 12 days fully justify the acknowledgment of an inventive step.

4.4. For the sake of completeness we note with regard to the auxiliary request that the feature of "reducing cell lysis to below 20% of normal serum complement activity" is a feature indicating the effective inhibition of haemolytic activity; see page 38, lines 11-13 and Figure 8. The effective inhibition of haemolytic activity is a key advantage being associated with the subject-matter claimed in the main and auxiliary requests. This advantage is expressly incorporated in the main claim of the auxiliary request.

4.5. In summary, we submit that the enclosed main and auxiliary requests meet the requirements of Article 56 EPC.

5. SUMMARY AND REQUESTS

In view of the above, we submit that the set of claims constituting the main request meets the requirements of the EPC. In case the patentability of the

main request cannot be acknowledged consideration of the auxiliary request is requested.

We further request that in view of our submissions, oral proceedings are cancelled and the proceedings be continued in writing. In case the Opposition Division comes to a different conclusion, we request that we be allowed to submit one or more additional claim request(s) on the occasion of the oral proceedings.

Dr. Joachim Wachenfeld
European Patent Attorney

Enclosure:
New main and auxiliary requests

VOSSIUS & PARTNER

Patentanwälte Rechtsanwälte

Via Telefax – 13 pages
Fax- No. 089-2399-4465 and confirmation copy

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European Patent Office

To the immediate attention of
Dr. Chapman

80298 M U N I C H

EP 05 71 2607.0 / 1720571
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

München, March 18, 2014
WA/ARO/FLG

This is further to the response to the Summons to attend oral proceedings submitted in the above-referenced opposition proceedings on March 17, 2014.

Enclosed are copies of the main request and auxiliary request discussed in the above-mentioned response.

Dr. Roger Absehe
European Patent Attorney

Enclosure:
Main and auxiliary requests (track mode and clean copy)

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EP 05 71 2607.0
Alexion Pharmaceuticals, Inc.
Our Ref.: M2367 EP

MAIN REQUEST

1. Eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered by intravenously 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).

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%;

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(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.

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 by intravenously 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).

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%;

(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.

EP 05 71 2607.0
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   (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).

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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 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).

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:

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(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.

EP 05 71 2607.0
Alexion Pharmaceuticals, Inc.
Our Ref.: M2367 OPP(EP)

AUXILIARY REQUEST

1. Eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), 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), and
   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).

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;

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(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

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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 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), and 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).

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,

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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:

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(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), and

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).

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(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.

VOSSIUS & PARTNER

Patentanwälte Rechtsanwälte

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18. März 2014

To the immediate attention of
Dr. Chapman

80298 M U N I C H

EP 05 71 2607.0 / 1720571
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

München, March 18, 2014
WA/ARO/FLG

Confirmation Copy

This is further to the response to the Summons to attend oral proceedings submitted in the above-referenced opposition proceedings on March 17, 2014.

Enclosed are copies of the main request and auxiliary request discussed in the above-mentioned response.

Dr. Roger Absehe
European Patent Attorney

Enclosure:
Main and auxiliary requests (track mode and clean copy)

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EP 05 71 2607.0
Alexion Pharmaceuticals, Inc.
Our Ref.: M2367 OPP(EP)

AUXILIARY REQUEST

1. Eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), 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), and
   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).

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

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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.

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 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), and 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).

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,

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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%;

(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.

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EP 05 71 2607.0
Alexion Pharmaceuticals, Inc.
Our Ref.: M2367 EP

MAIN REQUEST

1. Eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered by intravenously 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).

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%;

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(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.

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 by intravenously 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).

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.

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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%;

(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 80×10^9 per liter;

(s) the patient's reticulocyte count is greater than 120×10^9 per liter;

(t) the patient's reticulocyte count is greater than 150×10^9 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.

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EP 05 71 2607.0
Alexion Pharmaceuticals, Inc.
Our Ref.: M2367 EP

MAIN REQUEST

1. Eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), 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).

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%;

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(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.

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 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).

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.

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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%;

(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;

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(s) the patient's reticulocyte count is greater than 120×10^9 per liter;

(t) the patient's reticulocyte count is greater than 150×10^9 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.

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EP 05 71 2607.0
Alexion Pharmaceuticals, Inc.
Our Ref.: M2367 OPP(EP)

AUXILIARY REQUEST

1. Eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), 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), and
   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).

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

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Page 3524

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.

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 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), and

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).

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,

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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%;

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(o) the patient's platelet count is greater than 40,000 per microliter;

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(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.

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European patent application No. 05712607.0

II. To the Formalities Officer

1. ☒ The date / time fixed for oral proceedings is maintained.

   ☐ (if necessary) The reasons are indicated on enclosed EPO Form 2906/2906O, which is to be dispatched to the party with EPO Form 2008A/2310A.

2. ☐ At the instigation of the division the oral proceedings cannot take place on the arranged date for the reasons indicated on enclosed EPO Form 2906, which is to be dispatched to the party with EPO Form 2008A/2310A.

   Date _____ Director (see Internal Instructions IE-III, 1.2) _____

3. ☐ The summons to attend oral proceedings on ..... should be cancelled.

   3.1 ☐ The proceedings will be continued in writing.

   3.2 ☐ A new date will be set later.

   3.3 ☐ A new date/time is set as follows:

   date ..... at ..... hrs.
   Parties' written submissions and amendments in preparation for the oral proceedings, if any, should be made not later than

   ..... month(s) before the date of the oral proceedings.

4. ☐ The request for the oral proceedings to be held as a videoconference

   4.1 ☐ is allowed.

   4.2 ☐ is rejected for the reasons indicated on enclosed EPO Form 2906, which is to be dispatched to the party with EPO Form 2008A.

5. ☐ The application is deemed to be withdrawn. The oral proceedings are cancelled.

25.03.14
Date

Chairman _____ Second member _____ Primary Examiner _____ Legal member

III. Action taken by the formalities officer

☒ EPO Form 2008A/2310A has been dispatched, if applicable (see point II above) together with EPO Form 2906/2906O.

☐ A new summons has been dispatched.

☐ .....

25.04.14
Date

C. Cavestri
Formalities Officer

EPO Form 2088 04.10

CC20871

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Page 3527

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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

Formalities officer: Mr Consalvo Cavestri

Please specify the name of the formalities officer when contacting Customer Services by contact form or phone.

Vossius & Partner
Siebertstrasse 4
81675 München
ALLEMAGNE

FAXED

Date
30-04-2014

Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571
Applicant/Proprietor	
ALEXION PHARMACEUTICALS, INC.	

BRIEF COMMUNICATION

Oral Proceedings on 15.05.14

Subject: ☒ Your letter of 18.03.14

Communication:
- ☐ The summons to attend oral proceedings on 15.05.14 has been cancelled.
- ☐ The procedure will be continued in writing.
- ☒ The date fixed for oral proceedings is maintained.
- ☐ A new date will be set later.
- ☐

Please take note.

For the Opposition Division

Europäisches Patentamt • European Patent Office • Office européen des brevets

Registered letter
EPO Form 2008A 12.07 (30/04/14)

CC20871

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European Patent Office
Office européen des brevets

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Fax: +49 89 2399 4465

Application No.: 05 712 607.0

Patent No.: EP-B-1 720 571

Minutes of the oral proceedings before the OPPOSITION DIVISION

The proceedings were public.

Proceedings opened on 15.05.2014 at 09:15 hours

Present as members of the opposition division:

Chairman:	Cilensek, Zoran
1st member:	Chapman, Rob
2nd member:	Weikl, Martina
Minute writer:	Weikl, Martina

Present as or for the party or parties:

- For the Proprietor(s): ALEXION PHARMACEUTICALS, INC.

  Wachenfeld, Joachim (European Patent Attorney at Vossius & Partner)
  Abseher, Roger (European Patent Attorney at Vossius & Partner)
  Grasser, Florian (European Patent Attorney at Vossius & Partner)
  accompanied by
  Spalding, Todd N. (Vice President and Head of Intellectual Property at Alexion Pharmaceuticals, Inc.)
  Banks, Damon (Patent Agent at Alexion Pharmaceuticals, Inc
- For the Opponent 1: Pfizer Inc.

The identity of the person/s (as well as, if applicable, that of the witness or witnesses) and, where necessary, the authorisation to represent/authority to act were checked.

Essentials of the discussion and possible relevant statements of the parties:

1 The Chairman (CH) formally opened the Oral Proceedings (OP) for European patent EP1720571 with the title 'Method of Treating Hemolytic Disease' and informed the patentee about the presence of a paralegal assisting the proceedings in writing the minutes but not being part of the Opposition Division (OD) (see OJ 2014, A32).

2 Since the Opponent (O) had withdrawn the opposition with the letter dated 07. October 2013 and the proceedings were continued upon the OD's own volition, only the patentee (P) was represented at the OP.

3 The CH summarized that the opposition had been filed by the opponent Pfizer, Inc. on 12. March 2013 and that grounds of opposition under Articles 100(a), (b) and (c) had been mentioned in the notice of opposition. The CH explained further that the continuation of the opposition proceedings after withdrawal of opposition and upon the volition of the OD was based on the arguments of the opponent.

4 The CH confirmed with the representative (RP) of the P that there was a Main Request (MR) and an Auxiliary Request (AR1) on file, both filed with the letter dated 18. March 2014. The P furthermore requested to submit new documents into the OP before pleading their case. According to the P, these documents were destined to support the P's argumentation with respect to inventive step of the claimed subject-matter. The CH stated the OD would decide upon admission of said documents later in the proceedings, i.e. when the discussion would be turned to Article 56 EPC.

5 The CH then opened the discussion on Article 123(2) EPC and invited the P to indicate the basis under Article 123(2) EPC for the amendments which were present in the current MR (as opposed to the granted claims).

5.1 The P explained that the only amendment had been performed to the claimed route of administration in independent claims 1 and 5 of the MR. Basis for the new definition 'by intravenous infusion' could be found on page 32, line 13 of the description as filed, which related to a dosage regimen in the context of the claimed medical use. The P explained further that the new definition was narrower than the granted definition, hence no objections under Article 123(3) could arise. The P further stated that the amendments were a response to the objection of the O (Rule 80 EPC) and that there was no clarity issue (Article 84 EPC).

5.2 The P enquired whether further explanation concerning the valid combination of the newly introduced feature with the remaining features of the claim was necessary (compare point 1.14 of the summons dated 18. February 2014). The OD confirmed that it would be obvious that further antibody treatments would be administered by the same route and that no further discussion had to be opened on that subject.

5.3 Following the invitation of the CH, the P then submitted documents D14 (Annex 1) and D15 (Annex 2). D14 was a declaration of the inventor L. Bell. It was submitted to support the fact that dosing of the antibody had a specific effect on the health of the patient, and in particular that step (ii) of claims 1 and 5 was important for managing breakthrough patients (see point 4 of D14). D15 was a leaflet distributed with the marketed drug Solaris® (=eculizimab) which under points 2.1 and 5.6 summarized the same dosage regimen as claimed in the independent claims of the current MR. Hence, these documents should be admitted into the proceedings in order to further illustrate the relevance of the currently claimed dosage regimen.

6 After a break, the CH informed the P that the OD was of the opinion that the MR met the requirements of Articles 123(2), 123(3) and 84 EPC as well as of Rule 80 EPC. In addition, and following the line of reasoning as outlined in point 1.22 of the summons dated 18. February 2014, the CH informed the P that the OD was of the opinion that the MR did fulfil the requirements of Article 54 EPC.

7 The CH furthermore stated that D14 and D15 were allowed into the proceedings and opened the discussion on Article 56 EPC. It was agreed that D5 was the closest prior art (CPA).

7.1 The P opened his argumentation by summarizing the contribution to the art of the contested patent. The claimed subject-matter addressed the problem/risk of complement breakthrough in a group of patients undergoing eculizimab therapy for the treatment of Paroxysmal Nocturnal Hemoglobinuria (PNH). The claimed subject-matter was limited to a second-medical use including the dosage regimen required to efficiently overcome these problems. The P furthermore asked the OD to bear in mind that the skilled person in the field was a cautious, conservative person not willing to enter unpredictable areas.

7.2 With that in mind, the P identified two differences between the independent claims of the MR and D5 (which also dealt with the above described problem of breakthrough), the first one being step iv) having a dosing schedule feature of 900 mg every 12 days for patients exhibiting breakthrough whereas D5 did not recite an actual time interval but only disclosed slight decrease in the

dosing interval. The P argued that the interval reduction from 14 to 12 days was a substantial decrease of 14% and could not be referred to as a "slight" decrease.

The second difference was identified by the P as being the dosage regimen prior to the maintenance/extension phase, i.e. the definition of steps corresponding to steps (i) and (ii) of the current independent claims. The P emphasized that D5 itself was silent with respect to the dosage regimen prior to the extension study. According to the P, the subject-matter of steps (i) and (ii) (referred to as 'induction phase by the P) could also not be derived from a combination with other prior art documents which *had* disclosed details of the 'induction phase', such as D3, D4 and D6.

More particularly the P referred to D3 (abstract 257, line 5) and D4 (Abstract 154, line 10) and stated that both documents did not include step ii) but rather related to '900mg every other week'. Upon enquiry by the CH, the P declared that this had to be interpreted such that the first 900mg dose of antibody was given 2 weeks after the last administration of a 600mg dose, and not after one week as required by step (ii) of the claimed schedule.

7.3 The P conceded that D6 described an induction phase identical to steps (i) to (iii) of the claimed dosage regimen. He argued however that this disclosure was outweighed by the two contrasting disclosures of D3 and D4. Furthermore, the skilled person being cautious and conservative (see above) would be likely to choose the combination of the teaching of D5 with the induction phases as disclosed in D3 and D4. Hence, the skilled person would not end up with the dosage regimen as currently claimed. However, as was emphasized in D14, the presence of step (ii) was crucial for the subsequent prevention of breakthrough in the eculizimab treatment plan.

7.4 The P furthermore referred to D13 (page 1021, left column, 1st paragraph) and argued that D13 was teaching away from the presently claimed schedule since it suggested that the inhibition of C5 had to be cautiously balanced.

7.5 The P also noted that in the present context the decision T1979/09 cited by the OD (cited in the summons, point 2.23 of summons of 14. January 2014) was not applicable and referred to the corresponding arguments which had already been set out in his reply to the summons. The P furthermore referred to decision T1020/03 (point 72) in support of his arguments in favor of inventive step.

7.6 Before announcing a break, the CH asked the P if he had any further comments on the arguments which had been brought forth by the opponent. The P replied that the remaining documents cited by the opponent were regarded as less relevant so that he had no further comments.

8 After the break, the CH informed the P that the OD was of the opinion that the MR did not fulfil the requirements of Article 56 EPC. The claimed subject-matter was considered obvious over a combination of documents D5 (as CPA) and D6.

9 The CH opened the discussion on the AR1 and invited the P to indicate the basis under Article 123(2) EPC for the amendments.

9.1 The P referred to page 38 (lines 11-17) and Figure 8 as a basis for the amendments of the AR1.

10 After a break, the CH shared the opinion of the OD that the AR1 met the requirements of Articles 123(2), 123(3), 54 and 84 EPC as well as of Rule 80 EPC and opened the discussion on inventive step.

10.1 The P stated that the independent claims of the auxiliary request introduced both the parameter according to which complement breakthrough should be measured (namely cell lysis) and the threshold level which marks the efficient inhibition of breakthrough (namely less than 20%). The P argued that neither D5, let alone D6 (which did not even relate to complement breakthrough) did establish these parameters which allow the skilled person to efficiently evaluate the problem of breakthrough and the effect of counteracting measures.

10.2 Upon enquiry by the CH, the P explained that in the prior art breakthrough had rather been measured by means of urine color etc.

10.3 Before the CH announced a break, the first examiner (EX1) also noted that there was a contradiction between page 38, lines 11-17 which stated that two patients exhibited breakthrough and Figure 8 where 4 patients where observed to cross the 20% threshold transiently.

11 After the break, the P named further means of measuring complement breakthrough which had been used in the prior art, among them e.g. LDH and AST levels as well as EORTC QLC30 (quality of life) as disclosed in D6. The P emphasized that the method of the present application was superior to these measures since it provided a *direct measure* of complement breakthrough. With respect to the contradiction of page 38 and Figure 8, the P emphasized that the important conclusion of Figure 8 was that the long-term values for all patients are below 20%.

12 After a further break, the CH informed the P that the OD was of the opinion that the AR1 did not fulfill the requirements of Article 100(a) EPC in conjunction with Article 56 EPC

13 The P stated that he had no further requests or comments.

14 The C announced the decision of the OD to revoke the patent under Article 101(3)(b) for failure of the MR and the AR1 to comply with Article 100(a) EPC in conjunction with Article 56 EPC. The CH informed the P that the decision of the OD was open to appeal.

15 The CH closed the OP.

After deliberation of the opposition division,

- the chairman announced the following **decision**:

"The European patent is revoked."

Regarding the reasons for the decision, the chairman referred to:

Article 101(3)(b)EPC: Taking into consideration the amendments made by the proprietor of the patent during the opposition proceedings, the patent and the invention to which it relates do **not** meet the requirements of the Convention

The main request and auxiliary request do not meet the criteria of Article 56 EPC

The chairman **closed the oral proceedings** on 15.05.2014 at 14:15 hours.

Cilensek, Zoran
Chairman

Weikl, Martina
Minute Writer

Annex(es):

Annex 1

EP 05 71 2607.0 / 1720571
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

D14

Declaration of Leonard Bell, M.D.

1. I, Leonard Bell, M.D., a citizen of the United States, do reside at 59 Tumblebrook Road, Woodbridge, CT 06525. I am of the age of majority, and am aware that this Declaration is submitted in support of the European Patent EP 1 720 571 ("the Patent"), of which I am a co-inventor.

2. I am a founder and the present Chief Executive Officer of Alexion Pharmaceuticals, Inc. Prior to founding Alexion, I was an attending physician at Yale-New Haven Hospital and Assistant Professor of Medicine and Pathology at the Yale University School of Medicine, where I also received my M.D. degree. I remain on the faculty of the Yale University School of Medicine as an Adjunct Assistant Professor of Medicine and Pathology. I received my A.B. degree from Brown University.

3. Claim 1 of European Patent No. 1 720 571 is drawn to the use of the antagonist anti-C5 antibody eculizumab for treating a specific subgroup of patients afflicted with paroxysmal nocturnal hemoglobinuria (PNH). These patients are to be treated with eculizumab by intravenous infusion and 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, in the event of a breakthrough of complement activity, thereafter (iv) 900 mg every 12 days. Step (i) of this dosing schedule is a "loading" (or "induction") dose for eculizumab, whereas steps (ii), (iii), and (iv) are "maintenance" doses. Importantly, steps (i) and (ii) establish an initial, basal therapeutic level of complement inhibition that can be further maintained by step (iii), or step (iv) in the case of the subgroup of breakthrough patients recited in the claims. This schedule is described by, and exemplified in, the working examples of the Patent.

4. The dose recited in step (ii) – 900 mg of eculizumab administered to the PNH patient on week five – is especially important for the instantly-specified subgroup of breakthrough patients. This dose is important because, without it, the loading dose specified by step (i) does not provide an adequate baseline of

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complement inhibition that can be maintained by the maintenance dose of step (iii) or (iv).

5. The dose specified by step (ii) was included in the PNH dosing schedule based on the results of clinical studies investigating, among other things, the pharmacokinetic and pharmacodynamic properties of eculizumab in human participants. In a subset of those studies, the dosing schedule administered to the participants consisted of a loading dose – 600 mg each week for five weeks – followed by a maintenance dose of 600 mg every two weeks. On that schedule, complement inhibition was inconsistently blocked in the participants. This observation indicated that the final 600 mg loading dose of eculizumab was insufficient to maintain a therapeutic level of complement inhibition for the two weeks prior to the start of the maintenance dosing schedule. As noted above, addition of the step (ii) dose of 900 mg, an increased dose relative to the insufficient final loading dose of 600 mg above, provides a trough level of eculizumab sufficient to achieve complete terminal complement inhibition that can be maintained by step (iii), or for patients who experience breakthrough, step (iv). Maintaining complement suppression in PNH patients is particularly important because, as the specification of the Patent states at paragraphs [0002] to [0005], failure to do so results in, among other pathologic sequelae, complement-mediated hemolysis and thrombosis, the latter of which can be fatal, and is the leading cause of death in patients with PNH.

6. The U.S. product insert for Soliris®, the tradename under which eculizumab is sold, exemplifies the importance of the second loading dose specified by step (ii) of claim 1. Namely, Point 2.1 of the product insert provides the recommended dosage regimen of eculizumab for PNH patients as: "600 mg weekly for the first 4 weeks, followed by 900 mg for the fifth dose 1 week later, then 900 mg every 2 weeks thereafter."

7. All statements made herein made on the basis of my own knowledge are true; all statements made herein on information and belief are believed to be true.

5/14/2014.
Date

Leonard Bell, M.D.

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Annex 2

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use Soliris safely and effectively. See full prescribing information for Soliris.

Soliris® (eculizumab)

Concentrated solution for intravenous infusion
Initial U.S. Approval: 2007

WARNING: SERIOUS MENINGOCOCCAL INFECTIONS

See full prescribing information for complete boxed warning

Life-threatening and fatal meningococcal infections have occurred in patients treated with Soliris. Meningococcal infection may become rapidly life-threatening or fatal if not recognized and treated early (5.1).

- **Comply with the most current Advisory Committee on Immunization Practices (ACIP) recommendations for meningococcal vaccination in patients with complement deficiencies.**
- **Immunize patients with a meningococcal vaccine at least 2 weeks prior to administering the first dose of Soliris, unless the risks of delaying Soliris therapy outweigh the risks of developing a meningococcal infection. (See *Serious Meningococcal Infections* (5.1) for additional guidance on the management of the risk of meningococcal infection.)**
- **Monitor patients for early signs of meningococcal infections, and evaluate immediately if infection is suspected.**

Soliris is available only through a restricted program under a Risk Evaluation and Mitigation Strategy (REMS). Under the Soliris REMS prescribers must enroll in the program (5.2). Enrollment in the Soliris REMS program and additional information are available by telephone: 1-888-SOLIRIS (1-888-765-4747).

RECENT MAJOR CHANGES

Box Warning	9/2011
Indications and Usage (1.2)	9/2011
Contraindications (4)	9/2011
Dosage and Administration (2.2)	9/2011
Warnings and Precautions (5.3, 5.4 and 5.6)	9/2011
Adverse Reactions (6.1)	9/2011
Use in Specific Populations (8.4)	9/2011
Clinical Studies (14.2)	9/2011
Patient Counseling Information (17)	9/2011

INDICATIONS AND USAGE

Soliris is a complement inhibitor indicated for:

- The treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH) to reduce hemolysis (1.1).
- The treatment of patients with atypical hemolytic uremic syndrome (aHUS) to inhibit complement-mediated thrombotic microangiopathy (1.2).

The effectiveness of Soliris in aHUS is based on the effects on thrombotic microangiopathy (TMA) and renal function. Prospective clinical trials in additional patients are ongoing to confirm the benefit of Soliris in patients with aHUS.

Limitation of Use

Soliris is not indicated for the treatment of patients with Shiga toxin *E. coli* related hemolytic uremic syndrome (STEC-HUS).

DOSAGE AND ADMINISTRATION

Only administer as an intravenous infusion

PNH Dosage Regimen: (2.1)
aHUS Dosage Regimen: (2.2)

DOSAGE FORMS AND STRENGTHS

300 mg single-use vials each containing 30 mL of 10 mg/mL sterile, preservative-free solution (3).

CONTRAINDICATIONS

Soliris is contraindicated in:

- Patients with unresolved serious *Neisseria meningitidis* infection (4).
- Patients who are not currently vaccinated against *Neisseria meningitidis*, unless the risks of delaying Soliris treatment outweigh the risks of developing a meningococcal infection (5.1).

WARNINGS AND PRECAUTIONS

- Discontinue Soliris in patients who are being treated for serious meningococcal infections.
- Use caution when administering Soliris to patients with any other systemic infection (5.2).

ADVERSE REACTIONS

The most frequently reported adverse reactions in the PNH randomized trial (≥10% overall and greater than placebo) are: headache, nasopharyngitis, back pain, and nausea (6.1).

The most frequently reported adverse reactions in aHUS single arm prospective trials (≥15% combined per patient incidence) are: hypertension, upper respiratory tract infection, diarrhea, headache, anemia, vomiting, nausea, urinary tract infection, and leukopenia (6.1).

To report SUSPECTED ADVERSE REACTIONS, contact Alexion Pharmaceuticals, Inc. at 1-888-SOLIRIS (1-888-765-4747) or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

USE IN SPECIFIC POPULATIONS

Pregnancy: Based on animal data, Soliris may cause fetal harm (8.1).

Nursing Mothers: Caution should be exercised when administered to a nursing woman (8.3).

Pediatric Use: PNH: safety and effectiveness not established. aHUS: safety and effectiveness similar to adult patients (8.4).

See 17 PATIENT COUNSELING INFORMATION and the FDA-approved Medication Guide.

Revised: 09/2011

FULL PRESCRIBING INFORMATION: CONTENTS*

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- 1.2 Atypical Hemolytic Uremic Syndrome (aHUS)

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14 CLINICAL STUDIES
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16 HOW SUPPLIED/STORAGE AND HANDLING

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***Sections or subsections omitted from the full prescribing information are not listed.**

FULL PRESCRIBING INFORMATION

WARNING: SERIOUS MENINGOCOCCAL INFECTIONS

Life-threatening and fatal meningococcal infections have occurred in patients treated with Soliris. Meningococcal infection may become rapidly life-threatening or fatal if not recognized and treated early (5.1).

- Comply with the most current Advisory Committee on Immunization Practices (ACIP) recommendations for meningococcal vaccination in patients with complement deficiencies.
- Immunize patients with a meningococcal vaccine at least 2 weeks prior to administering the first dose of Soliris, unless the risks of delaying Soliris therapy outweigh the risk of developing a meningococcal infection. (See *Serious Meningococcal Infections (5.1)* for additional guidance on the management of the risk of meningococcal infection.)
- Monitor patients for early signs of meningococcal infections and evaluate immediately if infection is suspected.

Soliris is available only through a restricted program under a Risk Evaluation and Mitigation Strategy (REMS). Under the Soliris REMS, prescribers must enroll in the program (5.2). Enrollment in the Soliris REMS program and additional information are available by telephone: 1-888-SOLIRIS (1-888-765-4747).

1 INDICATIONS AND USAGE

1.1 Paroxysmal Nocturnal Hemoglobinuria (PNH)

Soliris is indicated for the treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH) to reduce hemolysis.

1.2 Atypical Hemolytic Uremic Syndrome (aHUS)

Soliris is indicated for the treatment of patients with atypical hemolytic uremic syndrome (aHUS) to inhibit complement-mediated thrombotic microangiopathy.

The effectiveness of Soliris in aHUS is based on the effects on thrombotic microangiopathy (TMA) and renal function. Prospective clinical trials in additional patients are ongoing to confirm the benefit of Soliris in patients with aHUS.

Limitation of Use

Soliris is not indicated for the treatment of patients with Shiga toxin *E. coli* related hemolytic uremic syndrome (STEC-HUS).

2 DOSAGE AND ADMINISTRATION

Healthcare professionals who prescribe Soliris must enroll in the Soliris REMS. [*see Warnings and Precautions (5.2)*].

Vaccinate patients according to current ACIP guidelines to reduce the risk of serious infection. [*see Warnings and Precautions (5.1) and (5.2)*].

1

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Only administer as an intravenous infusion.

2.1 Recommended Dosage Regimen - PNH

Soliris therapy consists of:

- 600 mg weekly for the first 4 weeks, followed by
- 900 mg for the fifth dose 1 week later, then
- 900 mg every 2 weeks thereafter.

Soliris should be administered at the recommended dosage regimen time points, or within two days of these time points *[see Warnings and Precautions (5.6)]*.

2.2 Recommended Dosage Regimen - aHUS

For patients 18 years of age and older, Soliris therapy consists of:

- 900 mg weekly for the first 4 weeks, followed by
- 1200 mg for the fifth dose 1 week later, then
- 1200 mg every 2 weeks thereafter.

For patients less than 18 years of age, administer Soliris based upon body weight, according to the following schedule (Table 1):

Table 1: Dosing recommendations in patients less than 18 years of age

Patient Body Weight	Induction	Maintenance
40 kg and over	900 mg weekly x 4 doses	1200 mg at week 5; then 1200 mg every 2 weeks
30 kg to less than 40 kg	600 mg weekly x 2 doses	900 mg at week 3; then 900 mg every 2 weeks
20 kg to less than 30 kg	600 mg weekly x 2 doses	600 mg at week 3; then 600 mg every 2 weeks
10 kg to less than 20 kg	600 mg weekly x 1 dose	300 mg at week 2; then 300 mg every 2 weeks
5 kg to less than 10 kg	300 mg weekly x 1 dose	300 mg at week 2; then 300 mg every 3 weeks

Soliris should be administered at the recommended dosage regimen time points, or within two days of these time points.

Supplemental dosing of Soliris is required in the setting of concomitant support with PE/PI (plasmapheresis or plasma exchange; or fresh frozen plasma infusion) (Table 2).

Table 2: Supplemental dose of Soliris after PE/PI

Type of Intervention	Most Recent Soliris Dose	Supplemental Soliris Dose With Each PE/PI Intervention	Timing of Supplemental Soliris Dose
Plasmapheresis or plasma exchange	300 mg	300 mg per each plasmapheresis or plasma exchange session	Within 60 minutes after each plasmapheresis or plasma exchange
	600 mg or more	600 mg per each plasmapheresis or plasma exchange session	
Fresh frozen plasma infusion	300 mg or more	300 mg per each unit of fresh frozen plasma	60 minutes prior to each 1 unit of fresh frozen plasma infusion

2.3 Preparation and 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 60 mL for 300 mg doses, 120 mL for 600 mg doses, 180 mL for 900 mg doses or 240 mL for 1200 mg doses (Table 3).

Table 3: Preparation and Reconstitution of Soliris

Soliris Dose	Diluent Volume	Final Volume
300 mg	30 mL	60 mL
600 mg	60 mL	120 mL
900 mg	90 mL	180 mL
1200 mg	120 mL	240 mL

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.

2.4 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

Soliris is contraindicated in:

- Patients with unresolved serious *Neisseria meningitidis* infection
- Patients who are not currently vaccinated against *Neisseria meningitidis*, unless the risks of delaying Soliris treatment outweigh the risks of developing a meningococcal infection *[see Warnings and Precautions (5.1)]*.

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). Life-threatening and fatal meningococcal infections have occurred in patients treated with Soliris.

Administer a polyvalent meningococcal vaccine according to the most current Advisory Committee on Immunization Practices (ACIP) recommendations for patients with complement deficiencies. Revaccinate patients in accordance with ACIP recommendations, considering the duration of Soliris therapy.

Vaccinate patients without a history of meningococcal vaccination at least 2 weeks prior to receiving the first dose of Soliris. If urgent Soliris therapy is indicated in an unvaccinated patient, administer the meningococcal vaccine as soon as possible. In clinical studies, 33/67 patients with aHUS were treated with Soliris less than 2 weeks after meningococcal vaccination and 31 of these 33 patients received antibiotics for prophylaxis of meningococcal infection until at least 2 weeks after meningococcal vaccination. The benefits and risks of antibiotic prophylaxis for prevention of meningococcal infections in patients receiving Soliris have not been established.

Vaccination reduces, but does not eliminate, the risk of 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)]*. In clinical studies among non-PNH patients, meningococcal meningitis occurred in one unvaccinated patient. In addition, a previously vaccinated patient with aHUS developed meningococcal sepsis during the post-study follow-up period *[see Adverse Reactions (6.1)]*.

Closely monitor patients for early signs and symptoms of meningococcal infection and evaluate patients immediately if an infection is suspected. Meningococcal infection may become rapidly life-threatening or fatal if not recognized and treated early. Discontinue Soliris in patients who are undergoing treatment for serious meningococcal infections.

5.2 Soliris REMS

Because of the risk of meningococcal infections, Soliris is available only through a restricted program under a Risk Evaluation and Mitigation Strategy (REMS). Under the Soliris REMS, prescribers must enroll in the program.

Prescribers must counsel patients about the risk of meningococcal infection, provide the patients with the REMS educational materials, and ensure patients are vaccinated with a meningococcal vaccine.

Enrollment in the Soliris REMS program and additional information are available by telephone: 1-888-SOLIRIS (1-888-765-4747).

5.3 Other Infections

Soliris blocks terminal complement activation; therefore patients may have increased susceptibility to infections, especially with encapsulated bacteria. Children treated with Soliris may be at increased risk of developing serious infections due to *Streptococcus pneumoniae* and *Haemophilus influenza* type b (Hib). Administer vaccinations for the prevention of *Streptococcus pneumoniae* and *Haemophilus influenza* type b (Hib) infections according to ACIP guidelines. Use caution when administering Soliris to patients with any systemic infection.

5.4 Monitoring After Soliris Discontinuation

Treatment Discontinuation for PNH

Monitor patients after discontinuing Soliris for at least 8 weeks to detect hemolysis.

Treatment Discontinuation for aHUS

After discontinuing Soliris, monitor patients with aHUS for signs and symptoms of thrombotic microangiopathy (TMA) complications for at least 12 weeks. In aHUS clinical studies, 18 patients (5 in the prospective studies) discontinued Soliris treatment. TMA complications occurred following a missed dose in 5 patients, and Soliris was reinitiated in 4 of these 5 patients.

Clinical signs and symptoms of TMA include changes in mental status, seizures, angina, dyspnea, or thrombosis. In addition, the following changes in laboratory parameters may identify a TMA complication: occurrence of two, or repeated measurement of any one of the following: a decrease in platelet count by 25% or more compared to baseline or the peak platelet count during Soliris treatment; an increase in serum creatinine by 25% or more compared to baseline or nadir during Soliris treatment; or, an increase in serum LDH by 25% or more over baseline or nadir during Soliris treatment.

If TMA complications occur after Soliris discontinuation, consider reinstitution of Soliris treatment, plasma therapy [plasmapheresis, plasma exchange, or fresh frozen plasma infusion (PE/PI)], or appropriate organ-specific supportive measures.

5.5 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.

5.6 Laboratory Monitoring

PNH

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 (14.1)]*.

aHUS

Early signs of thrombotic microangiopathy (TMA) include a decrease in platelet count, and

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increases in serum LDH and creatinine levels. Follow patients for signs of TMA by monitoring serial platelet counts, serum LDH, and creatinine during Soliris therapy and following discontinuation of Soliris.

5.7 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 patients experienced an infusion reaction which required discontinuation of Soliris. Interrupt Soliris infusion and institute appropriate supportive measures if signs of cardiovascular instability or respiratory compromise occur.

6 ADVERSE REACTIONS

6.1 Clinical Trial Experience

Meningococcal infections are the most important adverse reactions experienced by patients receiving Soliris. 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 one unvaccinated patient. Meningococcal sepsis occurred in one previously vaccinated patient enrolled in the retrospective aHUS study during the post-study follow-up period [*see Warnings and Precautions (5.1)*].

PNH

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 4 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 4: 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%).

aHUS

The safety of Soliris therapy in patients with aHUS was evaluated in two prospective, single-arm studies (aHUS Studies 1 and 2) and one retrospective study (aHUS Study 3). The data described below were derived from 37 adult and adolescent patients with aHUS enrolled in aHUS Study 1 and aHUS Study 2. All patients received the recommended dosage of Soliris. Median exposure was 38 weeks (range: 2-64 weeks).

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 5 summarizes all adverse events reported in at least 10% of patients in aHUS Studies 1 and 2 combined.

Table 5: Per Patient Incidence of Adverse Events in Adult and Adolescent Patients Enrolled in aHUS Study 1 and aHUS Study 2 Separately and in Total

MedDRA ver. 11.0	Number (%) of Patients Study 1 (n=17)	Study 2 (n=20)	Total (n=37)
Vascular Disorders			
Hypertension[a]	8(47)	5 (25)	13 (35)
Infections and Infestations			
Upper respiratory tract infection[b]	5 (29)	8 (40)	13(35)
Urinary tract infection	4 (24)	2 (10)	6 (16)
Gastrointestinal Disorders			
Diarrhea	6 (35)	6 (30)	12 (32)
Vomiting	5 (29)	3 (15)	8 (22)
Nausea	4 (24)	3 (15)	7 (19)
Abdominal pain	0	4 (20)	4 (11)
Nervous System Disorders			
Headache	7 (41)	4 (20)	11 (30)
Blood and Lymphatic System Disorders			
Anemia	6 (35)	3 (15)	9 (24)
Leukopenia	4 (24)	2 (10)	6 (16)
Psychiatric Disorders			
Insomnia	4 (24)	1 (5)	5 (14)
Respiratory, Thoracic and Mediastinal Disorders			
Cough	2 (12)	3 (15)	5 (14)
Pharyngolaryngeal pain	1 (6)	4 (20)	5 (14)
General Disorders and Administration Site Conditions			
Fatigue	3 (18)	1 (5)	4 (11)
Peripheral edema	3 (18)	1 (5)	4 (11)
Pyrexia	3 (18)	1 (5)	4 (11)
Ear and Labyrinth Disorders			
Vertigo	1 (6)	3 (15)	4 (11)
Musculoskeletal and Connective Tissue Disorders			
Pain in extremity	1 (6)	3 (15)	4 (11)

[a] includes the preferred terms hypertension, accelerated hypertension, and malignant hypertension.

[b] includes the preferred terms upper respiratory tract infection and nasopharyngitis.

In aHUS Studies 1 and 2 combined, 54% (20/37) of patients experienced a serious adverse event (SAE). The most commonly reported SAEs were hypertension (16%) and infections (14%). One patient discontinued Soliris due to adverse events deemed unrelated to Soliris.

Analysis of retrospectively collected adverse event data from pediatric and adult patients enrolled in aHUS Study 3 (N=30) revealed a safety profile that was similar to that which was observed in the two prospective studies. aHUS Study 3 included 19 pediatric patients less than 18 years of age. Overall, the safety of Soliris in pediatric patients with aHUS enrolled in Study 3 appeared similar to that observed in adult patients. The most common (≥15%) adverse events occurring in pediatric patients are presented in Table 6.

Table 6: Adverse Reactions Occurring in at Least 15% of Patients Less than 18 Years of Age Enrolled in aHUS Study 3

MedDRA ver. 11.0	Number (%) of Patients < 2 yrs (n=5)	2 to < 12 yrs (n=10)	12 to <18 yrs (n=4)	Total (n=19)
General Disorders and Administration Site Conditions				
Pyrexia	4 (80)	4 (60)	1 (25)	9 (47)
Gastrointestinal Disorders				
Diarrhea	1 (20)	4 (40)	1 (25)	6 (32)
Vomiting	2 (40)	1 (10)	1 (25)	4 (21)
Infections and Infestations				
Upper respiratory tract infection[a]	2 (40)	3 (30)	1 (25)	6 (32)
Respiratory, Thoracic and Mediastinal Disorders				
Cough	3 (60)	2 (20)	0 (0)	5 (26)
Nasal congestion	2 (40)	2 (20)	0 (0)	4 (21)
Cardiac Disorders				
Tachycardia	2 (40)	2 (20)	0 (0)	4 (21)

[a] includes the preferred terms upper respiratory tract infection and nasopharyngitis.

6.2 Immunogenicity

As with all proteins there is a potential for immunogenicity. The immunogenicity of Soliris has been evaluated using two different immunoassays for the detection of anti-eculizumab antibodies: a direct enzyme-linked immunosorbent assay (ELISA) using the Fab fragment of eculizumab as target was used for the PNH indication; and an electro-chemiluminescence (ECL) bridging assay using the eculizumab whole molecular as target was used for the aHUS indication. Low titers of antibodies to Soliris were detected in 3/196 (2%) of all PNH patients treated with Soliris by the ELISA assay. In patients with aHUS treated with Soliris, antibodies to Soliris were detected in 1/37 (2.7%) by the ECL assay. An ECL based neutralizing HAHA assay with a low sensitivity of 2 mcg/mL was performed to detect neutralizing antibodies for the 37 patients with aHUS. No neutralizing activity to Soliris was detected in patients with aHUS treated with Soliris. No apparent correlation of antibody development to clinical response was observed in both indications. The immunogenicity data reflect the percentage of patients whose test results were considered positive for antibodies to Soliris in an ELISA based assay and/or an ECL based assay are highly dependent on the sensitivity and specificity of the assay used. 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.

6.3 Postmarketing Experience

Cases of serious or fatal meningococcal infections have been reported.

7 DRUG INTERACTIONS

Drug interaction studies have not been performed with Soliris.

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8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Pregnancy Category C:

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 function.

8.3 Nursing Mothers

It is not known whether Soliris is excreted 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 antibodies in human milk 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 human milk feeding.

8.4 Pediatric Use

The safety and effectiveness of Soliris for the treatment of PNH in pediatric patients below the age of 18 years have not been established.

Three clinical studies assessing the safety and effectiveness of Soliris for the treatment of aHUS included a total of 25 pediatric patients (ages 2 months to 17 years). The safety and effectiveness of Soliris for the treatment of aHUS appear similar in pediatric and adult patients *[see Dosage and Administration (2.2), Adverse Reactions (6.1), and Clinical Studies (14.2)]*.

Administer vaccinations for the prevention of infection due to *Neisseria meningitidis, Streptococcus pneumoniae* and *Haemophilus influenza* type b (Hib) according to ACIP guidelines *[see Warnings and Precautions (5.1, 5.2)]*.

8.5 Geriatric Use

Sixteen patients 65 years of age or older (15 with PNH and 1 with aHUS) 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, a complement inhibitor, is a formulation of eculizumab which is a recombinant humanized monoclonal IgG2/4$_K$ 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 and complement-mediated thrombotic microangiopathy (TMA) in patients with aHUS.

A genetic mutation in patients with PNH 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.

In aHUS, impairment in the regulation of complement activity leads to uncontrolled terminal complement activation, resulting in platelet activation, endothelial cell damage and thrombotic microangiopathy.

12.2 Pharmacodynamics

In the PNH 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.

A second population PK analysis with a standard 1 compartmental model was conducted on the multiple dose PK data from 57 aHUS patients receiving the recommended Soliris regimen in studies 1, 2 and 3. In this model, the clearance of Soliris for a typical aHUS patient weighing 70 kg was 14.6 mL/hr and the volume of distribution was 6.14 L. The elimination half-life was 291 h (approximately 12.1 days).

The clearance and half-life of eculizumab were also evaluated during plasma exchange interventions. Plasma exchange increased the clearance of eculizumab to 3660 mL/hr and reduced the half-life to 1.26 hours. Supplemental dosing is recommended when Soliris is administered to aHUS patients receiving plasma infusion or exchange [*see Recommended Dosage Regimen (2.1)*].

Dedicated 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. Pediatric and adolescent patients (less than 18 years of age) and patients with renal impairment were included in the aHUS clinical studies [*see Clinical Studies (14)*]. Population PK analysis showed age, gender, race, and renal function do not influence the PK of eculizumab.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Long-term animal carcinogenicity studies of eculizumab have not been conducted.

Genotoxicity studies have not been conducted with eculizumab.

Effects of eculizumab 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

14.1 PNH

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 7).

Table 7: PNH Study 1 Patient Baseline Characteristics

Parameter	Study 1 Placebo N = 44	Study 1 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 treatments (%)	16 (36)	14 (33)
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 8). 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 8: PNH 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) (range)	10 (2 - 21)	0 (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

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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)*].

14.2 aHUS

Three single-arm studies [(two prospective (aHUS Studies 1 and 2) and one retrospective (aHUS Study 3)] evaluated the safety and efficacy of Soliris for the treatment of aHUS. Patients with aHUS received meningococcal vaccination prior to receipt of Soliris or received prophylactic treatment with antibiotics until 2 weeks after vaccination. In all studies, the dose of Soliris in adult and adolescent patients was 900 mg every 7 ± 2 days for 4 weeks, followed by 1200 mg 7 ± 2 days later, then 1200 mg every 14 ± 2 days thereafter. The dosage regimen for pediatric patients weighing less than 40 kg enrolled in aHUS Study 3 was based on body weight [*see Recommended Dosage Regimen (2.2)*]. Efficacy evaluations were based on thrombotic microangiopathy (TMA) endpoints.

Endpoints related to TMA included the following:

- platelet count change from baseline
- hematologic normalization *(maintenance of normal platelet counts and LDH levels for at least four weeks)*
- complete TMA response *(hematologic normalization plus at least a 25% reduction in serum creatinine for a minimum of four weeks)*
- TMA-event free status *(absence for at least 12 weeks of a decrease in platelet count of >25% from baseline, plasma exchange or plasma infusion, and new dialysis requirement)*
- Daily TMA intervention rate *(defined as the number of plasma exchange or plasma infusion interventions and the number of new dialyses required per patient per day)*.

aHUS Resistant to PE/PI (aHUS Study 1)

aHUS Study 1 enrolled patients who displayed signs of thrombotic microangiopathy (TMA) despite receiving at least four PE/PI treatments the week prior to screening. One patient had no PE/PI the week prior to screening because of PE/PI intolerance. In order to qualify for enrollment, patients were required to have a platelet count ≤150 x 10^9/L, evidence of hemolysis such as an elevation in serum LDH, and serum creatinine above the upper limits of normal, without the need for chronic dialysis. The median patient age was 28 (range: 17 to 68 years). Patients enrolled in aHUS Study 1 had an ADAMTS-13 level above 5%. Seventy-six percent of patients had an identified complement regulatory factor mutation or auto-antibody. Table 9 summarizes the key baseline clinical and disease-related characteristics of patients enrolled in aHUS Study 1.

Table 9: Baseline Characteristics of Patients Enrolled in aHUS Study 1

Parameter	aHUS Study 1 N = 17
Time from aHUS diagnosis until screening in months, median (min, max)	10 (0.26, 236)
Time from current clinical TMA manifestation until screening in months, median (min, max)	<1 (<1, 4)
Baseline platelet count (× 10^9/L), median (range)	118 (62, 161)
Baseline LDH (U/L), median (range)	269 (134, 634)

Patients in aHUS Study 1 received Soliris for a minimum of 26 weeks. After completion of the initial 26-week treatment period, most patients continued to receive Soliris by enrolling into an extension study. In aHUS Study 1, the median duration of Soliris therapy was approximately 38 weeks (range: 2 weeks to 64 weeks).

Reduction in terminal complement activity and an increase in platelet count relative to baseline were observed after commencement of Soliris. Soliris reduced signs of complement-mediated TMA activity, as shown by an increase in mean platelet counts from baseline to 26 weeks. In aHUS Study 1, mean platelet count increased from 109 ± 32 x10^9/L at baseline to 169 ± 72 x10^9/L by one week; this effect was maintained through 26 weeks (mean platelet count at week 26: 210 ± 68 x10^9/L). Table 10 summarizes the efficacy results for aHUS Study 1.

Table 10: Efficacy Results for aHUS Study 1 in PE/PI-Resistant aHUS

Efficacy Parameter	aHUS Study 1 N=17
Change in platelet count from baseline through week 26 (× 10^9/L), least square mean (95% CI)[1]	73 (40-105)
Hematologic normalization[2], n (%) Median Duration of hematologic normalization, weeks (range)[2]	13 (76) 37 (25, 62)
Complete TMA response, n (%) Median Duration of complete TMA response, weeks (range)[2]	11 (65) 38 (25, 56)
TMA event-free status, n (%)	15 (88)
Daily TMA intervention rate, median (range) Before eculizumab On eculizumab treatment	0.88 (0.04, 1.59) 0 (0, 0.31)
Change in eGFR (mL/min/1.73 m^2) at 26 weeks, median (range)	+20 (-1, 98)
eGFR improvement ≥15 mL/min/1.73 m^2, n (%)[2] Median duration of eGFR improvement, days (range)[2]	9 (53) 251 (70-392)

1. Calculated at each post-dose day of measurement (excluding Days 1 to 4) through Week 26 using a repeated measurement ANOVA model.
2. At data cut-off (September 8, 2010).

Renal function, as measured by median eGFR, was improved during Soliris therapy. Four of the five patients who required dialysis at study entry were able to discontinue dialysis for the duration of Soliris treatment, and one patient developed a new dialysis requirement.

In aHUS Study 1, responses to Soliris were similar in patients with and without identified mutations in genes encoding complement regulatory factor proteins.

aHUS Sensitive to PE/PI (aHUS Study 2)

aHUS Study 2 enrolled patients undergoing chronic PE/PI who generally did not display hematologic signs of ongoing thrombotic microangiopathy (TMA). All patients had received PT at least once every two weeks, but no more than three times per week, for a minimum of eight weeks prior to the first Soliris dose. Patients on chronic dialysis were permitted to enroll in aHUS Study 2. The median patient age was 28 years (range: 13 to 63 years). Patients enrolled in aHUS Study 2 had an ADAMTS-13 level above 5%. Seventy percent of patients had an identified complement regulatory factor mutation or auto-antibody. Table 11 summarizes the key baseline clinical and disease-related characteristics of patients enrolled in aHUS Study 2.

Table 11: Baseline Characteristics of Patients Enrolled in aHUS Study 2

Parameter	aHUS Study 2 N=20
Time from aHUS diagnosis until screening in months, median (min, max)	48 (0.66, 286)
Time from current clinical TMA manifestation until screening in months, median (min, max)	9 (1, 45)
Baseline platelet count (× 10^9/L), median (range)	218 (105, 421)
Baseline LDH (U/L), median (range)	200 (151, 391)

Patients in aHUS Study 2 received Soliris for a minimum of 26 weeks. After completion of the initial 26-week treatment period, most patients continued to receive Soliris by enrolling into an extension study. In aHUS Study 2, the median duration of Soliris therapy was approximately 40 weeks (range: 26 to 52 weeks). Table 12 summarizes the efficacy results for aHUS Study 2.

Table 12: Efficacy Results for aHUS Study 2 in PE/PI-Sensitive aHUS

Efficacy Parameter	aHUS Study 2 N=20
TMA Event-free status n (%) (95% CI)[1]	16 (80) (56-94)
Daily TMA intervention rate, median (range) Before eculizumab On eculizumab treatment	0.23 (0.05, 1.09) 0
Change in platelet count from baseline through week 26 (× 10^9/L), least square mean (95% CI)[1]	5 (-17.5 - 28)
Hematologic normalization[2], n (%) Median duration of hematologic normalization, weeks (range)[3]	18 (90) 38 (22, 52)
Complete TMA response, n (%) Median duration of complete TMA response, weeks (range)[3]	5 (25) 32 (12, 38)
Change in eGFR (mL/min/1.73 m^2) at 26 weeks, median (range)	+5 (-1, 20)
eGFR improvement ≥15 mL/min/1.73 m^2, n (%)	1 (5)

1. Calculated at each post-dose day of measurement (excluding Days 1 to 4) through Week 26 using a repeated measurement ANOVA model.
2. In aHUS Study 2, 85% of patients had normal platelet counts and 80% of patients had normal serum LDH levels at baseline, so hematologic normalization in this population reflects maintenance of normal parameters in the absence of PE/PI.
3. At data cut-off (October 10, 2010).

In aHUS Study 2, responses to Soliris were similar in patients with and without identified mutations in genes encoding complement regulatory factor proteins. Reduction in terminal complement activity was observed in all patients after commencement of Soliris. Soliris reduced signs of complement-mediated TMA activity, as shown by an increase in mean platelet counts from baseline to 26 weeks. Platelet counts were maintained at normal levels despite the elimination of PE/PI. The mean platelet count was 229 ± 78 x10^9/L at baseline, and 233 ± 69 x10^9/L at week 26. Renal function, as measured by median eGFR, was maintained during Soliris therapy. No patient required new dialysis with Soliris.

Retrospective Study in Patients with aHUS (aHUS Study 3)

The efficacy results for the aHUS retrospective study (aHUS Study 3) were generally consistent with results of the two prospective studies. Soliris reduced signs of complement-mediated TMA activity, as shown by an increase in mean platelet counts from baseline. Mean platelet count increased from 171 ± 83 x10^9/L at baseline to 233 ±109 x10^9/L after one week of therapy; this effect was maintained through 26 weeks (mean platelet count at week 26: 254 ± 79 x10^9/L).

A total of 19 pediatric patients (ages 2 months to 17 years) received Soliris in aHUS Study 3. The median duration of Soliris therapy was 16 weeks (range 4 to 70 weeks) for children < 2 years of age (n=5), 31 weeks (range 19 to 63 weeks) for children 2 to <12 years of age (n=10), and 38 weeks (range 1 to 69 weeks) for patients 12 to 17 years of age (n=4). Fifty three percent of pediatric patients had an identified complement regulatory factor mutation or auto-antibody.

Overall, the efficacy results for these pediatric patients appeared consistent with what was observed in patients enrolled in aHUS Studies 1 and 2 (Table 13). No pediatric patient required new dialysis during treatment with Soliris.

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Table 13: Efficacy Results in Pediatric Patients Enrolled in aHUS Study 3

Efficacy Parameter	<2 yrs (n=5)	2 to <12 yrs (n=10)	12 to <18 yrs (n=4)	Total (n=19)
Platelet count normalization, n (%)[1]	4 (80)	10 (100)	3 (75)	17 (89)
Hematologic Normalization, n (%)	2 (40)	5 (50)	1 (25)	8 (42)
Complete TMA response, n (%)	2 (40)	5 (50)	1 (25)	8 (42)
Daily TMA intervention rate, median (range)				
Before eculizumab	1 (0, 2)	<1 (0.07, 1.46)	<1 (0, 1)	0.31 (0.00, 2.38)
On eculizumab treatment	<1 (0, <1)	0 (0, <1)	0 (0, <1)	0.00 (0.00 , 0.08)
Patients with eGFR improvement ≥15 mL/min/1.73 m^2, n (%)[2]	2 (40)	6 (60)	1 (25)	9 (47)

1. Platelet count normalization was defined as a platelet count of at least 150,000 X 10^9/L on at least two consecutive measurements spanning a period of at least 4 weeks.
2. Of the 9 patients who experienced an eGFR improvement of at least 15 mL/min/1.73 m^2, one received dialysis throughout the study period and another received eculizumab as prophylaxis following renal allograft transplantation.

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 FDA-approved patient labeling (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:

- headache with nausea or vomiting
- headache and a fever
- headache with a stiff neck or stiff back
- fever of 103° F (39.4° C) or higher
- fever and a rash
- confusion
- muscle aches with flu-like symptoms
- eyes sensitive to light

Patients should be informed that they will be given a Soliris Patient Safety Information 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 also be informed that there may be an increased risk of other types of infections, particularly those due to encapsulated bacteria. Parents or caregivers of children receiving Soliris for the treatment of aHUS should be informed that their child should be vaccinated against *Streptococcus pneumoniae* and *Haemophilus influenza* type b (Hib) according to current medical guidelines.

Patients with PNH should be informed that they may develop hemolysis due to PNH when Soliris is discontinued and that they will be monitored by their healthcare professional for at least 8 weeks following Soliris discontinuation. Patients with aHUS should be informed that there is a potential for TMA complications due to aHUS when Soliris is discontinued and that they will be monitored by their healthcare professional for at least 12 weeks following Soliris discontinuation. Patients who discontinue Soliris should be instructed to keep the Soliris Patient Safety Information Card with them for three months after the last Soliris dose, because the increased risk of meningococcal infection persists for several weeks following discontinuation of Soliris.

Manufactured by:

Alexion Pharmaceuticals, Inc.
352 Knotter Drive
Cheshire, CT 06410 USA

US License Number 1743

This product, or its use, may be covered by one or more US patents, including U.S. Patent No. 6,355,245 in addition to others including patents pending.

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MEDICATION GUIDE

Soliris® (so-leer-is)
(eculizumab)

Read the Medication Guide before you start Soliris and before each infusion. This Medication Guide does not take the place of talking with your doctor about your medical condition or your treatment. Talk to your doctor if you have any questions about your treatment with Soliris.

What is the most important information I should know about Soliris?

Soliris is a medicine that affects your immune system. Soliris can lower the ability of your immune system to fight infections.

- **Soliris increases your risk of getting serious and life-threatening meningococcal infections.**

  Meningococcal infections may quickly become life-threatening and cause death if not recognized and treated early.

1. **You must receive a meningococcal vaccine at least 2 weeks before your first dose of Soliris unless you have already had this vaccine. If your doctor decides that urgent treatment with Soliris is needed, you should receive a meningococcal vaccine as soon as possible.**
2. **If you had a meningococcal vaccine in the past, you might need a booster dose before starting Soliris. Your doctor will decide if you need another dose of a meningococcal vaccine.**
3. **A meningococcal vaccine does not prevent all meningococcal infections. Call your doctor or get emergency medical care right away if you get any of these signs and symptoms of a meningococcal infection:**
   - **headache with nausea or vomiting**
   - **headache and a fever**
   - **headache with a stiff neck or stiff back**
   - **fever of 103° F (39.4° C) or higher**
   - **fever and a rash**
   - **confusion**
   - **muscle aches with flu-like symptoms**
   - **eyes sensitive to light**

Your doctor will give you a **Patient Safety Card** about the risk of meningococcal infection. Carry it with you at all times during treatment and for 3 months after your last Soliris dose. Your risk of meningococcal infection may continue for several weeks after your last dose of Soliris. It is important to show this card to any doctor or nurse who treats you. This will help them diagnose and treat you quickly.

Soliris is only available through a program called the Soliris REMS. Before you can receive Soliris, your doctor must:

- enroll in the Soliris REMS program
- counsel you about the risk of meningococcal infection
- give you information about the symptoms of meningococcal infection
- give you a **Patient Safety Card** about your risk of meningococcal infection, as discussed above.
- make sure that you are vaccinated with a meningococcal vaccine

Soliris may also increase the risk of other types of serious infections. If your child is treated with Soliris, make sure that your child receives vaccinations against *Streptococcus pneumoniae* and *Haemophilus influenza* type b (Hib).

What is Soliris?

Soliris is a prescription medicine called a monoclonal antibody. Soliris is used to treat people with:

- a disease called Paroxysmal Nocturnal Hemoglobinuria (PNH). PNH affects red blood cells.
- a disease called atypical Hemolytic Uremic Syndrome (aHUS). aHUS affects the blood system, kidney, and sometimes other body organs.

Soliris works by blocking part of your immune system. This can help your symptoms but it can also increase your chance for infection.

It is important that you:

- have all recommended vaccinations before you start Soliris
- stay up-to-date with all recommended vaccinations during treatment with Soliris

Who should not receive Soliris?

Do not receive Soliris if you:

- have a meningococcal infection
- have not been vaccinated against meningitis infection, unless your doctor decides that urgent treatment with Soliris is needed. See "What is the most important information I should know about Soliris?"

What should I tell my doctor before receiving Soliris?

Before receiving Soliris, tell your doctor if you:

- have an infection or fever
- are pregnant or plan to become pregnant. It is not known if Soliris will harm your unborn baby.
- are breastfeeding or plan to breastfeed. It is not known if Soliris passes into your breast milk.

Tell your doctor about all the medicines you take, including prescription and non-prescription medicines, vitamins, and herbal supplements.

Know the medicines you take. Keep a list of them to show your doctor and pharmacist when you get a new medicine.

How will I receive Soliris?

- Soliris is given through a vein (I.V. or intravenous infusion) usually over 35 minutes. If you have an allergic reaction during your Soliris infusion, your doctor may decide to give Soliris more slowly or stop your infusion.
- If you are an adult, you will usually receive a Soliris infusion by your doctor:
  - weekly for five weeks, then
  - every 2 weeks
- If you are less than 18 years of age, your doctor will decide how often you will receive Soliris depending on your age and body weight.
- After each infusion, you should be monitored for one hour for allergic reactions. See "What are the possible side effects of Soliris?"
- If you forget or miss a Soliris infusion, call your doctor right away.
- **If you have PNH, your doctor will need to monitor you closely for at least 8 weeks after stopping Soliris. Stopping treatment with Soliris may cause breakdown of your red blood cells due to PNH.**

  Symptoms or problems that can happen due to red blood cell breakdown include:
  - drop in the number of your red blood cells
  - drop in your platelet count
  - confusion
  - chest pain
  - kidney problems
  - blood clots
  - difficulty breathing
- **If you have aHUS, your doctor will need to monitor you closely during and for at least 12 weeks after stopping treatment for signs of worsening aHUS symptoms or problems related to abnormal clotting (thrombotic microangiopathy).**

  Symptoms or problems that can happen with abnormal clotting may include:
  - stroke
  - confusion
  - seizures
  - chest pain (angina)
  - difficulty breathing
  - kidney problems
  - swelling in arms or legs
  - a drop in your platelet count

What are the possible side effects of Soliris?

Soliris can cause serious side effects, including:

- **See "What is the most important information I should know about Soliris?"**

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- **Serious allergic reactions.** Serious allergic reactions can happen during your Soliris infusion. Tell your doctor or nurse right away if you get any of these symptoms during your Soliris infusion:
  - chest pain
  - trouble breathing or shortness of breath
  - swelling of your face, tongue, or throat
  - feel faint or pass out

  If you have an allergic reaction to Soliris, your doctor may need to infuse Soliris more slowly, or stop Soliris. See "How will I receive Soliris?"

Common side effects in people with PNH treated with Soliris include:
- headaches
- runny nose and colds
- sore throat
- back pain
- nausea

Common side effects in people with aHUS treated with Soliris include:
- high blood pressure
- common cold (upper respiratory infection)
- diarrhea
- headache
- nausea and vomiting
- low red blood cell count
- low white blood cell count
- urinary tract infection

Tell your doctor about any side effect that bothers you or that does not go away. These are not all the possible side effects of Soliris. For more information, ask your doctor or pharmacist.

Call your doctor for medical advice about side effects. You may report side effects to FDA at 1-800-FDA-1088.

General information about Soliris

Medicines are sometimes prescribed for conditions other than those listed in a Medication Guide. This Medication Guide summarizes the most important information about Soliris. If you would like more information, talk with your doctor. You can ask your doctor or pharmacist for information about Soliris that is written for healthcare professionals.

What are the ingredients in Soliris?

Active ingredient: eculizumab

Inactive ingredients: sodium phosphate monobasic, sodium phosphate dibasic, sodium chloride, polysorbate 80 (vegetable origin) and Water for Injection.

This Medication Guide has been approved by the U.S. Food and Drug Administration.

Manufactured by Alexion Pharmaceuticals, Inc. 352 Knotter Drive, Cheshire, CT 06410 USA.

Revised: 09/2011

100172v4.0
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Europäisches Patentamt
European Patent Office
Office européen des brevets

Anmeldenummer	Application No.	Numéro de la demande:
	05712607.0	

INFORMATION

Die mündliche Verhandlung am:	The oral proceedings of:	La procédure orale du:
	15.05.14	
hat ergeben:	resulted in:	fut conclue comme suit:
☐ Das europäische Patent wird widerrufen, da wenigstens ein Einspruchsgrund der Aufrechterhaltung des europäischen Patents entgegensteht (Art. 101(2) EPÜ).	The European patent is revoked because at least one ground for opposition prejudices the maintenance of the European patent (Art. 101(2) EPC)	Le brevet européen est révoqué car au moins un motif d'opposition s'oppose au maintien du brevet européen (art. 101(2) CBE).
☒ Das europäische Patent wird widerrufen, da unter Berücksichtigung der vom Patentinhaber im Einspruchsverfahren vorgenommenen Änderungen das europäische Patent und die Erfindung, die es zum Gegenstand hat, den Erfordernissen des EPÜ nicht genügen (Art. 101 (3) b) EPÜ).	The European patent is revoked because, account being taken of the amendments made by the patent proprietor during opposition proceedings, the patent and the invention to which it relates were found not to meet the requirements of the EPC (Art. 101(3)(b) EPC).	Le brevet européen est révoqué car il a été établi que, compte tenu des modifications apportées par le titulaire du brevet au cours de la procédure d'opposition, le brevet et l'invention qui en fait l'objet ne satisfont pas aux exigences de la Convention sur le brevet européen (art. 101(3)(b) CBE).
☐ Der Einspruch wird/Die Einsprüche werden zurückgewiesen (Art. 101(2) EPÜ).	The opposition(s) is/are rejected (Art. 101(2) EPC).	L'opposition est/Les oppositions sont rejetée(s) (art. 101(2) CBE).
☐ Es wird festgestellt, dass unter Berücksichtigung der vom Patentinhaber im Einspruchsverfahren vorgenommenen Änderungen das Patent und die Erfindung, die es zum Gegenstand hat, den Erfordernissen des Europäischen Patentübereinkommens genügen (Art. 101(3)(a) EPÜ).	Account being taken of the amendments made by the patent proprietor during the opposition proceedings, the patent and the invention to which it relates are found to meet the requirements of European Patent Convention (Art. 101(3)(a) EPC).	Il est établi que, compte tenu des modifications apportées par le titulaire du brevet au cours de la procédure d'opposition, le brevet et l'invention qui en fait l'objet satisfont aux exigences de la Convention sur le brevet européen (art. 101(3)(a) CBE).
☐ Die während der mündlichen Verhandlung eingereichten Änderungen sind beigefügt.	Amendments filed during oral proceedings are annexed.	Les modifications soumises pendant la procédure orale sont annexées.

15.05.2014
Datum / Date / Date

Unterschrift / Signature / Signature

NB: Dieses Formblatt ist nur als Information zu sehen. Die schriftliche Entscheidung hat Vorrang.
This form is provided for the sake of information only. The written decision prevails.
Le présent formulaire n'a qu'une valeur informative. La décision écrite prévaut.

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80298 MUNICH
GERMANY
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Application No.: 05 712 607.0

Patent No.: EP-B-1 720 571

Direct Decision:

☐ yes ☒ no

Revocation of the European Patent (Art. 101(3)(b) EPC)

The Opposition Division - at the oral proceedings dated 15.05.2014 - has decided:

European Patent No. EP-B- 1 720 571 is revoked.

The Grounds for the decision (Form 2916) are enclosed.

24. 06. 14
Date

Chairman	1st Examiner	2nd Examiner	Legally qualified member
Cilensek, Zoran	Chapman, Rob	Weikl, Martina	

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Vossius & Partner
Siebertstrasse 4
81675 München
ALLEMAGNE

Date: 01-07-2014

Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571

Applicant/Proprietor
ALEXION PHARMACEUTICALS, INC.

Decision revoking the European patent (Art. 101(2), (3)(b) EPC)

The Opposition Division - at the oral proceedings dated 15.05.14 - has decided:

The European Patent No. 1720571 is revoked.

☐ Additional decision:
The opposition of the opponent(s) ____ is rejected as inadmissible.
Apportionment of costs (Art. 104(1) EPC) as indicated in the grounds for the decision.

The reasons for the decision are enclosed.

Appeal
This decision is open to appeal. Attention is drawn to the attached text of Articles 106 to 108 EPC and Rules 97 and 98 EPC.

Opposition Division:

1st Examiner:	2nd Examiner:	Chairman:
Chapman R	Weikl M	Cilensek Z

Enclosures: Reasons for the decision (Form 2916, 10 pages)
Text of Articles 106 - 108 and Rules 97 - 98 EPC (EPO Form 2019)
☒ Minutes of oral proceedings

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Registered letter with advice of delivery
EPO Form 2331 12.07 (26/06/14)

to EPO postal service: 26.06.14

CC20871

1 Facts and Submissions

Reference is made to the following documents cited by the Opponent; the numbering will be adhered to for the rest of the proceedings.

D1 Hillmen et al., (2004) The New England Journal of Medecine, 350:552-9

D2 Jane Salodof MacNeil (2002) Medscape Medical News

D3 Hillmen et al., (2003) British Journal of Hematology 121 (Suppl. 1) 87-95, Abstract 257

D4 Hillmen et al., (2002) Blood. 100(11), Abstract 154

D5 Hillmen et al., (2003) Journal of The American Society of Hematology 102(11) Abstract 1858

D6 Rother et al., (2003) Molecular Immunology. 40, p 197, Abstract 54

D7 Rosse et al., (2004) American Society of Hematology, p 48-62

D8 Biotechnology News (2004) 24(30), p 10

D9 US 6,355,245

D10 Thomas et al., (1996) Molecular Immunology, Vol. 33, No. 17/18, p 1389-1401

D11 Drugs of the Future (2004) 29(7): 673-676

D12 Hillmen et al., British Journal of Haematology, 125 (Suppl. 1), 1-72, Abstract 5

D13 Kaplan, (2002) Current Opinion in Inv. Drugs, vol. 3, no. 7, pages 1017-1023

D14 Declaration of Leonard Bell (2014)

D15 Packaging insert/Datasheet from Eculizumab (Alexion, 2011)

1.1 European patent EP1720571 (hereinafter designated the patent) entitled "Method of treating hemolytic disease", was published in the official bulletin on 13.06.2012 and a corrigendum was issued on 24.10.2012. The Proprietor (hereafter designated 'P') is Alexion Pharmaceuticals, Inc., Cheshire, US.

Said patent is based on European application 05712607.0, filed on 03.02.2005 and claims the priority from US 10/771,552 filed on 03.02.2004. The International publication number is WO2005/074607.

1.2 Notice of opposition against the above-mentioned patent was received from the following party (hereinafter named the Opponent or 'O').

1. Pfizer Inc., 235 East 42nd Street, New York 10017, US (filed online with letter dated 12.03.2013, received on 12.03.2013).

1.3 The O requested the revocation of the patent as granted in its entirety on the basis of Articles 100(a)(b)(c) because the subject-matter granted was not novel, inventive, or disclosed in a manner sufficiently clear and complete to be carried out by a skilled person (Articles 52(1),54,56 and 83 EPC), and comprised subject-matter that extended beyond the application as originally filed (Article 123(2) EPC). The O filed D1-D12 therewith.

1.4 In their letter dated 07.10.2013, the O withdrew their request of opposition against the above-mentioned patent.

1.5 With their letter dated 09.10.2013, the P requested that their patent be maintained as granted and that the opposition procedure be closed. The P requested oral proceedings in case their respective request would not be granted.

1.6 The Opposition Division (OD) issued a non-binding preliminary opinion with their official communication of 14.01.2014, based on the grounds of opposition raised by the O, and which the OD considered pertinent to pursue of its own motion, in accordance with Article 114 EPC. Oral proceedings were appointed for 15.05.2014.

1.7 The P submitted document D13 and arguments in support of maintenance of the patent as granted with their letter of 30.12.2013.

1.8 The OD reissued their non-binding preliminary opinion in order to account for the P's arguments that had not been presented to the OD before issuance of the summons to oral proceedings. The date scheduled for oral proceedings was maintained.

1.9 In their letter of 17.013.2014, the P submitted further arguments, a new main request (MR) and an auxiliary request (AR1) to replace the claims of the opposed patent. The P requested maintenance of the scheduled oral proceedings, in case the OD still not consider that any of the new requests overcome the objections raised.

1.10 Oral proceedings took place on 15.05.2014 in the presence of the P alone, since the O had withdrawn from proceedings. At the proceedings the Chairman (C) informed the P that the examination of the opposition was thus being continued of the OD's own motion. The P maintained their requests on file and submitted a declaration (D14), and a datasheet (D15) to support the subject-matter claimed.

1.11 At the end of oral proceedings, the OD announced the decision to revoke the patent in full in the absence of further allowable requests, because AR1 did not fulfill the requirements of Article 56 EPC.

2 Reasons for the Decision

Admissibility

2.1 The opposition complied with Articles 99 and 100 EPC. No objections were raised. The opposition is allowable.

Procedural matters

2.2 The O formally withdrew their opposition to the patent on 07.10.2013.

2.3 The OD considered that the arguments and documents submitted by the O to be valid grounds for opposition and thus decided to pursue opposition *proceedings of its own motion* (Article 114(1) EPC; GL D, VII-5.2;5.3).

2.4 The P filed D14, a declaration of a technical expert, and D15, a datasheet, during oral proceedings. The OD decided to admit D14 and D15 in to the proceedings, since they provided background information to the subject-matter claimed.

The decision is being carried out on the **following requests:**

Main Request

Description, Pages

3-13	of the patent specification			

Claims, Numbers

1-8	received on	18-03-2014	with letter of	18-03-2014

Drawings, Sheets

1/11-11/11	of the patent specification

Auxiliary Request 1

Description, Pages

3-13 of the patent specification

Claims, Numbers

1-8 received on 18-03-2014 with letter of 18-03-2014

Drawings, Sheets

1/11-11/11 of the patent specification

Main Request (MR)

The MR differs from the patent specification through the modification of the route of administration to explicitly state 'intravenous infusion', thus replacing the term 'intravenously' in claim 1 of the granted patent.

Rule 80, Articles 84, 123(2) and 123(3) EPC

2.5 The OD decided that the MR complies with Rule 80, Articles 84, 123(2) and 123(3) EPC since the term 'intravenous infusion' is clearly stated as the route of administration in the example on which it is based (p.32, li.13) in the application. The OD found that it would be obvious that all other steps in the claim comprising the same substance would be administered via the same route unless otherwise stated in the example. All objections raised were thus overcome.

Lack of novelty (Articles 100(a) and 54 EPC)

2.6 Objections were raised on grounds of lack of novelty of claims 1 and 5 with regard to D1.

2.7 D1 published on 05.02.2004 discloses all the features of claims 1-8 (see Methods and section 'Biochemical indicators of hemolysis' on p.552-556 and Fig.3A) but is published after the date of priority of the patent.

2.8 The patent claims priority from US10/771552, filed on 03.02.2004, and appears to disclose identical subject-matter to the application filed on 03.02.2005 on which the patent is based.

2.9 The OD considered that since the priority document discloses the claimed dosage regimen for treatment of complement breakthrough via intravenous infusion, D1 is not considered prior art and the subject-matter of claims 1 and 5 is new.

Lack of inventive step (Articles 56 and 100(a) EPC)

2.10 The OD objected to the MR on grounds of a lack of inventive step, in light of D5 alone as the closest prior art to the subject matter of claims 1 and 5, or in combination with any of D3, D4 or D6.

2.11 The P concurred that D5 should be considered the closest prior art, as it is directed to the same technical purpose, i.e. it is directed to treating a patient afflicted with PNH suffering a breakthrough in hemolytic activity.

2.12 Document D5 discloses the continuation of a pilot study comprising:

1. the use of eculizumab
2. the treatment of a patient affected with PNH
3. intravenous infusion
4. administration of 900mg bi-weekly
5. reducing the dosing interval slightly in response to breakthrough and thus resolving the symptoms of PNH.

2.13 The P stated that the difference between claim 1 and document D5 can therefore be considered to be that D5 does not disclose a complete dosage schedule. D5 is directed only to the maintenance stage of treatment, i.e. stages (iii) and (iv) of claim 1, and does not teach the essential induction phase stages (i) and (ii).

2.14 The technical effect associated with this difference is a complete dosage schedule for treating PNH and modifications for the alleviation of symptoms associated with a breakthrough in hemolytic activity.

2.15 The problem to be solved can therefore be considered to be the provision of a complete dosage regimen for maintaining patients with PNH symptom free.

2.16 The solution provided in the granted claims is not obvious, since the essential pre-loading stage is not taught in D5.

2.17 The P referred to the declaration D14, §4, wherein it is stated that the administration of 900 mg eculizumab at week five is essential for achieving an adequate baseline of complement inhibition that can be maintained with bi-

weekly 900 mg eculizumab doses thereafter. The P referred to the dosage regimen and provisions for its use, stated in the packaging datasheet D15 that accompanies eculizumab, as proof that this is the regimen used in practice.

The P thus concluded that D5 alone does not teach the features necessary to provide a complete dosage regimen for maintaining patients with PNH symptom free.

2.18 Furthermore, the P stated that D5 combined with any of D3,D4 or D6 would not permit the skilled person to arrive at the regimen claimed:

Documents D3 and D4 disclose a pilot study comprising the following features:

1. the use of eculizumab
2. the treatment of a patient affected with PNH
3. intravenous infusion
4. administration of 600mg each week for 4 weeks
5. administration of 900mg bi-weekly thereafter

The skilled person combining the teaching of D5 with any of D3 or D4 would not arrive at the dosage regimen claimed, since the administration of 900 mg eculizumab at week 5 is not taught.

2.19 The P explained that the skilled person would not arrive at the claimed dosage regimen when combining the teaching of D5 with D6:

Document D6 discloses the following features:

1. the use of eculizumab
2. the treatment of a patient affected with PNH
3. intravenous infusion
4. administration of 600mg each week for 4 weeks
5. administration of 900mg a week later
6. administration of 900mg bi-weekly thereafter

2.20 D6 teaches the administration of 900 mg eculizumab at week 5. However, the skilled person following the dosage schedule of D6 and experiencing breakthrough of hemolytic activity in PNH patients would not reduce the dosage interval to 900 mg eculizumab every 12 days: D5 merely teaches the 'slight' reduction of the dose in this situation. The P claimed that a reduction in dosage interval from 14 to 12 days cannot be considered slight, as it amounts

to around 15%. Moreover, the skilled person with a knowledge of D13 (p. 1021, col.1, §1 - 4) would know that the behaviour of the complement system is difficult to predict, and would not reduce the intervals by so much, and certainly not to 12 days, since shortening of dosing intervals may not result in an effective treatment by eculizumab.

2.21 The OD argued that the replacement of one regimen with another is a matter of routine experimentation, as supported by case law of the Technical Board of Appeal T1979/09, wherein under point 7.3.1 it is stated *'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.'*

and further under point 7.3.2, *'In the present case the amount of active ingredient remains the same as the amount used in D5 and there is no evidence on file of any unexpected technical effect caused by administering the red vine leave extract once daily. 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.'*

2.22 The P disagreed with this interpretation, since the present regimen comprises 4 different steps as stated in the claim, and thus cannot be compared to the simple regimen considered in T1979/09.

2.23 The OD argued that D13 is not directed to the same technical problem posed. Claim 1 is clearly directed to patients suffering breakthrough who are already receiving a standard regimen, as disclosed in D6. D5 clearly discloses how cases in which breakthrough is experienced can be resolved. D5, alike D6 was published after D13 and represents the actual stand of knowledge at the time of priority filing. The OD therefore considers that given the nature of D13 in reporting preliminary research results, and the subsequent publications D5 and D6, the skilled person would not consider D13 when selecting a dosage regimen for the indication presently claimed.

2.24 The P argued that neither D5 alone or combined with D6 any other cited document would anticipate the subject-matter of claim 1, however, if the skilled person were to combine D5 with other documents, he would select D3 or D4 as these relate to less complicated teachings.

2.25 The OD considers D6 to be the most recent and detailed teaching of the cited prior art D3,D4 and D6. The skilled person would therefore not consider D6 too complicated, rather to be the most up-to-date teaching for the treatment of PNH based on the pilot studies described in D2,D3 and D4.

2.26 The OD finds that according to D5, transient breakthroughs in complement activity associated with increased LDL, hemoglobinuria and mild symptoms in an extension trial of patients receiving the dosage regimen described in D6, were known to be preventable by a slight decrease in the dosing interval. The skilled person would have chosen and combined the teachings of D5 and D6 because they both describe the treatment of PNH with eculizumab. D6 discloses the latest details of a pilot study comprising the dose loading stages of parts (i) - (iii) of claim 1, whereas D5 describes an extension to what appears to be the same pilot study, wherein patients experiencing breakthrough of hemolytic activity are treated by a slight reduction in dosing interval, which successfully resolves their symptoms.

2.27 The OD thus concludes that the skilled person would be taught by D5 to reduce the dosage interval to solve the problem of breakthrough in PNH patients receiving the priming dosage regimen described in D6. The skilled person would reduce the interval 'slightly', i.e. by at say least 10%, to 12 days to resolve the symptoms, as suggested by D5.

The subject-matter of the MR therefore does not meet the requirements of Article 56 EPC.

Auxiliary Request 1 (AR1)

AR1 differs from the MR in that a result to be achieved by the dosage regimen of claim 1 of the MR has been added, i.e. a reduction of serum hemolytic activity (cell lysis) below 20%.

Rule 80, Articles 84, 123(2) and 123(3) EPC

2.28 The OD decided that AR1 complies with Rule 80, Articles 84, 123(2) and 123 (3) EPC since the term 'intravenous infusion' is clearly stated as the route of administration in the example on which it is based (p.32, li.13) in the application. The OD found that it would be obvious that all other steps in the claim comprising the same substance would be administered via the same route unless otherwise stated in the example. The feature that cell lysis is reduced by at least 20% was deemed to find a basis on p.38, li.11-17.

Lack of inventive step (Articles 56 and 100(a) EPC)

2.29 The OD objected to AR1 on grounds of a lack of inventive step, in light of D5 as the closest prior art to the subject matter of claims 1 and 5, in combination with D6.

2.30 The P argued that neither D5 nor D6 refer to any threshold value to be used to assess breakthrough of hemolytic activity.

2.31 The problem to be solved may thus be considered to be the provision of a dosage regimen that permits the maintenance of complement activity below a threshold that prevents breakthrough.

2.32 The solution is the dosage regimen of AR1. Neither D5 or D6 teach how to assess breakthrough by the parameters claimed. The application clearly discloses how serum hemolytic activity can be used to identify patients experiencing breakthrough, and that reducing this activity below 20% correlates resolves the symptoms of PNH (p.38 and Fig.8).

2.33 The OD noted that Fig.8 shows at least 4 patients with hemolytic activity above 20%, yet only 2 are described as experiencing breakthrough. The claimed parameter would thus appear to be a non-technical feature of the claim whose relevance is unclear, since patients with serum hemolytic activity above this threshold do not necessarily appear to demonstrate breakthrough.

2.34 The P explained that classic symptoms such as urine colour (hemoglobinuria) were signs of hemolytic breakthrough. In D5, LDH, hemoglobinuria and other mild symptoms were measured, but these are not direct methods of measuring serum complement activity. The parameter claimed thus identifies patients that are successfully treated.

2.35 The OD finds that the technical effect of the dosage regimen claimed is the treatment of PNH. The claim is directed to a dosage regimen and not to a method of diagnosis.

Fig.8 of the application demonstrates that patients with 0 - 30% serum hemolytic activity do not exhibit symptoms of PNH in the group tested. Not all patients are maintained below 20% activity, suggesting that the regimen claimed may not even achieve the technical effect claimed.

Furthermore, alternative methods for monitoring complement breakthrough are disclosed in Fig.4 of the application, which are the same as those used in D5.

The parameter of reducing hemolytic activity below 20% does not appear to provide a further technical feature associated with any surprising technical effect, but may at best be another read-out to confirm the same resolution of breakthrough already recorded in D5.

2.36 The OD therefore concludes that AR1 does not meet the requirements of Article 56 EPC.

Decision

3 Taking into account the amendments made by the P, the patent according to the Main Request or Auxiliary Request 1 was found not to comply with the requirements of Article 56 EPC. The OD decided to revoke the amended patent in accordance with the provisions of Article 101(3)(b) EPC.

Tracking Information

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Name:	Vossius & Partner
Adress:	Siebertstrasse 4
City:	81675 München
Country:	DE
Formtype:	2331/2019/2916
Send Date:	Tuesday, July 01, 2014

Delivery Information:

Tracking Number:	1Z01F1210459362963
Status:	DELIVERED
Pickup Date:	Tuesday, July 01, 2014
Delivery Date:	Wednesday, July 02, 2014
Deposited at:	OFFICE
Signed by (Name):	LEAXNER
Signature:	

Samsung Bioepis Exhibit No. 1002
Page 3560

Europäisches Patentamt
European Patent Office
Office européen des brevets

European Patent Office
80298 MUNICH
GERMANY

EPO - Munich
82

03. Juli 2014

Questions about this communication ?
Contact Customer Services at www.epo.org/contact

Formalities officer: Mr Consalvo Cavestri

Please specify the name of the formalities officer when contacting Customer Services by contact form or phone.

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ALLEMAGNE

Date
01-07-2014

Reference	Application No./Patent No.
M2367 EP	05712607.0 - 1412 / 1720571
Applicant/Proprietor	
ALEXION PHARMACEUTICALS, INC.	

EPA/EPO/OEB Formblatt/Form/Formulaire : 2936

Empfangsbescheinigung über den Zugang des vorstehend bezeichneten Schriftstücks
Acknowledgement of receipt of the document specified above
Récépissé du document spécifié ci-dessus

Unter Bezugnahme auf die Mitteilung im ABl EPA 7/2010, 377 wird gebeten, die Empfangsbescheinigung mit Empfangsdatum und Unterschrift zu versehen und **umgehend** an das EPA zurückzusenden:

With reference to the Notice in OJ EPO 7/2010, 377, you are requested to date and sign the acknowledgement of receipt and return it to the EPO **immediately**:

Conformément au communiqué paru au JO OEB 7/2010, 377, vous êtes prié d'indiquer sur le récépissé la date de réception du document, de signer le récépissé et de le renvoyer **sans délai** à l' OEB:

- **über die Online-Dienste des EPA** (als Anlage zu EPA Form 1038) / **through EPO Online Services** (as annex to EPO Form 1038) / **par les services en ligne de l'OEB** (en tant que pièce jointe au formulaire OEB 1038),
- **per Fax / by fax / par téléfax (+49 (0) 89 2399-4465 or +31 (0) 70 340-3016)**
- oder per Post / or by post / ou par courrier.

Empfangen am / Received on / Reçu le :

02. Juli 2014

Unterschrift / Signature /

RAINER VIKTOR
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DEUTSCHLAND

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EPA/EPO/OEB Form 2936 08.10

CC20871

Samsung Bioepis Exhibit No. 1002
Page 3561

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Patentanwälte Rechtsanwälte

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EPO - Munich
75

08. Sep. 2014

European Patent Office

80298 MUNICH

EP 05 71 2607.0 / 1720571
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

München, September 8, 2014
WA/FLG/BUS

On behalf of Alexion Pharmaceuticals, Inc., 352 Knotter Drive, Cheshire, CT 06410, U.S.A.

and in accordance with Art. 106(1) EPC

Zur Kasse

1860,00 €

APPEAL

is lodged against the decision to revoke the European Patent No. 1720571.

Enclosed please find EPO Form 1010 covering the official fee for appeal.

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Samsung Bioepis Exhibit No. 1002
Page 3562

It is requested that the Decision of July 1, 2014 be reversed and the patent be maintained.

As an auxiliary measure only, it is requested that oral proceedings according to Art. 116 EPC be held.

Dr. Joachim Wachenfeld
European Patent Attorney

Enclosure:
EPO Form 1010

Samsung Bioepis Exhibit No. 1002
Page 3563

Payment of fees and expenses

EPO - Munich
75
08. Sep. 2014

European Patent Office
Treasury and Accounts
80298 München
Germany

Please complete in typescript only

Alexion Pharmaceuticals, Inc.

01 Name of Payer: Vossius & Partner (No. 31)

Payer's reference: M2367 OPP(EP)

Mode of payment: ☐ Bank/Giro transfer [1]

Bank/Giro Office:

02 Address: Siebertstraße 4, 81675 München

☒ Debit from deposit account with the EPO is requested [2]

Deposit account No.: 2800.0321

Patent application / Patent No. (please use a separate form for each application)

03 EP 05 71 2607.0 - 1412 PCT 03

	Code		Currency	Amount
04	001	Filing fee – EP direct	EUR	
05	002	Search fee	EUR	
06	005	Designation fee(s)	EUR	
07	015	Claims fee(s) (Rules 45(1), 162(1) EPC)	EUR	
08	055	Additional copy	EUR	
09	006	Examination fee	EUR	
10	007	Fee for grant	EUR	
11	008	Additional fee for printing specification (more than 35 pages)	EUR	
12	033	Renewal fee for the 3rd year	EUR	
13	034	Renewal fee for the 4th year	EUR	
14	035	Renewal fee for the 5th year	EUR	
15	020	Filing fee – entry EP phase:	EUR	
16		Extension fee(s) For [4]:	EUR	
17	011	Appeal fee	EUR	1.860,00
18			EUR	
19			EUR	
20			EUR	
21			EUR	
22		Total	EUR	1.860,00

Signature: Dr. Joachim Wachenfeld
European Patent Attorney

Place, Date: Munich, September 8, 2014, WA/FLG/BUS

EPO Form 1010 04.09

Samsung Bioepis Exhibit No. 1002
Page 3564

Europäisches Patentamt
European Patent Office
Office européen des brevets

European patent application No. 05712607.0

APPEAL for immediate attention

I. Findings

1. ☒ Appeal in which **more than one party** is involved (opposition procedure, except when all oppositions have been withdrawn). **Refer the case to the Board of Appeal without delay using EPO Form 2703.**

2. ☐ Appeal in which **only one party** is involved (examination procedure and opposition procedure if all oppositions have been withdrawn).

2.1 Requirements of Article 108 EPC

The **notice of appeal** has been filed within the time limit.	yes ☐	no ☐
The **appeal fee** has been paid within the time limit.	yes ☐	no ☐
The **statement of grounds** has been filed within the time limit.	yes ☐	no ☐

If **one** of the answers set out above is 'no' and if no request for re-establishment of rights under Article 122 EPC has been filed (see I.2.2), **refer the case to the Board of Appeal without delay using EPO Form 2703.** The Board of Appeal decides on the admissibility of the appeal.

If **all** the answers set out above are "yes", continue under II/III.2.

2.2 ☐ A request for re-establishment of rights into the appeal period has been filed. Refer the case to the SCAPE of the SIS Unit without delay.

2.3 Special cases which have to be referred to the Board of Appeal without delay using EPO Form 2703

☐ Withdrawal of appeal
☐ Request for correction of appeal (R. 139 EPC)
☐ Expiry of three-month time limit for interlocutory revision (Art. 109(2) EPC)

15-09-2014	Germano, Almut	
Date	Formalities Officer	Director/SCAPE (for information)

II. Appeal against the decision of the Formalities Officer

1. ☐ Re-establishment of rights (Guidelines E-VII, 2.2.7)

☐ The conditions for granting re-establishment of rights are fulfilled. The request can be granted if this can be done within the three-month time limit of Article 109(2) EPC and if the conditions for granting interlocutory revision are fulfilled. Continue under II.2.

☐ The request for re-establishment of rights cannot be granted. **Refer the case to the Board of Appeal without delay using EPO Form 2703.**

2. ☐ The appeal is allowable and well-founded. The decision under appeal is rectified (Art. 109(1) EPC). EPO Form 2710 is to be dispatched.

☐ The decision under appeal is not rectified. **Refer the case to the Board of Appeal without delay using EPO Form 2703 (Art. 109(2) EPC).**

3. Reimbursement of appeal fee

☐ No request for reimbursement of the appeal fee has been filed.

☐ The Formalities Officer orders reimbursement of the appeal fee of his own motion (R. 103 EPC, Guidelines E-X, 7.3).

☐ A request for reimbursement of the appeal fee has been filed.

☐ Reimbursement of the appeal fee is ordered (R. 103 EPC).

☐ The request for reimbursement of the appeal fee cannot be allowed. **Refer the case to the Board of Appeal without delay using EPO Form 2703 (R. 103(2) EPC).**

.....	.....	.....
Date	Formalities Officer	SCAPE (for information)

III. Appeal against the decision of the Examining/Opposition Division

1. ☐ Re-establishment of rights (Guidelines E-VII, 2.2.7)

☐ The conditions for granting re-establishment of rights are fulfilled. The request can be granted if this can be done within the three-month time limit of Article 109(2) EPC and if the conditions for granting interlocutory revision are fulfilled. Continue under III.2.

☐ The request for re-establishment of rights cannot be granted. **Refer the case to the Board of Appeal without delay using EPO Form 2703.**

2. ☐ **To the Examining Division** ☐ **To the Opposition Division**

.....	.....
Date	Formalities Officer

To the Formalities Officer

3. ☐ The appeal is allowable and well-founded. The decision under appeal is rectified (Art. 109(1) EPC). EPO Form 2710 is to be dispatched.

☐ The decision under appeal is not rectified. **Refer the case to the Board of Appeal without delay using EPO Form 2703 (Art. 109(2) EPC).**

4. Reimbursement of appeal fee

☐ No request for reimbursement of the appeal fee has been filed.

☐ The Division orders reimbursement of the appeal fee of its own motion (R. 103 EPC, Guidelines E-X, 7.3).

☐ A request for reimbursement of the appeal fee has been filed.

☐ Reimbursement of the appeal fee is ordered (R. 103 EPC).

☐ The request for reimbursement of the appeal fee cannot be allowed. **Refer the case to the Board of Appeal without delay using EPO Form 2703 (R. 103(2) EPC).**

.....	☐ Examining Division	☐ Opposition Division
Date		

.....	.....	.....	.....
Chairman	2nd Examiner	Primary Examiner	Legal Member

Europäisches Patentamt
European Patent Office
Office européen des brevets

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Datum/Date
24.09.14

Zeichen/Reference/Référence	Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°
M2367 EP APPR	05712607 / 1720571
Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire	
ALEXION PHARMACEUTICALS, INC.	

File number: **T1897/14-3.3.04**

Commencement of proceedings before the Board of Appeal

The letter dated 08.09.14 filed by the patent proprietor
against the decision of the European Patent Office of 01.07.14
has been referred to Board of Appeal 3304.

The reference number is mentioned above.

Any further communications should be addressed to Directorate-General 3 of the European Patent Office and should quote this reference number.

The Registrar P. Cremona
Tel.: 089 / 2399 - 3341

Annex: procedural guidance
Registered letter

European Patent Office - Board of Appeal 3.3.04

These informal notes are provided by Board 3.3.04 for the guidance of the parties. They must be read in conjunction with and subject to the EPC and the Rules of Procedure of the Boards of Appeal (RPBA). Parties are expected to comply with them.

1. Board 3.3.04 endeavours to expedite the appeal procedure so far as possible. Parties are asked to assist the board in achieving this. To that end parties should observe the provisions of Articles 12, 13, 15 und 16 RPBA.

In particular parties should comply with the specific requirement in Article 12 (2) RPBA that the statement of grounds of appeal - and in *inter partes* cases the reply - should contain a party's complete case, specify expressly all the facts, arguments and evidence relied on, and be clearly and concisely presented.

Requests, both for sets of claims and for procedural measures, should also be filed with the statement of grounds of appeal or - as the case may be - the reply.

2. Extensions of time limits will in accordance with Article 12(5) RPBA only be exceptionally allowed following a written request explaining the exceptional circumstances and made sufficiently in advance.

The four month time limit provided in Article 12(1)(b) RPBA for filing the respondent's reply to the statement of grounds of appeal will usually not be extended for reasons of fairness to the appellant who also has a four month non-extendable time limit for filing the statement of grounds of appeal.

3. Communications will be sent to the parties if the board considers it appropriate to do so. Such communications may deal with some but not necessarily all procedural and/or substantive matters. Any opinions of the board shall be regarded as provisional and non-binding for the board.

4. The date set for oral proceedings reflects the requirement to give at least two months notice, the availability of the three members assigned to the case and the availability of a suitable hearing room (the board has no permanently allocated room).

Therefore, the date will in the ordinary course only be changed if the board is satisfied that the reasons advanced are so serious as to justify the change (see "Notice of the Vice-President of Directorate-General 3 of the European Patent Office dated 16 July 2007 concerning oral proceedings before the boards of appeal of the EPO"). According to point 2.3 of the "Notice" the parties are moreover required to state why another representative cannot substitute for the representative prevented from attending the oral proceedings.

Oral proceedings will normally be appointed for a maximum length of one working day. It is the parties' responsibility to comply with the given time frame.

5. Despite the board's efforts to process pending appeals efficiently, the high number of pending cases has the consequence that the board is at present mainly dealing with cases filed during the years 2009 and 2010. Parties should currently allow for a delay of at least three years between filing and disposal of their appeals (unless acceleration was requested and allowed, see "Notice from the Vice-President Directorate-General 3 dated 17 March 2008 concerning accelerated processing before the Boards of Appeal").

Gabriele Alt
Chairwoman, Board 3.3.04

VOSSIUS & PARTNER
Patentanwälte Rechtsanwälte

VOSSIUS & PARTNER * POB 86 07 67 * 81634 München * Germany

EPO - Munich
58

11. Nov. 2014

European Patent Office

80298 M U N I C H

T1897/14-3.3.04
(05 71 2607.0 / 1720571)
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

München, November 11, 2014
WA/FLG

Further to the appeal filed on September 8, 2014, against the decision of the Opposition Division to revoke the above-referenced European patent entitled "Method for treating haemolytic disease" issued on July 1, 2014, we now provide the grounds of appeal in accordance with Article 108 EPC.

It is requested that the patent is maintained for all designated contracting states based on the enclosed Main Request. Its subject-matter is patentable within the terms of the EPC. If the Board of Appeal does not see itself in a position to comply with our main request on the sole basis of our written submissions, we request consideration of auxiliary request 1.

The main request and auxiliary request 1 are identical to the main request and auxiliary request 1 dealt with in the impugned decision.

Should the Board be minded not to accept patentability of any of the enclosed requests, we request that the Board exerts its discretion to allow further claim amendments.

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If the Board does not see itself in a position to comply with any of our claim requests on the sole basis of our written submissions, we request oral proceedings in accordance with Article 116(1) EPC.

An adapted description can be provided at short notice.

I. MAIN REQUEST

1. INVENTIVE STEP (Article 56 EPC)

1.1. The Opposition Division (OD) concluded in section 2.27 of the contested decision that the skilled person is taught by D5 to reduce the dosage interval to solve the problem of complement breakthrough in paroxysmal nocturnal hemoglobinuria (PNH) patients receiving the priming dosage described in D6. The OD took the position that the skilled person would have reduced the interval "slightly", to 12 days to resolve the symptoms, as allegedly suggested by D5.

The OD stated by referring to T1979/09 that the replacement of one dosing regimen with another is a matter of "routine experimentation", regardless of the nature of the agent(s) being administered, the condition being treated, the relevant patient population or the complexity of the dosing regiment being "replaced".

1.2. Contribution of the invention to the art

Claim 1 of the main request is directed to eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), 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).

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Hence, the claims of the main request cover a specific and narrow four-stage treatment schedule for PNH patients with the anti-C5 antibody eculizumab.

As is well-known PNH is a rare, genetically acquired, life-threatening disease of the blood characterized by destruction of red blood cells by the complement system. The primary clinical manifestations of PNH range from hemolytic anemia, thrombosis or other deficiencies in hematopoiesis PNH patients may develop thrombosis (a blood clot) at some point in their illness, which is the main cause of severe complications and death in PNH. In fact, **about 50% of the patients die within 10 years** after diagnosis of the disease.

In 2007, eculizumab was approved for the treatment of PNH and dramatically changed the outlook for patients with PNH. Depending on the underlying extent of bone marrow failure certain patients can experience a breakthrough in hemolytic activity toward the end of the dosing interval (see discussion in paragraphs 76-78 of the patent). In order to ensure that PNH patients benefit from the treatment with eculizumab to the desired effect that **complement breakthrough is prevented** it is of utmost importance that eculizumab is administered in an appropriate dosage regimen.

The four-stage treatment schedule set forth in claim 1 of the main request is tailored to this appropriate dosage regimen of eculizumab. Eculizumab has the trade name Soliris®. The leaflet of Soliris® has been submitted as D15 during the opposition hearing. The claimed four-stage treatment schedule can be taken from sections 2.1 and 5.6 of D15.

The claims are therefore directed to the exact dosage regimen of eculizumab which saved lives of PNH patients and will do so in the future. These PNH patients have a decreased or even no need for blood transfusions, as well as a significantly decreased incidence of thrombosis. The contribution of the present invention to the art therefore should not be underestimated.

1.3. Difference between the closest prior art and the main claim

The claims of the main request provide for an improved treatment schedule of PNH patients showing complement breakthrough when previously treated using

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the established dosage schedule (i.e. steps (i) to (iii) of claim 1). As D5 addresses the problem of complement breakthrough, D5 has been considered as closest prior art.

D5 is a conference abstract authored by inventors of the revoked patent. The following is an excerpt of D5:

> "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 900 mg eculizumab by i.v. infusion over 30 minutes every other week for one year. [...] Two patients experienced transient breakthroughs in complement activity associated with LDH, hemoglobinuria and mild symptoms; these resolve immediately upon next dose of eculizumab and further breakthroughs were prevented by a slight decrease in dosing interval." (emphasis added)

Details on the 3-months pilot study as well as the slightest information on what is meant by a "slight decrease" are absent from D5. The difference between D5 and the claims is therefore two-fold:

(A) D5 is silent about the dosage schedule applied prior to the biweekly administration of 900 mg (i.e. steps (i) and (ii) of claim 1).

(B) D5 is silent about the feature of administering 900 mg every 12 days (step (iv) of claim 1 of the main request).

In view of D5 the technical problem underlying the claims may be seen as the provision of a complete eculizumab dosage regimen which is capable of preventing complement breakthroughs in all PNH patients, including those PNH patients which suffered from a complement breakthrough in the extension trial; see also section 2.15 of the impugned decision.

1.4. Distinguishing feature (A)

1.4.1. Two possible dosage schedules – both being applied prior to the biweekly administration of 900 mg (i.e. step (iii) of claim 1) - can be found in the prior art; see sections 2.18 and 2.19 of the impugned decision. While D6 describes a schedule which comprises steps (i) and (ii) of claim 1, D3 and D4 only describe

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step (i) but not step (ii). The skilled person is consequently confronted with the necessity to choose from two options, being a two step or a one step dosing schedule prior to step (iii) of claim 1.

In its assessment of inventive step the OD found that the skilled person would have reverted to D6 as secondary reference, since only D6 but neither D3 nor D4 discloses a dosing schedule comprising the administration of (i) 600 mg eculizumab each week for four weeks, (ii) 900 mg eculizumab 1 week later and (iii) 900 mg eculizumab on a biweekly basis as set forth in claim 1. The OD justified its position that the skilled person would have preferably turned to D6, by stating that D6 allegedly describes "the most recent and detailed teaching"; see section 2.25 of the impugned decision.

1.4.2. The skilled person can be defined in the present case as a medical doctor, more specifically a clinician or a team of clinicians specializing in the treatment of haemological disorders.

The oral communication corresponding to the abstract of D3 has been presented in April 2003, the abstract D4 is from November 2002, and the abstract D6 dates September 2003. Thus, from the outset, all three references were published within the same one year period. D6 would not necessarily have been considered more recent in any meaningful way which would have led the skilled artisan to have favored this reference. Moreover, while D6 mentions step (ii) of claim 1, neither D3 nor D4 mentions this step. Hence, the teachings of D3 and D4 are consistent, while D6 teaches something else.

Importantly, D6 assigns no particular relevance to the presence or absence of step (ii). However, step (ii) 900 mg eculizumab 1 week later – is **essential** in order to provide an effective and optimal dosage regimen, as evidenced by section 2.1 of D15 (i.e. the post-filing product leaflet of Soliris®). Indeed, this could only be known through hindsight. In this respect reference is made to section 4 of the declaration D14. The technical expert Dr. Bell explains in section 4 that

> "This dose is important because, without it, the loading dose specified by step (i) does not provide an adequate baseline of complement inhibition that can be maintained by the maintenance dose of step (iii) or (iv)"

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It follows that the omittance of step (ii) puts the PNH patients at an undesired increased risk of developing complement breakthrough.

In view of the contents of D3, D4 and D6 is not entirely clear whether D3 and D4 on the one hand and D6 on the other hand describe the same clinical study on PNH patients with eculizumab or two different studies. At face value and in view of the discussed differences there is some evidence that there are two different studies. However, even if considering that all three documents describe the **same** study all PNH patients would already have received the prior dosing schedule prior to the submission of the three abstracts by their authors. The OD's assumption that D6 describes something more recent and more detailed than D3 or D4 is therefore not correct under the assumption that the same study is described.

Irrespectively of whether assuming that the same clinical study or two different studies are described, the conclusion of the OD that the skilled person immediately and unquestionably knows that only D6 but not D3 or D4 describe the correct prior dosing schedule can only be drawn based on hindsight.

The OD in selecting D6 as a secondary reference obviously applied an "ex-post facto" analysis. As the Board is well aware, the Boards of Appeal have ruled that an "ex-post facto" approach when assessing inventive step is impermissible (see, for example, T 24/81, T 564/89, T 645/92 or T 795/93).

A correct application of the problem and solution approach has to avoid the inadmissible "ex-post facto analysis", the OD's biased selection of D6 as secondary reference already indicates that the decision to revoke the patent for an alleged lack of inventive step is not correct.

1.5. Distinguishing feature (B)

As it can be taken from the examples of the patent at paragraph [0073]

> "During the extension study, 2 patients did not sustain levels of eculizumab necessary to consistently block complement. [...] A reduction in the dosing interval from 14 to 12 days was sufficient to maintain levels of eculizumab

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> above 35 µg/ml and effectively and consistently blocked serum hemolytic activity for the remainder of the extension study for both patients."

It will be detailed below that the OD's assumption that the skilled person in view of D5 – even if considering D6 as the "preferred" secondary reference - would have arrived at administering 900 mg every 12 days (step (iv) of claim 1 of the main request) without inventive skills is based on unjust oversimplifications.

1.5.1 *Optimizing the eculizumab dosing schedule is not a matter of routine*

First, there is nothing routine about treating PNH patients with eculizumab, which is a humanized monoclonal antibody that binds to complement protein C5 and prevents its cleavage into C5a and C5b.

As is well-known, the complement protein C5 is a plasma protein. In order to fulfill its function it must be present throughout the body and at a relatively high concentration of 75 µg/ml (0.4 µM) in normal serum; see paragraph [0031] of the patent.

The skilled person is particularly cautious when it comes to interfering with the complement system which is a key component of the human immune system. This is also expressed in D13:

> "Since complement activation has both positive and negative effects, the risk associated with complement inhibition is probably not negligible and long term studies will be necessary to assess possible risks. In particular, a theoretical concern is the role that C5 plays in hepatic regeneration and in combating granulomatous infections such as tuberculosis. Long term studies will determine whether C5 inhibition could have deleterious effects in these processes." (see page 1021; left hand column; top)

The skilled person would have correctly proceeded very cautiously when either increasing the dose or reducing the dose of eculizumab. For example, D15 carries a prominent "Black Box" warning at the top of the first page. The warning states the following:

> "Life-threatening and fatal meningococcal infections have occurred in patients treated with soliris and may become rapidly life-threatening or fatal if not recognized and treated early"

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Furthermore, at the time the invention was made, those skilled in the art were fearful that treatment with eculizumab, which causes the PNH red cell clone size to increase from 36.7% to 58.9% could leave the patient vulnerable to a massive haemolytic attack if treatment was withdrawn (see paragraph [0069] of the patent). This fear is expressed in D7 as follows:

> "The reduction or cessation of transfusions and control of hemolysis in patients on eculizumab leads to an increase in the proportion of PNH red cells toward the level of PNH neutrophils [...] This raises the concern that stopping eculizumab for any reason might render the patient susceptible to a massive haemolytic attack and profound anemia." (see D7, page 53, lower left column).

Moreover, we refer to the sentence bridging pages 52 and 53 of D7, where it is stated:

> "The only apparent sequela [of treatment with eculizumab] is an increased risk of infection by encapsulated organisms, namely *Hemophilus influenza*, *Neisseria meningitides* and *N. gonorrhoea*." (information in brackets added)

As such, the skilled person would be highly concerned about the possibility of severe bacterial infection, and thus had manifest reasons to be exceptionally cautious in view of the specific facts of the present case, namely the potentially dangerous side effects of the anti-C5 antibody eculizumab.

As the Board is well aware, there is settled case law about the attitude of the skilled person in the field of life sciences. The skilled person is cautious and maintains a conservative attitude. She or he does not take unpredictable risks. This point applies to an exceptional extent and should be emphazised in the present case. Choices which carry minimal risk and thus might be considered worth trying or obvious to try (as they are less dangerous) in a particular context, cannot be equated with choices which carry potentially devastating, life threatening risks. The risks posed in the present case, involving a complex biologic drug, i.e. eculizumab, involved in a fundamental, life-depending pathway would have required a significantly heightened evaluation of the potentially available options.

This point is highlighted by a different drug of the same class, alemtuzumab, a humanized anti-CD52 antibody which was first approved in 2001 for refractory B-cell chronic lymphocytic leukemia (B-CLL). Alemtuzumab has exceptional lympholytic activity, driven by complement activation antibody-dependent cytotoxicity, and apoptosis. Accordingly, it was well known that this drug often causes severe side effects, particularly when administered intravenously. For example, as reported at page 6 of the enclosed monograph (BC Cancer Agency Cancer Drug Manual, D16[1]) for alemtuzumab, intravenous administration can cause "serious and potentially fatal complications including myocardial infarction, cardiomyopathy, cardiac arrest, arrhythmias, bronchospasm, and acute respiratory distress". Thus, several pre-treatment protocols involving immunosuppressants are recommended prior to administering alemtuzumab, as well as starting with low doses of the drug (3 mg) and escalating the dosage while carefully monitoring patients. In fact, the subcutaneous route has been studied in an attempt to reduce side effects and make the treatment more manageable (see Hale et al. (2004) *Blood* 224(4):948-955 being enclosed herewith as D17[2]). Thus, like eculizumab, optimizing the dosage for alemtuzumab was anything but routine and is still being explored, even at dosages on a magnitude of 200 to 300 times lower than the presently claimed dosages for eculizumab.

D17 goes on to discuss how alemtuzumab may cause substantial "first dose" reactions as a result of cytokine release when it is given intravenously at 3,18 and 19 days. The standard treatment regimen involves dose escalation and frequent, relatively small doses. A full treatment course for resistant CLL consists of approximately one gram of the antibody (see page 954 of D17). This is in stark contrast to the presently claimed, far higher 600 mg and 900 mg dosages (0.9 g every two weeks) for eculizumab, which would have been considered to hold a much greater risk. Moreover, many factors can affect the immunogenicity of alemtuzumab, including the dose and length of treatment, the underlying disease, prior exposure to chemotherapy, concomitant immunosuppressive drugs, and route of administration or the formulation of the drug (see discussion on page 954 of D17). Again, this highlights the fact that optimizing the dosages of

[1] This document is only submitted for providing the Board with an example of a drug which caused fatal complications due to overdosing.
[2] This document is only submitted for providing the Board with an example of a drug which caused fatal complications due to overdosing.

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complex, antibody-based therapeutics depends on a number of complex, varying factors, and was far from "routine" at the priority date of the present application, even at dosages far lower than those presently claimed.

Indeed, the authors conclude that alemtuzumab provides a useful therapeutic option for patients with CLL. It has excellent activity as first-line treatment and can give good responses in patients who are unresponsive to conventional chemotherapy. However, "the optimal dose route and regimen are not yet known" (see page 955 of D17).

When C5 regulation is perturbed as in PNH the results can be catastrophic. As discussed in paragraph 5 of the declaration D14 of the technical expert Dr. Bell and paragraphs [0002]-[0005] of the patent, failure to **completely** suppress C5 in PNH leads to hemolysis and thrombosis. As mentioned above, thrombosis is the leading cause of death in PNH patients and 50% of PNH patients die within 10 years.

Solving the problem of completely suppressing C5 requires the use of very large doses of eculizumab. However, modifying large doses of any biologic, especially to increase levels of the drug in a patient's serum (e.g., by increasing dosages or shortening dosing intervals), was a risky and dangerous proposition at the priority date, i.e. 2004, not to mention the added complexity in the case of eculizumab where complement C5 plays a **fundamental role in the human immune system**. At that time, and largely still today, the 900 mg maintenance dose was unique among biologic drugs. The dose for most biologic drugs is less than 300 mg. A typical example is the therapeutic anti-TNF antibody adalimumab which is administered at 40 mg every two weeks (see, for example, http://www.drugs.com/dosage/humira.html).

1.5.2. *Possible paths for optimization*

The contents of the prior art do not create a one-way street situation contrary to the position obviously taken by the OD. As mentioned, adjusting the dosing of a biological which interferes with the complement system was and is a most sensitive issue and cannot be equated with a routine measure. This is simply because there was no routine to be relied upon. D5 does not provide a complete

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teaching how to prevent complement breakthrough in PNH patients because what a "slight decrease in dosing interval" means is entirely open. Hence, at the priority date different paths for optimization were available, such as a 13.5 day interval or 13 day interval. There was no pointer to a 12 days interval. Rather the skilled person would have shied away from such a significant decrease in the time interval.

In the case of the present invention, it was PK/PD modeling and data that led the inventors to identify the 12 days interval as being the **precise and critical** solution to the problem, among other available, potentially useful options. Paragraph [0078], Figures 4 and 8 of the patent exemplify the pharmacodynamic data driven analysis used to choose 12 days. It is again only through hindsight – i.e. starting from the work of the inventors and physicians conducting the eculizumab clinical trials - to assume that the skilled person could have known that the specific changes to the clinical protocol that resulted in the present claims were safe and highly effective at preventing breakthrough. Importantly, it was not obvious or possible to know in advance whether a dangerous inflammatory event would occur at the increased dose resulting from the shortened dose interval. Nor was it obvious or possible to know in advance whether these new conditions would trigger an increased human anti-drug antibody response. Only by way of inventive work resulting in the presently claimed invention (i.e., a highly specific and unpredictible change in dosing frequency among other options) was this discovered.

The fact that the inventors first performed labor intense and inventive PK/PD modeling before actually applying dosing step (iv) to PNH patients underscores how critical any dosing change of eculizumab is. It is in particular no option to use a "try-and-see" approach in PNH patients, given that such an approach would involve irresponsible risks for the PNH patients.

1.5.3. *D5 fails to render decreasing the dosing interval from 14d to 12d obvious*

Appellant strongly urges the Board to reconsider the patentability of the main request on the grounds of an alleged lack of inventive step. The OD has significantly erred in hinging its decision on the vague statement in D5 of "slightly decreasing" the dosing interval to support the assertion that the claimed invention

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lacks inventive step. Indeed, the OD offers no basis whatsoever for its conclusion that the skilled person would have automatically equated a "slight decrease" in dosing interval with a decrease of considerably more than 10% (i.e., from 14 days to 12 days). Again, this is an ex-post facto approach.

A change from a 14 day interval to a 12 day interval for a drug administered at 900 mg for maintenance dosing results in a **significant increase** in exposure to the patients of 3.9 grams or **16.7%** of therapeutic antibody annually. The magnitude of this change is not commensurate with the phrase "slight decrease" as suggested in D5 (see section 2.20 of the contested decision) but would have carried great risks.

The OD decision should be reversed in this appeal, because the selection of the 12 day dosing interval for patients experiencing breakthrough required inventive skills. As discussed in the above-cited paragraph [0073] of the patent, the dose decision was made to maintain a minimum target level of 35 µg/ml of eculizumab in the affected patient's serum. This change was supported scientifically by examining the PK/PD data generated in patients with PNH and being treated with eculizumab. The OD's reliance on the statement in D5 trivializes the significance of this non-obvious and precise solution to a clinically devastating problem. As discussed above, the change of dosing interval from 14 days to 12 days to prevent breakthrough was not made by simple process of elimination or a try-and-see approach, but rather by inventive reasoning and a carefully selected scientific approach.

1.5.4. *Decision T1979/09 is not applicable*

In addition, we wish to submit that analogizing the decision T1979/09 to the facts and circumstances of the present patent is incorrect. The claims at issue in T1979/09 were directed to a dietary supplement for preventing or treating discomfort associated with venous insufficiency by administering flavonoids by water extracting crushed and dried red vine leaves. This case is distinguishable from the present matter and the decision states that

> "the skilled person would carry out these steps according to the known standard technologies for the processing of medicinal plants" (see page 17 of T1979/09).

It cannot be the case that dose considerations made in the context of suppressing complement protein C5 by administering a high dose of a biologic drug to patients with a potentially fatal disease by intravenous administration are routine experimentation on the level of administering flavonoids extracted from leaves of medicinal plants. The consequences of making a mistake in either of the two situations are certainly in no way equivalent.

Cleavage of complement C5 plays a central role in the immune system; see Rother et al. *Nature Biotechnology* 25 (11) 1256-1264 (2007) which is provided herewith as document D18[3]. There are three distinct complement pathways known as alternative pathway, classical pathway, and lectin pathway and they differ in the mechanism of activation. But no matter the manner of activation, all three pathways converge at the point of C5 cleavage. C5 is cleaved into C5a and C5b. C5a is a potent anaphylatoxin that mediates leukocyte chemotaxis, increases vascular permeability and induces other secondary inflammatory mediators. The simultaneous release of C5b triggers the recruitment of terminal complement components C6, C7, C8 and C9 to form the terminal complement complex which results in lysis and destruction of a target cell. Individuals who are deficient in other complement components such as C1, C2 or C4 develop severe autoimmune symptoms such as Lupus-like syndrome and are at recurrent risk of developing sepsis. Therefore, the complete inhibition of complement C5 was rightly viewed in 2004 as a very uncertain and medically consequential activity.

According to the European Medicines Agency (EMA) in their Assessment Report on *Vitis vinifera* L.

> "Since ancient times, beneficial effects on health have been ascribed to wine and vine leaves, as confirmed by numerous "recipes" reported in Egyptian papyri, the Sumerian Tablets, the writings of Hippocrates of Cos (5th-4th Century BC), Celcus (1st century AD), Galen (130-201 AD) and Paracelsus (1493-1541). (see EMA/HMPC/16633/2009 section 2, Historical Data on Medicinal Use provided herewith as document D19[4]).

[3] This document is only submitted for providing the Board with a short and concise overview of the scientific background of the invention.
[4] This document is only submitted for providing the Board with background information on the case at stake in T1979/09.

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The incorporation of the flavonoids into in a dose range of 300-800 mg and incorporated into a dietary supplement tablet does not change the fact that the flavonoids were known to be generally safe and beneficial. The flavonoids were orally administered and allowed to diffuse into the blood stream in the digestive process. The EMA found that for red vine leaf converted from a tea administration to a tablet

> "there is no safety specific information that is relevant for medical products." (see EMA/HMPC/16633/2009 section 5.3, Adverse Events).

It follows that the administration of flavonoids has no severe side effect and overdosing flavonoids in a patient is not life-threatening for the patient. By contrast, overdosing the first in class biologic protein drug eculizumab may result in the blocking the complement system of the immune system to an extent that puts the patient in a life-threatening situation. In the first case optimizing of the dosing schedule is a matter of routine, whereas in the latter case it is not.

Thus, we contend that dosing through intravenous administration of a first in class biologic protein drug, at an usually high dose, with the intention of suppressing the activity of an immune system component does involve inventive activity. Where the consequences of dosing changes may be catastrophic, the inventors must invest greater judgment and thought about dose changes or dose interval changes. The inventors for a drug such as eculizumab are evaluating the effects of dose changes on the immune system in a very quantitative fashion. Any statements made in the context of dosing decisions for dietary supplements should not apply with equal force to the clinical trials of a first in class drug like eculizumab. We therefore consider that T1979/09 is not applicable. The facts of the case underlying this decision are different from that of the present case.

1.6. In summary, we submit that the reasons of the OD for revoking the patent for an alleged lack of inventive step are without merit. The Board of Appeal is therefore requested to set the decision of the OD aside and to give the order to maintain the initially granted patent based on the main request.

II. AUXILIARY REQUEST 1

In case the Board of Appeal cannot accept that the main request meets the requirements of Article 56 EPC, consideration of auxiliary request 1 is requested. The arguments and facts presented herein above in connection with the main request are equally applicable to the auxiliary request 1.

As compared to the main request claim 1 of auxiliary request 1 further comprises the feature of "reducing cell lysis to below 20% of normal serum complement activity" which is a feature indicating the effective inhibition of haemolytic activity; see page 38, lines 11-13 and Figure 8. The effective inhibition of haemolytic activity is a key advantage being associated with the subject-matter claimed in the main and auxiliary requests. This advantage is expressly incorporated in the main claim of auxiliary request 1.

Dr. Joachim Wachenfeld
European Patent Attorney

Enclosure:
D16 (Monograph from the BC Cancer Agency Cancer Drug Manual)
D17 (Hale et al. (2004) *Blood* 224(4):948-955)
D18 (Rother et al. *Nature Biotechnology* 25 (11) 1256-1264 (2007))
D19 (EMA/HMPC/16633/2009 section 2, Historical Data on Medicinal Use)
Main Request
Auxiliary Request 1

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(05 71 2607.0 / 1720571)
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

PERSPECTIVE

nature biotechnology

Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria

Russell P Rother[1], Scott A Rollins[1], Christopher F Mojcik[1], Robert A Brodsky[2] & Leonard Bell[1]

The complement system provides critical immunoprotective and immunoregulatory functions but uncontrolled complement activation can lead to severe pathology. In the rare hemolytic disease paroxysmal nocturnal hemoglobinuria (PNH), somatic mutations result in a deficiency of glycosylphosphatidylinositol-linked surface proteins, including the terminal complement inhibitor CD59, on hematopoietic stem cells. In a dysfunctional bone marrow background, these mutated progenitor blood cells expand and populate the periphery. Deficiency of CD59 on PNH red blood cells results in chronic complement-mediated intravascular hemolysis, a process central to the morbidity and mortality of PNH. A recently developed, humanized monoclonal antibody directed against complement component C5, eculizumab (Soliris; Alexion Pharmaceuticals Inc., Cheshire, CT, USA), blocks the proinflammatory and cytolytic effects of terminal complement activation. The recent approval of eculizumab as a first-in-class complement inhibitor for the treatment of PNH validates the concept of complement inhibition as an effective therapy and provides rationale for investigation of other indications in which complement plays a role.

The complement system: a potential target for therapeutics

The complement system consists of more than 20 serum proteins that interact in a precise series of enzymatic cleavage and membrane binding events leading to the generation of products with immunoprotective, immunoregulatory, proinflammatory and cytolytic properties[1,2].

Proximal complement. The cascade proceeds through three distinct pathways—alternative, classical and lectin—which all result in the generation of C3 convertase complexes that mediate the cleavage of C3 to C3a and C3b[1,3] (**Fig. 1**). Recent studies have delineated the structure of C3 and C3 fragments and provided important insights into their function[4–7]. The classical pathway is generally initiated by the interaction of C1q with antigen-antibody complexes. The lectin pathway is activated by the interaction of mannose-binding lectin (MBL) with specific mannose-containing carbohydrate structures. The alternative pathway is initiated by deposition of preformed C3b on a variety of substrates such as bacteria and cell membranes, including erythrocytes. This C3b is continuously available due to the interaction of C3 with water, a process that represents an important part of the innate immune system called complement 'tick-over'. In addition to its role in the initiation of alternative pathway activation, C3b is also necessary for the amplification and progression of the complement cascade through all pathways of activation and serves as a key immunoprotective and immunoregulatory molecule.

The importance of proximal complement components in immunity is illustrated by the clinical effects observed in people with genetic complement deficiencies. For example, individuals with C3 deficiency are at particular risk of infection from polysaccharide-coated bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Neisseria meningitidis*, and frequently die at a young age from overwhelming sepsis[8]. The absence of C3 is generally thought to prevent the progression of complement through all pathways of activation, thereby reducing the body's ability to opsonize pathogens and mount an inflammatory response, although some studies suggest that C5 cleavage can occur in the absence of C3 by thrombin[3,9]. Individuals deficient in C1, C2 or C4 develop severe autoimmune symptoms such as a lupus-like syndrome and are also susceptible to recurrent overwhelming sepsis. Thus, deficiencies in early components of the complement cascade lead to impairment in the ability to generate C3b, which is critical for immune complex clearance and is the primary opsonin for many pathogenic microorganisms.

Terminal complement. All pathways of complement activation converge at the cleavage of C5 into C5a and C5b by the C5 convertase enzyme complexes. This cleavage event initiates the terminal complement cascade (**Fig. 1**). C5a is a potent anaphylatoxin that mediates leukocyte chemotaxis, increases vascular permeability, alters smooth muscle tone and induces secondary inflammatory mediators such as hydrolytic enzymes, reactive oxygen species, arachidonic acid metabolites and cytokines[10]. C5a receptors are present on a variety of cell types that directly contribute to inflammation, including monocytes, macrophages and neutrophils. The C5a receptor CD88 is a member of the N-formyl peptide receptor family, which transduces signals through a G protein–dependent pathway resulting in biochemical proinflammatory

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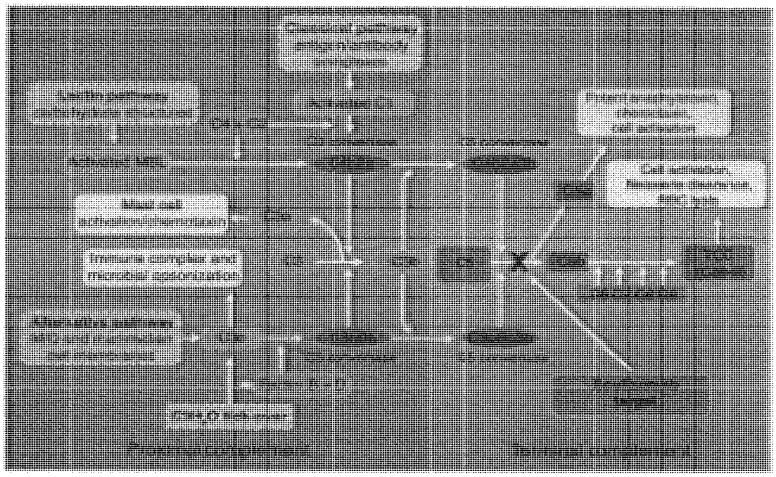


Figure 1 Targeted blockade of complement protein C5. The complement cascade can be activated via the classical, lectin and alternative pathways. The proximal components of complement (proteins upstream of C5) are essential for microbial opsonization and immune complex clearance. All pathways of complement activation converge at the cleavage of the terminal complement protein C5 leading to the generation of molecules with proinflammatory and cell lytic properties. Targeted blockade at C5 with eculizumab therefore prevents the deleterious properties of terminal complement activation while preserving the immunoprotective and immunoregulatory functions of proximal complement. M/O, microorganisms. TCC, terminal complement complex.

responses[11]. A second C5a receptor has been identified (C5L2) that also facilitates C5a-mediated signaling in a variety of cell types[12]. C5b recruits the terminal complement components C6, C7, C8 and C9 to form C5b-9 or the terminal complement complex (TCC) on the surface of cells. The generation of the TCC at sublytic concentrations stimulates the release of many of the same proinflammatory molecules described for C5a. Most importantly, unimpeded assembly of the TCC on the cell surface results in cell lysis. Deposition of TCC on erythrocytes results in the destruction of these cells in hemolytic diseases such as PNH[13,14].

The importance of terminal complement components in immunity is demonstrated by individuals with genetic deficiencies of C5, C6, C7, C8 or C9, who show an increased incidence of infections with *N. meningitidis*, a bacterium that can cause meningitis[8]. Vaccines against *N. meningitidis* are recommended for terminal complement–deficient individuals, although breakthrough infections have been reported[15]. Interestingly, *N. meningitidis* infections in terminal complement–deficient people are typically less severe than in normal individuals, possibly due to the lack of TCC-mediated endotoxin release from the bacteria[15].

Targeted blockade of the complement cascade at C5. In the rational design of a therapeutic complement inhibitor, C5 is an attractive target[1]. Because C5 is common to all pathways of complement activation, blockade at this point stops the progression of the cascade regardless of the stimuli. In addition, prevention of C5 cleavage effectively blocks the generation of the potent proinflammatory molecule C5a and the cell lytic TCC. Importantly, C5 blockade preserves the critical immunoprotective and immunoregulatory functions of upstream components that culminate in C3b-mediated opsonization and immune complex clearance.

Development of eculizumab

To generate potent inhibitors of complement component C5, panels of murine anti-human C5 monoclonal antibodies were generated and initially screened for the ability to inhibit TCC-mediated lysis of antibody-sensitized chicken erythrocytes by human complement in a standard hemolytic assay[16]. The most potent candidates were cloned and purified, and functional activity was further characterized to identify anti-C5 antibodies that could also effectively block the generation of C5a. Of ~30,000 hybridomas screened, one monoclonal antibody (mAb), designated murine 5G1.1 (m5G1.1 mAb), was identified that effectively blocked both TCC-mediated hemolysis and the generation of C5a, at a 0.5:1 molar ratio of antibody to C5. Murine 5G1.1 mAb binding to human C5 mapped to the N-terminal region of the alpha chain.

Engineering. To reduce the potential for immunogenicity, we cloned and grafted the complementarity-determining regions of m5G1.1 mAb into human heavy and light chain antibody frameworks (**Fig. 2**)[16,17]. The human heavy-chain framework was derived from the H20C3H antibody variable region[18], which contained no amino acid changes relative to the original germline sequence. The human light-chain framework was derived from the I.23 antibody variable region[19], which contained only one amino acid change from the original germline sequence; the germline amino acid residue was restored at this position. The use of purely germline framework acceptor sequences should further minimize potential immunogenicity of the humanized antibody, because the immune system of every individual should be tolerant to these sequences.

The use of antibodies as therapeutics in some clinical settings is complicated by effector functions of such antibodies that serve to activate complement and/or to bind to antibody receptors (Fc receptors) on inflammatory cells after they have engaged their target. To reduce the potential for eculizumab to elicit proinflammatory responses, we replaced the heavy-chain constant region of the parental antibody with components of both human IgG2 and IgG4 constant regions (**Fig. 2**)[20]. The human IgG2 antibody isotype does not bind Fc receptors[21,22], whereas the human IgG4 isotype does not activate the complement cascade[23,24]. The IgG2/IgG4 hybrid constant region of eculizumab includes the CH1 and hinge regions of human IgG2 fused to the CH2 and CH3 regions of human IgG4, and lacks the ability to bind Fc receptor and to activate complement[20]. To avoid the generation of an antigenic site during the fusion, we used a restriction endonuclease cleavage site common to both IgG2 and IgG4 to join the two constant regions. Indeed, a total of 31 amino acids flanking the fusion site are identical between IgG2 and IgG4.

Binding characteristics. The humanized monoclonal antibody eculizumab maintained its ability to block the cleavage of C5 during complement activation as the antibody potently inhibited both the generation of C5a and C5b-9–mediated human serum hemolytic activity[16]. Eculizumab is highly species-restricted, with minimal activity against any other primate or mammalian C5. No nonspecific or unexpected

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binding of eculizumab to any human tissue examined in a tissue cross-reactivity study was observed. In addition, the affinity of eculizumab for human C5 (K_d = 120 pM) is similar to that of the parental m5G1.1-mAb, indicating that the binding properties of the antibody were not compromised during the humanization process.

The eculizumab pharmacokinetic profile was analyzed using a one-compartmental model. When administered by intravenous infusion, eculizumab has a half-life of 272 ± 82 h, and its distribution appears to be primarily limited to the vascular space. Eculizumab serum concentrations appear to reach steady state after ~57 days. At steady state, the eculizumab accumulation ratio (R_{acc}) was calculated to be 1.075, which is indicative of minimal accumulation over time. Pharmacodynamic activity correlates directly with eculizumab serum concentrations and maintenance of trough levels above 35 μg/ml results in essentially complete blockade of hemolytic activity *in vivo*[25].

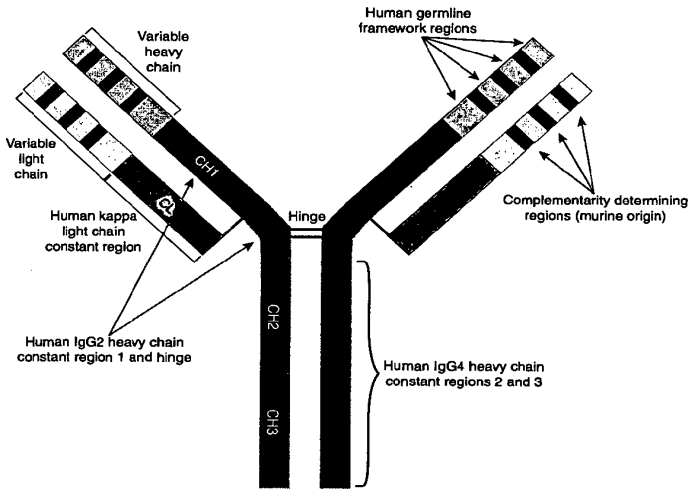


Figure 2 Engineering of eculizumab to reduce immunogenicity and eliminate effector functions. To minimize immunogenicity, we grafted murine complementarity-determining regions into human heavy and light chain germline antibody framework sequences. Additionally, human IgG2 and IgG4 heavy chain sequences were combined to form a hybrid constant region that is unable to bind Fc receptors or to activate the complement cascade. Eculizumab exhibits high affinity for human C5, effectively blocking its cleavage and downstream proinflammatory and cell lytic properties.

Preclinical development. Because of the species-restricted nature of eculizumab, pharmacological activities *in vivo* were explored with a surrogate anti-mouse C5 antibody (BB5.1) that demonstrated terminal complement–inhibitory activity comparable to that of eculizumab. As no adequate mouse models of PNH exist, *in vivo* studies targeting other therapeutic indications with potential involvement of terminal complement activation were used. In a collagen-induced model of arthritis, BB5.1 was shown to prevent the onset of disease and to improve the course of previously established disease[26]. Treatment with BB5.1 also resulted in an improvement in renal disease and in dramatic prolongation of survival in a lupus-like autoimmune model[27]. These models provided substantial evidence that a functionally blocking anti-C5 antibody could effectively and consistently block terminal complement activation leading to a reduction in terminal complement-mediated proinflammatory sequelae. The surrogate BB5.1 antibody was also used in mouse toxicity studies in which no compound-related mortalities or significant abnormalities were observed.

Early clinical development. Initial human clinical studies of eculizumab, and a single-chain antibody variant of eculizumab called pexelizumab, were performed in individuals with rheumatoid arthritis and systemic lupus erythematosus[28,29] and in individuals with coronary artery bypass graft surgery and myocardial infarction, respectively[30,31]. The initial eculizumab studies served two purposes, to determine a dosing schedule for future trials and to establish initial safety of terminal complement inhibition in humans. Participants received a single dose of eculizumab ranging from 0.1 mg/kg to 8 mg/kg; all doses were observed to be safe and well-tolerated. The 8 mg/kg dose (~600 mg total) provided complete complement blockade for 7–14 days. Studies exploring multiple chronic dose regimens with eculizumab were also performed in individuals with membranous nephritis and rheumatoid arthritis[32,33].

PNH—a disease in search of a complement inhibitor

Etiology of PNH. PNH is a rare form of hemolytic anemia with an estimated incidence of 1.3 cases per million per year and a prevalence of 15.9 cases per million[34]. The disease is manifest by an acquired genetic deficiency of endogenous complement inhibitors on the surface of blood cells; these cells are referred to as the PNH clone(s). PNH clones are deficient in proteins that are normally linked to the cell surface by a glycosylphosphatidylinositol (GPI)-anchor. The inability of cells to attach proteins through this lipid moiety is caused by somatic mutations occurring in the X-linked gene, phosphatidylinositol glycan–complementation class A (*PIGA*)[35–37], a critical molecule in the GPI-anchoring pathway.

In individuals with PNH, cells deficient in GPI-anchored proteins exist in all blood lineages including erythroid, myeloid and lymphoid cells. These PNH cells are clonal and harbor matching *PIGA* mutations indicating that the mutations occur in multipotent hematopoietic stem cells[38,39]. Granulocytes from the blood of healthy controls also possess *PIGA* mutations at an exceedingly low frequency (~1 in 50,000), suggesting that random mutations in this gene are important in the pathogenesis of PNH[40]. Thus, it has been hypothesized that the development of PNH requires two independent events to occur; hematopoietic stem cells must acquire a *PIGA* mutation and an immune attack that preferentially targets normal stem cells must be present[41,42]. This proposal presumes that the bone marrow insult targets a GPI-anchored protein on the hematopoietic stem cell, thereby providing a survival advantage to GPI-anchoring–deficient stem cells (that is, cells possessing a *PIGA* mutation). It should be noted however that *PIGA* mutations characterized in healthy controls appear to occur in more differentiated colony-forming cells rather than multipotent hematopoietic stem cells[39]. These colony-forming cells would not have self-renewal capacity and therefore would not be propagated beyond their terminally differentiated progeny.

There is clinical evidence to support a survival advantage of *PIGA* mutated hematopoietic stem cell clones over normal stem cells in damaged bone marrow. The mAb alemtuzumab (Campath) is used to treat

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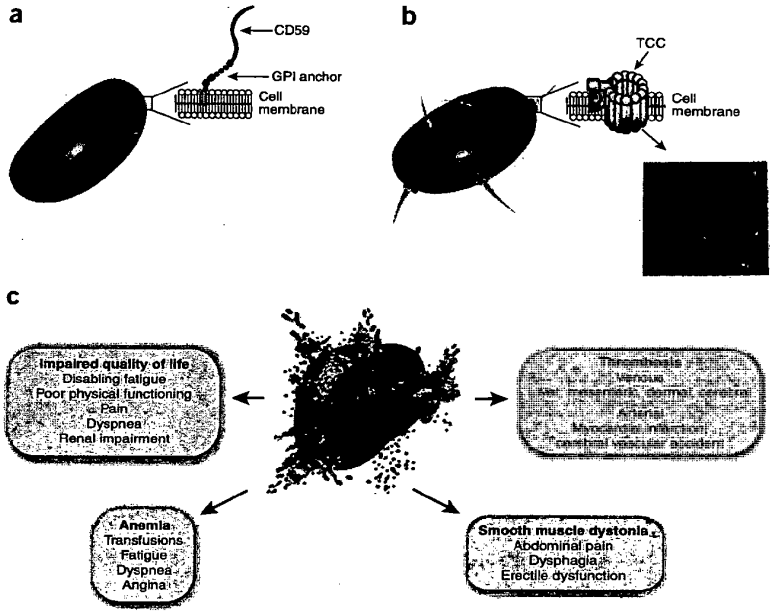


Figure 3 Terminal complement deposition on the surface of PNH RBCs results in chronic hemolysis and serious clinical morbidities. (**a**) Normal RBCs express complement regulatory proteins including the terminal complement inhibitor CD59. CD59 is tethered to the RBC lipid bilayer of the cell membrane by a GPI anchor. The presence of CD59 protects these cells from autologous complement-mediated destruction. (**b**) PNH RBCs lack GPI-anchored proteins including CD59. Continuous complement activation leads to formation of the terminal complement complex (TCC) that transverses the lipid bilayer. These complexes form numerous pores in the RBC membrane, as shown by the electron micrograph (inset). As pores form in the membrane, water enters the RBC (arrows), resulting from osmotic pressure. (**c**) As the concentration of TCCs increases, the PNH RBC swells and ultimately ruptures (hemolysis). Intravascular hemolysis causes the release of the RBC contents such as hemoglobin into the plasma. Hemolysis in PNH is chronic and leads to multiple clinical sequelae, including severe anemia requiring transfusions, disabling fatigue, dyspnea, impaired quality of life, recurrent pain associated with smooth muscle dystonia, and thrombosis. Artwork by Edmond Alexander, ©2007 Edmond Alexander, printed with permission. Micrograph image is from E.R. Podack. Molecular mechanisms of cytolysis by complement and cytolytic lymphocytes. *J. Cell. Biochem.* **30**, 133–170 (1986). Copyright © 1986 Wiley-Liss. Reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

chronic lymphocytic leukemia by targeting and destroying malignant T- and B-cells that express the GPI-anchored protein CD52. In many individuals, a PNH T-cell clone expands after alemtuzumab treatment. In this case alemtuzumab supplies the bone marrow insult and the GPI-anchoring deficient progenitor T-cells that lack CD52 escape immune destruction and expand[43].

Pathophysiology of PNH. Although PNH blood cells of all lineages are missing the wide array of GPI-anchored proteins from their surface, they are remarkably normal with regard to their physiological functions (e.g., fighting infection, clotting, carrying oxygen). However, the PNH red blood cell (RBC) is exquisitely sensitive to destruction because of the inability to regulate autologous terminal complement assembly (**Fig. 3**). This defect is primarily triggered by the absence of terminal complement inhibitor function[13,14,44,45]. CD59 normally blocks formation of the TCC on the RBC surface, thereby preventing intravascular hemolysis[46–48]. Hemolysis in PNH is chronic because of a continuous state of complement activation (tick-over; **Fig. 1**), but brisk episodes of hemolysis (paroxysms) coincident with increases in complement activation triggered by infections, surgery, strenuous exercise, excessive alcohol intake and blood transfusions frequently occur[49].

Chronic hemolysis is central to the morbidities in PNH and contributes to mortality in those with the disease (**Fig. 3**). It may result in severe anemia requiring transfusion, and refractory transfusion-dependent hemolytic anemia has been considered a major complication of PNH warranting consideration of bone marrow transplantation[35]. Fatigue in PNH patients is tied directly to hemolysis, strikingly disproportionate to the degree of anemia, frequently debilitating and similar to the severity observed in anemic cancer patients[25,49–52]. Hemolysis is also directly linked to diminished health status and functioning[25,49]. Other disabling morbidities evidenced by individuals with PNH that are likely a direct result of hemolysis include dysphagia, abdominal pain, erectile dysfunction, pulmonary hypertension and renal failure[49,53,54]. Thromboembolism, the most feared complication in PNH, is also directly associated with hemolysis and may be induced by the release of free hemoglobin, consumption of nitric oxide, and subsequent clotting[53,55–63].

Thrombosis is clinically evident in ~40% of individuals with PNH and is the leading cause of premature mortality[64–68]. Although venous thrombosis is more typical in PNH, arterial thrombosis, particularly in the cerebral circulation, is not uncommon[56,64]. Thrombosis is evident in individuals with small and large PNH clone sizes and varying degrees of hemolysis, although it appears to be more common in those with larger clones[56,57,66,68]. Individuals with hemolysis but without a history of transfusion also experience thrombosis, including fatal first thrombosis[57]. Subclinical thrombosis has been reported in 60% of a cohort of PNH patients, despite use of anticoagulation, and unrelated to transfusion requirements[69].

Complement inhibitors in PNH. As PNH is defined by the acquired genetic deficiency of the endogenous terminal complement inhibitor CD59 on the surface of blood cells, restoration of terminal complement inhibition should effectively reduce hemolysis in PNH and abrogate the serious clinical morbidities associated with this life-threatening disease. Initial attempts to circumvent the functional defect in PNH cells focused on replacing CD59 on the surface of PNH cells through gene therapy. A recombinant, transmembrane form of CD59 (CD59-TM) was generated and analyzed for the ability to regulate complement activity[70]. A GPI-anchoring deficient complement-sensitive B-cell line derived from an individual with PNH was virally transduced with CD59-TM, resulting in protection against classical complement-mediated membrane damage. These data established that a functional transmembrane form of CD59 can be expressed on the surface of PNH cells

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through a gene therapy approach. Retroviral transduction of PNH erythroid cells with a functional *PIGA* gene has also been shown to restore GPI-anchor protein expression on the surface of these cells, thereby protecting them from complement-mediated destruction[71]. However, to provide a renewable source of complement-protected erythrocytes in individuals with PNH through a gene therapy approach, efficient targeting of early erythroid progenitor cells would be required, which has not been achieved to date. This led to the development of the complement inhibitor eculizumab, which binds to and blocks the cleavage of C5 in plasma, thereby reestablishing terminal complement regulation in the plasma of individuals with PNH. The potential effect of terminal complement blockade on hemolysis in PNH was observed in a person with coexistent C9 deficiency and PNH who had no signs of hemolysis[72]. PNH was diagnosed in this individual only after the administration of a whole blood transfusion during a routine surgery, which served to reconstitute C9 and trigger hemolysis.

Eculizumab in PNH: clinical trials

Eculizumab was evaluated in PNH in three separate parent studies—a phase 2 pilot study and two phase 3 studies. All participants in these trials were allowed to continue eculizumab in follow-on extension studies.

Pilot study. The first eculizumab PNH study (beginning in May 2002) evaluated terminal complement inhibition in a 3-month open-label phase 2 pilot study in 11 participants with hemolytic PNH at two sites in the United Kingdom[73]. In this study, participants with at least four transfusions in the previous 12 months received 600-mg infusions of eculizumab every week for 4 weeks, followed 1 week later by a 900-mg dose and then by 900 mg every other week through week 12. In this study and all subsequent trials, participants were vaccinated against *N. meningitidis* at least 2 weeks before the first dose of eculizumab. Clinical and biochemical indicators of hemolysis were measured throughout the trial. Mean lactate dehydrogenase (LDH) levels decreased from 3111 U per liter before treatment to 594 U per liter during treatment ($P = 0.002$; **Table 1**). The mean percentage of PNH erythrocytes increased from 36.7% of the total erythrocyte population to 59.2% ($P = 0.005$). The mean and median transfusion rates decreased from 2.1 and 1.8 units per individual per month to 0.6 and 0.0 units per individual per month, respectively ($P = 0.003$, for the comparison of the median rates). Episodes of hemoglobinuria were reduced by 96% ($P < 0.001$), and measurements of the quality of life substantially improved. From this study, it appeared that eculizumab was safe and well-tolerated in individuals with PNH. This terminal complement inhibitor reduced hemolysis, hemoglobinuria, and the need for transfusion, with an associated improvement in quality of life.

After completion of the initial 12-week study, all 11 participants from the initial PNH study chose to continue treatment in a 52-week extension study; all participants completed the study[25]. The dramatic reductions in hemolysis and the need for transfusion that were observed in the pilot study were maintained throughout the extension study, and significant improvements in quality-of-life measures also continued. In this study, long-term eculizumab administration continued to be safe and well-tolerated. Clinical breakthrough occurred in two individuals with insufficient eculizumab levels; shortening the dosing interval (increasing the total dose) resulted in rapid restoration of complement suppression, abrogation of hemolysis, and resolution of disease signs and symptoms. These data demonstrate the close relationship between sustained terminal complement inhibition, hemolysis, and symptoms in individuals with PNH. Further, dramatic clinical improvements in signs and symptoms attributed specifically to hemolysis including hemoglobinuria, and the smooth muscle dystonias—abdominal pain, dysphagia and erectile dysfunction—were observed in these participants[54]. Ten of the original 11 participants have remained on eculizumab for more than 5 years of continuous treatment.

Phase 3 pivotal program. The results from the initial open-label phase 2 pilot study were sufficiently compelling that discussions were initiated with the FDA and the EMEA in order to plan phase 3 studies for registration in the United States and Europe, respectively. In 2003,

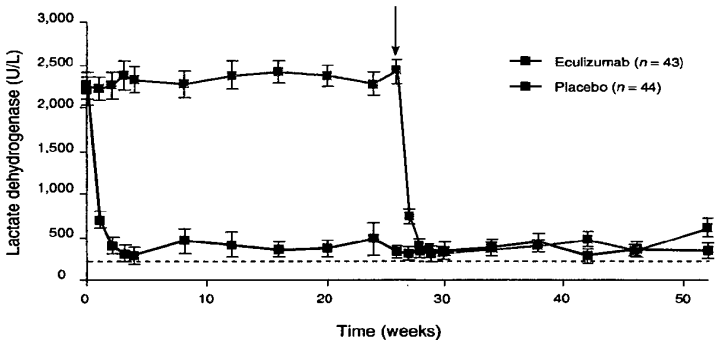


Figure 4 Reduction in hemolysis during treatment with eculizumab. Mean levels of lactate dehydrogenase reflect the degree of hemolysis from baseline to week 52. The dashed line indicates the upper limit of the normal range for lactate dehydrogenase (normal range, 103 to 223 U per liter). In eculizumab-treated patients, the mean level of lactate dehydrogenase was rapidly reduced to just above the upper limit of the normal range. In the placebo group, the mean level of lactate dehydrogenase remained highly elevated. The arrow depicts the transition of placebo-treated patients in TRIUMPH to eculizumab treatment in the phase 3 extension study at which time levels of lactate dehydrogenase rapidly reduced to near normal values.

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Table 2 Primary and secondary endpoints of the phase 3 pivotal efficacy study TRIUMPH

	Treatment period		
	Placebo	Eculizumab	*P* value
Stabilization of hemoglobin levels (percent of patients)[a]	0	49	<0.000001
Units transfused/patient[a]			
Median	10	0	<0.000001
Mean ± s.e.m.	11.0 ± 0.83	3.0 ± 0.67	
Transfusion Avoidance (percent of patients)	0	51	<0.000001
FACIT-Fatigue score (change from baseline)	−4.0 ± 1.7	6.4 ± 1.2	0.000006
LDH (area under the curve, U/L × day)	411,822	58,587	<0.000001

[a]Co-primary endpoints.

eculizumab received orphan designation for PNH in both the United States and Europe. The phase 3 eculizumab in PNH program consisted of: TRIUMPH, the pivotal trial that examined eculizumab safety and efficacy in a hemolytic PNH population with at least 4 transfusions in 12 months; SHEPHERD, which examined safety and efficacy of eculizumab in a broader and more heterogeneous hemolytic PNH population in individuals with a history of transfusion; and a phase 3b extension study to evaluate long-term safety and efficacy, including the potential anti-thrombotic effect of eculizumab.

TRIUMPH was a double-blind, randomized, placebo-controlled, multicenter, phase 3 trial[74]. Individuals were first enrolled and treated in 2004 and received either placebo or eculizumab intravenously; eculizumab was given at a dose of 600 mg weekly for 4 weeks, followed 1 week later by a 900-mg dose and then 900 mg every 14 ± 2 days through week 26. Eighty-seven participants (43 eculizumab, 44 placebo) at 34 international sites were enrolled. The two primary endpoints were the stabilization of hemoglobin levels and the number of units of packed red cells transfused. Biochemical indicators of hemolysis and the participant's fatigue and quality of life were also assessed. After enrollment, participants were observed for up to 13 weeks. Those who did not require a transfusion during the observation period were considered ineligible for randomization. Eighty-seven individuals underwent randomization. Every participant treated with eculizumab experienced an objective response. Across all treated participants, a significant reduction in hemolysis (as measured by LDH) was observed after 1 week of treatment and maintained throughout the 26 week study in the eculizumab group but not in placebo (**Fig. 4**). There was an 85.8% lower median area under the curve for LDH plotted against time (in days) in the eculizumab group, as compared with the placebo group (58,587 versus 411,822 U per liter, respectively; $P < 1 \times 10^{-6}$; **Table 2**). After completion of the study, a similar reduction in hemolysis was demonstrated with placebo participants transitioning to eculizumab. Stabilization of hemoglobin levels in the absence of transfusions was achieved in 49% (21 of 43) of the individuals assigned to eculizumab and none (0 of 44) of those assigned to placebo ($P < 1 \times 10^{-6}$). During the study, a median of 0 units of packed red cells was administered in the eculizumab group, as compared with 10 units in the placebo group ($P < 1 \times 10^{-6}$). A total of 51% of the eculizumab group remained transfusion independent during the entire 26-week study, whereas no one in the placebo group achieved this status. Even among participants receiving eculizumab in whom transfusion independence was not achieved, transfusion requirements were reduced by 44% as these individuals also demonstrated a clinically and statistically significant reduction in hemolysis. The reduced but continued requirement for transfusion support in some individuals may reflect more severe bone marrow aplasia and/or residual low-level hemolysis through the extravascular compartment[74]. Clinically and statistically significant improvements were also found in fatigue, as measured by scores on the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) instrument ($P = 6 \times 10^{-6}$) and the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire (EORTC QLQ-C30; $P < 1 \times 10^{-6}$). Participants that did not achieve complete transfusion independence during eculizumab treatment also experienced significant improvement in fatigue and other measures of quality of life. Of the 87 participants, 4 in the eculizumab group and 9 in the placebo group had serious adverse events, none of which were considered to be treatment-related; all these patients recovered without sequelae. The most common adverse events reported in the eculizumab group were headache, nasopharyngitis, back pain and nausea. Headache and back pain occurred more frequently in the eculizumab group than in the placebo group. Anti-eculizumab antibody responses were of low titer, transient, and occurred at a frequency similar to that of placebo. The TRIUMPH study demonstrated that eculizumab is a well-tolerated and effective therapy for PNH.

After initiation of the TRIUMPH study, eculizumab was evaluated in SHEPHERD, an open-label, non-placebo controlled, phase 3 clinical safety and efficacy study in a more diverse PNH population including patients with marked thrombocytopenia and minimal transfusion requirements. Participants were first enrolled and treated in 2005, and eculizumab was administered for a total of 52 weeks using a dosing schedule identical to that of TRIUMPH. Ninety-seven individuals at 33 international sites were enrolled. Adverse events were similar to those with placebo in the TRIUMPH study. Hemolysis was significantly reduced leading to improved anemia with a reduction in or elimination of transfusion requirement, lessened fatigue and improved overall health-related quality of life[75]. Compared to the overall population, efficacy with eculizumab was similar in individuals who would be expected to have less severe disease, including patient subgroups with lower baseline hemolysis, mild anemia, and minimal or no pretreatment transfusion requirement[76].

Of the 195 individuals entering the pilot, TRIUMPH or SHEPHERD studies, 187 participants completed these studies and elected to receive eculizumab in a common 104-week phase 3b open-label extension study. Overall, there was no evidence of an increased incidence of infection across PNH studies with eculizumab as compared to placebo, including serious infections, severe infections or multiple infections. Two individuals vaccinated against *N. meningitidis* experienced meningococcal sepsis; they were treated and recovered without clinical sequelae. Serious hemolysis was not observed in any of the initial 16 participants who discontinued treatment. Long-term eculizumab treatment was associated with a reduction in hemolysis up to more than 54 months of continuous treatment. Importantly, the design of the phase 3b extension study specified that the thrombosis rate for each participant would be compared before and during eculizumab treatment. Early evaluation shows that the 7.37 thrombotic events per 100 patient-years experienced before eculizumab was reduced to 1.07 events per 100 patient-years with eculizumab treatment ($P < 1 \times 10^{-6}$)[77].

Approval of eculizumab. After completion of TRIUMPH and after the pre-specified interim 26-week analysis in SHEPHERD, applications for marketing authorization of eculizumab in the treatment of PNH were submitted to the FDA and EMEA in September 2006. The FDA

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accepted the application for priority review and in March 2007, less than 6 months after the completion of SHEPHERD, FDA approved eculizumab for the treatment of patients with PNH to reduce hemolysis. The EMEA granted review of the eculizumab application under the Accelerated Assessment procedure, the fastest evaluation time frame for full approval awarded by EMEA. In June 2007, eculizumab became the first medicinal product to receive approval by the European Commission within the Accelerated Assessment procedure and it is indicated for the treatment of patients with PNH.

Conclusions and perspectives

Clinical development of eculizumab spanned a full decade before its first approval in the rare, disabling and life-threatening, clonal blood disorder PNH (**Fig. 5**). The studies before initiation of the PNH clinical program provided an important assessment of the safety profile and immunomodulatory effects of eculizumab. Once initiated, the PNH program has been one of the most extensive pre-approval clinical programs for an 'ultra-orphan' disease; the design of the multinational trials enrolling 195 participants allowed sequential, controlled and long-term evaluation of both biochemical and clinically validated measurements in the PNH population. This paradigm of careful evaluation may be used in the consideration of eculizumab in other uncommon, debilitating and life-threatening diseases for which there is evidence suggesting that terminal complement may significantly contribute to the pathophysiology of the disease, including antibody-mediated transplant rejection[78], myasthenia gravis and other peripheral neuropathies[79–83], fulminant lupus[27,29], catastrophic anti-phospholipid syndrome[84,85] and pattern 2 multiple sclerosis[86]. The understanding of critical pharmacokinetic and pharmacodynamic properties in PNH patients and the accomplishment of large-scale commercial manufacturing of eculizumab may also permit the evaluation of new formulations of eculizumab in future development programs for still other diseases with evidence suggesting complement-mediated pathology, including asthma[87] and age-related macular degeneration[88].

Clinical trials have demonstrated that eculizumab dramatically and reproducibly abrogates intravascular hemolysis, the primary clinical manifestation of PNH. These trials have shown that the reduction in hemolysis is independently associated with improvements in major morbidities in individuals with PNH including anemia, fatigue and health-related quality of life, as well as fewer thrombotic events. Because of the extremely rare nature of the disease and the ethical dilemma posed by the efficacy evident with eculizumab in the treatment of PNH, a prospective randomized controlled clinical trial is not a feasible means to examine the long-term impact of eculizumab on survival in individuals with PNH. However, given the evident anti-hemolytic effect and attendant clinical benefit of eculizumab administration, it is possible that eculizumab could beneficially impact several of the leading causes of premature mortality in PNH including venous and arterial thrombosis, hemorrhage that may be worsened by platelet consumption, renal failure and liver failure. In a *post hoc* analysis of the survival of eculizumab-treated PNH patients compared to historical controls[64], ~15 deaths would have been expected during the 2-year period of eculizumab treatment; however, two deaths were observed during this period, or 87% fewer than expected ($P = 0.0005$). This preliminary analysis suggests that long-term eculizumab treatment may beneficially affect survival in individuals diagnosed with PNH.

The humanization strategy used to construct eculizumab has resulted in only very rare evidence of responses to eculizumab, with an incidence similar to that found with placebo treatment. There is no evidence of tolerance or a neutralizing antibody response in any treated individual to date and infusion-related immune effects are similar in frequency to placebo. It is interesting to speculate that the selection of purely germline framework acceptor regions, seen as native by all recipients, has allowed for this important safety benefit.

Rigorous evaluation of eculizumab in PNH has helped tease out the role of hemolysis in mediating critical patient-reported outcomes, including fatigue and patient functioning. The reproducible demonstration that patient improvement with eculizumab, due to the abrogation

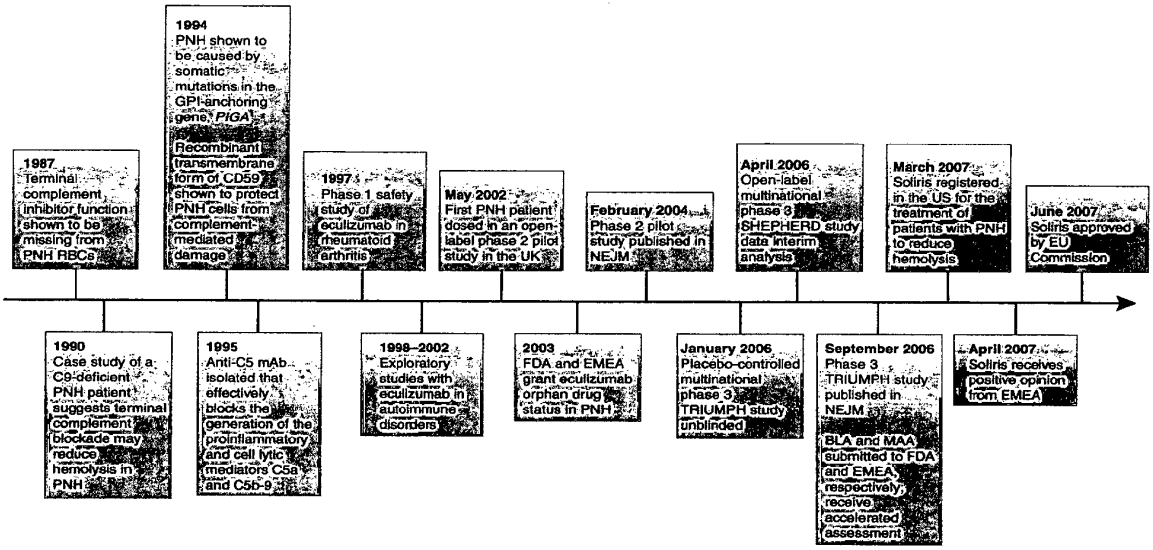


Figure 5 Eculizumab in PNH development timeline. BLA, biologic license application; MAA, marketing authorization application.

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of the underlying hemolysis, is distinct from associated improvements in anemia may have applications to understanding the potential multifactorial etiology of fatigue in individuals with anemia in other disease settings, such as cancer. Similarly, evident improvement in smooth muscle dystonias and thrombosis with eculizumab treatment in PNH has helped to establish new and important clinical paradigms, particularly with regard to the role of cell-free plasma hemoglobin and nitric oxide scavenging in thrombosis, smooth muscle dystonia and vasomotor abnormalities[53]. In this case, focus on a rare disease may not only lead to benefit for afflicted individuals but may also contribute to the overall foundation of knowledge. Such knowledge may provide insight into more common diseases with disordered nitric oxide regulation, including systemic and pulmonary hypertension, diabetes, sickle cell disease, and other genetic or acquired hemolytic anemias.

In summary, data from six clinical studies, enrolling a total of 195 participants, demonstrate the robust efficacy of eculizumab for the treatment of individuals with PNH. Every individual treated with eculizumab to date has shown an objective improvement in hemolysis. Treatment with eculizumab modifies the clinical course of PNH and markedly reduces the serious morbidities associated with the disease. In particular, therapy with eculizumab reduces anemia, blood transfusion requirements, and the risk for life-threatening thrombosis. Eculizumab also improves other morbidities that are often serious, including fatigue, pain, dyspnea and impaired overall quality of life. Treated individuals will continue to be monitored in a global registry. Overall, the therapeutic benefit and safety of eculizumab in the treatment of patients with the rare blood disease PNH validates the utility of terminal complement inhibitor therapeutics for the treatment of medical conditions where complement activation is involved.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/.

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Erratum: Funding crunch forces stem cell company to abandon therapies

Monya Baker
Nat. Biotechnol. 25, 951–952, 2007; published online 3 September 2007

In the version of this article originally published, in the penultimate paragraph, the name of the Monash University scientist was misspelled; the correct spelling is Colin Pouton. In addition, the statement in paragraph 2 "the SSCC, which is being supported by the same group that funded ESI, the Agency for Science, Technology and Research (A*STAR)" is incorrect. ESI is not funded by A*STAR. Its major investor is Bio-One Capital, a venture capital fund controlled through the Singapore Government's Economic Development Board. The SSCC is funded by A*STAR.

Erratum: Mice with a human touch

Christopher Thomas Scott
Nat. Biotechnol. 25, 1075–1077, 2007; published online 6 October 2007

In the version of this article initially published, it was incorrectly stated on p. 1076, paragraph 2, line four from bottom, that "Aya Jakobovits, originally at Cell Genesys and later at Abgenix, shepherded the XenoMouse through its entire development." Five of the six XenoMouse strains developed at Abgenix were initiated after Jakobovits left in 1997.

Erratum: Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria

Russell P Rother, Scott A Rollins, Christopher F Mojcik, Robert A Brodsky & Leonard Bell
Nat. Biotechnol. 25, 1256–1264 (2007); published online 7 November 2007; corrected after print 7 December 2007

In the version of this article initially published, on p. 1258, paragraph 2, the incorrect serum concentrations to reach steady state and the incorrect accumulation ratio (R_{acc}) for eculizumab were given. The corrected sentence reads "Eculizumab serum concentrations appear to reach steady state after ~57 days. At steady state, the eculizumab accumulation ratio (R_{acc}) was calculated to be 1.075." In addition, in Figure 5, in the PDF version only, the word "assessment" was cut off the bottom of the box beginning "September 2006." The final sentence should read "BLA and MAA submitted to FDA and EMEA, respectively; receive accelerated assessment." And in Table 1, the heading "Units of packed RBCs transfused/patient/month" now appears in a separate row. The errors have been corrected in the HTML and PDF versions of the article.

Corrigendum: A diverse family of thermostable cytochrome P450s created by recombination of stabilizing fragments

Yougen Li, D Allan Drummond, Andrew M Sawayama, Christopher D Snow, Jesse D Bloom & Frances H Arnold
Nat. Biotechnol. 25, 1051–1056 (2007); published online 26 August 2007; corrected after print 7 December 2007

In the version of this article initially published, the sentence starting with "The most thermostable 450 P450 chimera" contains an extra "450." The correct sentence should start as follows "The most thermostable P450 chimera". The error has been corrected in the HTML and PDF versions of the article.

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Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

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Blood concentrations of alemtuzumab and antiglobulin responses in patients with chronic lymphocytic leukemia following intravenous or subcutaneous routes of administration

Geoff Hale, Peppy Rebello, Lee R. Brettman, Chris Fegan, Ben Kennedy, Eva Kimby, Mike Leach, Jeanette Lundin, Håkan Mellstedt, Paul Moreton, Andy C. Rawstron, Herman Waldmann, Anders Osterborg and Peter Hillmen

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Blood concentrations of alemtuzumab and antiglobulin responses in patients with chronic lymphocytic leukemia following intravenous or subcutaneous routes of administration

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Alemtuzumab is a humanized anti-CD52 antibody licensed for refractory B-cell chronic lymphocytic leukemia (B-CLL), when given intravenously at 30 mg thrice weekly. However, the intravenous route is associated with infusion-related reactions and is inconvenient. We measured blood concentrations in 30 relapsed patients treated with intravenous alemtuzumab and in 20 patients from a previously untreated group who received similar doses subcutaneously. Highest trough samples in the intravenous group were less than 0.5 μg/mL to 18.3 μg/mL (mean 5.4 μg/mL). The cumulative dose required to reach 1.0 μg/mL was 13 mg to 316 mg (mean 90 mg). Higher blood concentrations correlated with the achievement of better clinical responses and minimal residual disease. The highest measured concentrations in the subcutaneous group were similar (0.6 μg/mL to 24.8 μg/mL, mean 5.4 μg/mL). However, the cumulative dose to reach 1.0 μg/mL was higher: 146 mg to 1106 mg (mean 551 mg). No antiglobulin responses were detected in 30 patients given intravenous alemtuzumab whereas 2 of 32 patients given subcutaneous alemtuzumab made substantial anti-idiotype responses. Thus, subcutaneous alemtuzumab achieved concentrations similar to those for intravenous alemtuzumab, although with slightly higher cumulative doses. Subcutaneous alemtuzumab is more convenient and better tolerated but may be associated with some patients forming anti–alemtuzumab antibodies, particularly those patients who were previously untreated. (Blood. 2004;104:948-955)

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Introduction

Alemtuzumab (CAMPATH-1H, Campath; ILEX Pharmaceuticals, San Antonio, TX) is a humanized immunoglobulin G1 (IgG1) antibody that recognizes the CD52 antigen, a lipid-anchored glycoprotein, on lymphocytes.[1-3] Alemtuzumab is exceptionally lympholytic and has been tested as an immunosuppressive agent in transplantation and autoimmune diseases. It is active against a range of lymphoid malignancies and in 2001 was approved for the treatment of B-cell chronic lymphocytic leukemia (B-CLL) in patients who have been treated with alkylating agents and who failed fludarabine therapy. This was based largely on a trial in 93 patients, most of whom had advanced disease with an extremely poor prognosis.[4] After a short dose escalation, alemtuzumab was given intravenously at 30 mg thrice weekly for 4 to 12 weeks. This gave an overall response rate (complete remission [CR] + partial remission [PR]) of 33%. Other trials in chemoresistant CLL reported comparable response rates of 42% and 29% respectively.[5,6]

The dose regimen was developed empirically without the benefit of detailed pharmacodynamic or pharmacokinetic studies. Alemtuzumab often causes substantial first-dose reactions, and although the reactions can now be minimized by appropriate premedication, they are the reason why an initially escalating dose was followed by frequent small doses. This regimen is not the most convenient for patients or physicians. Therefore, the subcutaneous route has been studied in an attempt to reduce side effects and make the treatment more manageable.[7,8]

The different routes of administration can be compared by analysis of the biodistribution of antibody, on the principle that treatments that give comparable blood concentrations should produce similar clinical effects. Some information is available about the pharmacokinetics of intravenous alemtuzumab in patients undergoing stem cell transplantation who have no substantial leukemic burden.[9,10] The terminal half-life was estimated to be approximately 15 to 20 days. However, in patients with advanced CLL, tumor cells could absorb antibody from the circulation.

The humanization of alemtuzumab reduced the risk of antiglobulin responses.[11] However, the antigen binding sites are still potentially immunogenic. The ability to provoke a response depends on the target antigen, the immune status of the patient, the use of immunosuppressive drugs, and other factors, perhaps including the route of administration. There is a suggestion that subcutaneous administration might be more immunogenic than

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intravenous administration in patients with rheumatoid arthritis,[12,13] though it is well known that such patients are particularly able to make anti–Ig antibodies.

Here we measured blood concentrations of alemtuzumab and antiglobulin responses against it during 2 different clinical trials. In the first, alemtuzumab was administered intravenously to patients who had previously failed alkylating agents and fludarabine as in the licensed indication. In the second, alemtuzumab was administered subcutaneously to patients who required therapy but had received no prior treatment.[8] We compared the blood concentrations and antiglobulin responses and investigated the possible relationship between alemtuzumab concentrations and clinical responses.

Patients, materials, and methods

Patients

Clinical studies were carried out with the approval of local and, in the United Kingdom, regional ethical committees. All patients gave written informed consent prior to enrolment.

Study design: intravenous study

An open-label phase 2 trial was carried out at 5 centers in the United Kingdom (Aberdeen, Birmingham, Bournemouth, Glasgow, Leeds). Primary objectives included the assessment of safety and efficacy as well as measurement of pharmacokinetics. Patients were eligible if they were at least 18 years old, had confirmed B-CLL requiring treatment, had failed previous treatment with purine analogs, had a World Health Organization (WHO) performance status of 2 or less, and had creatinine and bilirubin concentrations less than twice the upper limit of normal (unless due to disease). Exclusion criteria were active infection, HIV positivity, pregnancy or lactation, history of anaphylaxis to antibodies, less than 3 weeks since chemotherapy or less than 6 weeks since investigational therapy, central nervous system (CNS) involvement or persisting severe pancytopenia, other severe concurrent diseases, secondary malignancy, or mental disorders. Patients were premedicated with paracetamol and chlorpheniramine 30 minutes before the first infusion. Septra (sulfamethoxazole/trimethoprim 800 mg/160 mg twice a day, 3 times per week) and aciclovir (200 mg thrice daily) were given prior to therapy and continued until normal blood lymphocytes returned to the normal range. Allopurinol was given during the first 4 weeks of treatment. Alemtuzumab (M&I Partnership, Cambridge, MA) was diluted in 100 mL of 0.9% saline and administered intravenously over approximately 2 hours. A dose of 3 mg was given on day 1 and if it was well tolerated, a 10-mg dose was given on day 2 and a 30-mg dose on day 3. If severe reactions occurred, the same dose was repeated daily until it was well tolerated and then the dose was escalated. Subsequent 30-mg doses were given thrice weekly for up to 12 weeks, though treatment was occasionally extended if clinically indicated.

Before therapy, a full history, physical examination, and laboratory evaluation were performed, including assessment of lymph node, liver, and spleen size, computed tomographic (CT) scan, Rai staging, blood analysis, bone marrow aspirate, and trephine and immunophenotyping by flow cytometry. Disease parameters were reassessed and blood and bone marrow were reanalyzed by flow cytometry for residual CLL cells every 4 weeks. Therapy was stopped if there was no evidence of CLL or if sequential bone marrow samples showed no fall in the level of CLL. Blood samples were collected weekly to measure alemtuzumab concentrations and antiglobulins. After the last dose, samples were collected daily for 4 days and then weekly for 8 weeks. This was not always possible for logistic or clinical reasons.

During follow-up, patients were assessed monthly for 6 months. Responders were assessed every 6 months thereafter until disease progression. Disease response and toxicity were graded according to the criteria of the National Cancer Institute (NCI).

Study design: subcutaneous study

An open-label phase 2 trial was conducted at 4 clinics at the Karolinska Institute, Stockholm, Sweden.[8] The primary objective was to assess the efficacy of alemtuzumab administered subcutaneously. Patients were eligible if they had a diagnosis of B-CLL, were age 18 to 75, had a WHO performance status of 1 or less, life expectancy at least 12 weeks, required treatment, and had not been treated previously. Other details were comparable to the intravenous study except the treatment regimen. Alemtuzumab was obtained from the Therapeutic Antibody Centre, Oxford, United Kingdom (patients 1-29) or from M&I Partner (patients 30-41). The 2 preparations were similar although the formulations were slightly different and antibody from the Therapeutic Antibody Centre was stored frozen.[14] On day 1, 3 mg alemtuzumab was administered by subcutaneous injection in the thigh. If well tolerated, 10 mg was given on day 3 and 30 mg (in 2 injection sites) was given on day 5. In the event of erythema or edema, the same dose was repeated until well tolerated. After dose escalation, most patients self-administered the antibody. The 30-mg dose was given thrice weekly for up to 18 weeks. If treatment was interrupted for more than 7 days the dose was reinitiated at 3 mg or 10 mg. Therapy was stopped if patients achieved a complete remission (CR) or fulfilled NCI criteria for progressive disease (PD).

Measurement of alemtuzumab in patient serum

Serum samples were collected before doses and at various times afterward and stored at −70°C until analysis by immunofluorescence.[15] Test samples were incubated at 56°C for 30 minutes to inactivate complement then incubated with HUT-78 cells at room temperature for 30 minutes. The cells were washed and resuspended in fluorescein isothiocyanate (FITC)–labeled polyclonal anti–human IgG Fc domain (F-9512; Sigma, Poole, United Kingdom), incubated for 30 minutes, washed and fixed with formaldehyde prior to flow cytometry. Alemtuzumab concentrations were calculated from the median fluorescence by interpolation on a standard curve. The lower limit of quantitation was 0.5 μg/mL (allowing for 2-fold sample dilution), overall precision was plus or minus 13%, and overall accuracy was 109%. There was no interference by normal or patient control sera and no reactivity with $F(ab')_2$ fragments of alemtuzumab.[15]

Measurement of antiglobulin responses

Antiglobulin responses to alemtuzumab were measured in serum samples by sandwich enzyme-linked immunosorbent assay (ELISA) as previously described[11,16] with some modifications. Microtiter plates were coated with alemtuzumab (M&I Partner) and blocked with diluent (phosphate-buffered saline [PBS] containing 2% bovine serum albumin [BSA]). Standards, quality-control samples, and test samples were added, incubated for 1 hour at room temperature, and rinsed 4 times with wash buffer (PBS containing 0.05% Tween 20). Biotin-labeled alemtuzumab was added to each well, incubated for 1 hour, and rinsed as before. The assay was developed with extravidin-peroxidase (E-2886; Sigma) and ortho-phenylenediamine. Color development was measured at 450 nm for 20 minutes by Genesis II software (Thermo Life Sciences, Basingstoke, United Kingdom). The standard was YID13, a monoclonal anti-idiotype specific for alemtuzumab.[16] One U/mL of antiglobulin activity is the signal equivalent to 1 ng/mL of YID13 reference standard. The limit of detection (LOD) and lower limit of quantitation (LLOQ) was 488 U/mL serum (allowing for 2-fold sample dilution), overall precision was plus or minus 14%, and overall accuracy was 94%. There were no false positives and no interference by normal or patient control sera. Positive signals were obtained with serum containing rheumatoid factor or spiked with native CD52 antigen. The assay was reproducible despite variations in reagent concentrations, incubation and washing conditions, blood clotting conditions, and sample storage conditions within the specified ranges. Up to 3-fold enhancement of antiglobulin activity was seen in samples that had been heat-treated or stored for over a month at 4°C. This might be due to aggregation, since aggregates of the control anti-idiotype reagent also gave enhanced activity. Alemtuzumab and anti-alemtuzumab interact with each other to reduce the signal in either assay if both were present in the same sample. Thus, the

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antiglobulin assay measures only the concentration of free anti–alemtuzumab antibody, that is, the excess over the levels of alemtuzumab itself. Considering that alemtuzumab concentration was never more than 26 μg/mL, then it might interfere with measurement of antiglobulin responses less than about 26 000 U/mL. However, the antiglobulin levels in a patient who made a clear-cut response exceeded 10^6 U/mL. We report data for all samples measured, both during and, where available, after treatment. For complete assessment of antiglobulin responses, it is always preferable to analyze samples over an appropriate period of time after the end of treatment.

Measurement of minimal residual disease by flow cytometry

Residual CLL cells were quantified by sensitive 4-color flow cytometry; a method more sensitive than consensus-primer polymerase chain reaction (PCR) and more practicable than sequence-specific quantitative PCR.[17] At least 5×10^6 bone marrow leukocytes were incubated with a mixture of fluorescent-labeled antibodies, washed twice, and analyzed using a FACSort (Becton Dickinson, Oxford, United Kingdom) with CELLQuest v3.1 software. The antibodies used were CD45/CD48 (Chemicon, Chandlers Ford, United Kingdom)/CD19/CD3, kappa/lambda/CD19/CD5, CD20 (Beckman Coulter, High Wycombe, United Kingdom)/CD79b (Beckman Coulter)/CD19/CD5, and CD20 (Beckman Coulter)/CD38 (BD Biosciences, Oxford, United Kingdom)/CD19/CD5 conjugated to FITC/phycoerythrin (PE)/PE-Cy5/allophycocyanin (APC), respectively. Reagents without named manufacturer were produced in-house. Between 50 000 and 500 000 cells were analyzed. B cells were identified by setting a region on CD19 versus side scatter (SSC), followed by a forward scatter region, and then ensuring that no $CD3^+$ events fell within the combined gate. CLL cells were discriminated from mature B cells by their stronger expression of CD5 and weaker expression of CD20 and CD79b, and from B progenitors by their weaker expression of CD38. This assay can detect 1 CLL cell in approximately 10^4 to 10^5 leukocytes.

Results

Collection and analysis of samples

Serum samples were obtained from all 30 patients in the intravenous study and 20 of the 41 patients in the subcutaneous study, almost exclusively from a single clinic (Karolinska Hospital). Samples were tested in duplicate and the mean concentration was calculated. If the mean was less than 0.5 μg/mL (LLOQ), a level of less than 0.5 μg/mL was recorded. If either result was more than 20 μg/mL or a measurement was technically faulty, then the test was repeated, using a higher dilution if appropriate, and the repeat results were used.

Optimal time for collection of peak samples following intravenous administration

A preliminary experiment was carried out to identify the time following intravenous administration when the peak antibody concentration might be found. Samples were collected from 3 patients at 15 minutes, 30 minutes, and 60 minutes after the end of one dose each week for 4 to 7 weeks. The peak sample occurred at 15 minutes, significantly more often (21 times) than at 30 minutes (6 times) or 60 minutes (2 times) ($P < .01$; paired Wilcoxon signed rank test). Therefore, for subsequent patients a single sample was collected at 15 minutes to determine the peak concentration.

Serum concentrations of alemtuzumab during intravenous treatment

A total of 1561 patient samples were tested. Terminal samples (after the last dose) were not available for some patients, and in a few cases only a small number of terminal samples were available.

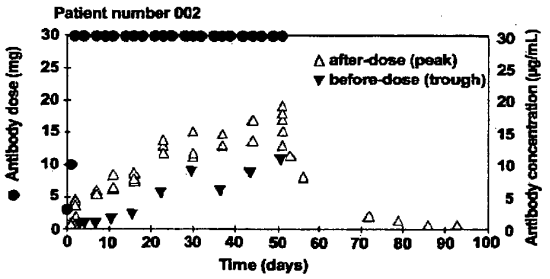


Figure 1. Example of alemtuzumab concentrations in patient treated with intravenous antibody. After an initial dose escalation, 30 mg of alemtuzumab was administered 3 times a week for 8 weeks (●). Serum samples were taken before (Δ) and after (▼) the dose once a week. This patient made a good clinical response to the therapy.

All samples were tested in duplicate; the overall mean coefficient of variation (CV) was 10%. Many of the early samples and many trough samples were below the limit of quantitation (< 0.5 μg/mL). Five samples gave anomalous results, substantially diverging from other results for the same patients. These could be explained by accidental mix-ups at the clinical centers and were omitted from analysis. An example of the doses and blood concentrations throughout the course of treatment for one patient is shown in Figure 1.

In contrast to patients who had received transplants,[9,10] there were wide variations in alemtuzumab concentrations between different patients with CLL. The highest peak ranged from 2.8 μg/mL to 26.4 μg/mL (mean 10.7 μg/mL). The highest trough ranged from less than 0.5 μg/mL to 18.3 μg/mL (mean 5.4 μg/mL). The cumulative dose before the trough concentration reached 1.0 μg/mL ranged from 13 mg to 316 mg (mean 90 mg; Table 1). In 9 of 21 evaluable patients, the highest trough concentration was measured just before the last dose, indicating that a steady state had not been reached. Total cumulative doses varied between patients, but did not correlate with the final antibody concentrations (Table 1).

Comparison of the starting lymphocyte count with the peak concentration following the first 30-mg infusion showed a modest negative correlation, that is, the higher the initial lymphocyte count, the lower tended to be the peak concentration of alemtuzumab (Figure 2).

In Figure 3 the highest trough concentration for each patient is plotted against the response measured by NCI criteria for 27 patients where the clinical response was reported within 4 months of the end of treatment. There was a significant increase in trough

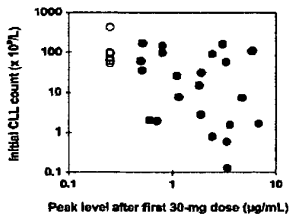


Figure 2. Relationship between initial CLL count and alemtuzumab blood concentration: intravenous study. The numbers of CLL cells in the blood directly before intravenous therapy are plotted against the peak concentration of alemtuzumab measured directly after the first 30-mg dose (●). In 8 patients the concentration was below the limit of quantitation, and these have all been plotted at an arbitrary level of 0.25 μg/mL (○). Pearson correlation coefficient for log (initial CLL count) versus log (alemtuzumab concentration) was −0.44, $P = .01$.

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Table 1. Alemtuzumab dose and serum concentrations: intravenous study

Patient no.	Cumulative dose, mg	Number of samples	Terminal samples	Dose to reach 1 μg/mL	Maximum peak, μg/mL	Maximum trough, μg/mL	Before last dose, μg/mL	After last dose, μg/mL
001	1020	80	12	140	16.3	8.3	7.7	11.7
002	873	56	12	13	19.1	13.0	13.0	19.1
003	1160	91	23	43	8.7	1.1	6.9	5.9
004	638	54	0	103	25.4	18.3	ND	ND
005	1064	69	7	46	16.3	6.2	6.2	16.3
006	736	55	8	16	9.5	3.7	2.8	8.5
007	1061	75	11	133	19.8	11.9	11.9	16.4
008	291	19	3	111	5.8	0.7	0.7	0.7
009	1008	73	12	83	13.1	7.7	7.7	12.2
010	178	21	0	46	4.0	0.8	ND	ND
011	1070	71	11	123	8.3	1.9	1.9	8.3
012	473	28	0	53	4.4	0.2	ND	ND
013	940	60	13	193	4.0	0.5	< 0.5	2.9
014	435	31	0	46	12.5	9.9	ND	ND
015	2063	89	0	43	6.4	2.8	ND	ND
016	1016	58	11	103	9.5	4.9	4.5	9.0
017	1065	55	8	133	6.6	1.7	1.2	6.0
018	733	40	1	43	8.2	2.6	ND	ND
019	465	27	1	73	19.9	14.8	ND	ND
020	1060	56	12	43	17.7	9.4	7.7	12.9
021	560	32	0	163	4.0	1.1	ND	ND
022	1000	46	6	43	7.1	1.0	0.8	6.3
023	1060	47	6	43	26.0	17.0	ND	24.1
024	1126	67	12	316	8.0	2.8	2.8	5.8
025	1060	64	11	73	8.9	5.2	3.1	6.7
026	868	57	13	133	9.4	1.6	1.4	4.7
027	1066	45	10	73	2.8	0.9	0.5	2.5
028	673	42	13	43	8.5	4.0	4.0	6.9
029	416	40	10	133	7.6	3.3	3.3	7.6
030	763	35	5	133	12.2	3.7	3.7	12.2
Minimum	179	19	0	13	2.8	< 0.5	< 0.5	0.7
Mean	873	52	8	90.4	10.7	5.4	4.0	9.4
Maximum	2063	91	23	316	26.4	18.3	13.0	24.1

ND indicates not determined.

concentrations with increasing clinical response ($P = .006$, Kruskal-Wallis test). There was an even more striking correlation between trough concentrations and the attainment of minimal residual disease (MRD) negativity in 7 patients (filled symbols) compared with the 17 patients who still had residual CLL cells in the bone marrow at the end of treatment as measured by 4-color flow cytometry ($P < .0001$, Kruskal-Wallis test)

In Figure 4 the mean trough concentration is plotted throughout the treatment for 2 groups of patients: (1) 8 responders who reached MRD less than 0.4%, and (2) 22 others. The mean trough concentration was significantly higher in the responders throughout the whole course of treatment from week 2 to week 11 ($P < .02$ at each time point, Kruskal-Wallis test).

Clearance of alemtuzumab following the last dose showed a similar correlation with residual disease. In patients with undetectable CLL cells, there was a single clearance phase characterized by

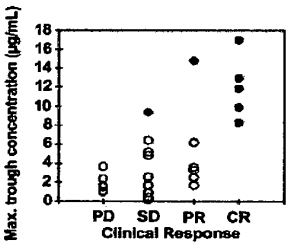


Figure 3. Relationship between maximum trough concentrations of alemtuzumab and clinical response: intravenous study. The highest trough concentration of alemtuzumab (which generally occurred following the penultimate or last dose) is plotted against the clinical response at the end of intravenous treatment as determined by NCI criteria. PD indicates progressive disease; SD, stable disease; PR, partial remission; CR, complete remission. A better clinical outcome was significantly associated with higher alemtuzumab concentrations ($P = .006$, Kruskal-Wallis test). There was an even stronger correlation between high trough concentrations (> 8 μg/mL) and good responses measured by less than 0.1% CLL cells in the bone marrow (●; $P < .0001$). Three patients who died before the end of the planned treatment course are not included in this figure. Of those patients, 2 had low alemtuzumab concentrations (≤ 1.1 μg/mL) and progressive disease at the time of death. The third had a high antibody concentration (18.3 μg/mL) and low levels of residual disease (0.21%). Each symbol represents a single patient; ● indicates a patient with no detectable MRD; ○, a patient with detectable MRD.

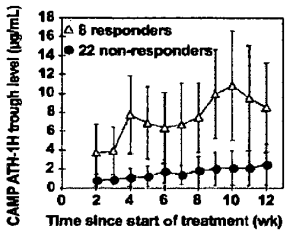


Figure 4. Mean trough concentrations of alemtuzumab during intravenous therapy. The mean trough concentrations during treatment (measured 48 hours after a dose, once a week) are plotted, with standard deviations, for 8 patients who ultimately reached less than 0.4% CLL cells in the bone marrow (△) and compared with 22 patients who still had residual CLL cells at the end of treatment (●).

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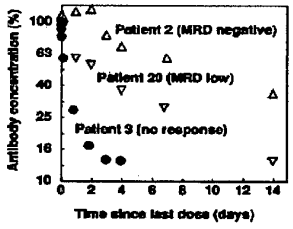


Figure 5. Clearance of alemtuzumab in selected patients treated intravenously. In patients with minimal residual disease (by flow cytometry) at the end of therapy, clearance was slow and single phase (△). Patients who still had a small amount of CLL cells showed biphasic clearance (▽) and patients who still had bulky disease cleared the antibody rapidly (●). However, in each case the terminal half-life was roughly similar.

a relatively long half-life. In patients who still had a substantial tumor burden, most of the antibody was rapidly cleared from the blood. Patients with low levels of residual disease showed an intermediate pattern. An example of each is shown in Figure 5.

Sixteen patients contributed informative terminal samples after the last 30-mg dose. From these data the following mean pharmacokinetic parameters were calculated (ILEX Oncology, unpublished data, September 2001). The apparent steady-state volume of distribution (V_{SS}) was 0.185 L/kg and the apparent volume of distribution during the terminal phase (VZ) was 0.252 L/kg. These are larger than for other monoclonal antibodies in humans but are consistent with the notion that alemtuzumab distributes through the plasma compartment and a substantial extravascular lymphocyte compartment. The mean terminal phase half-life ($t_{1/2,\ z}$) was 6.1 days, which is consistent with previous measurements for alemtuzumab and other chimeric antibodies in humans.

Serum concentrations of alemtuzumab during subcutaneous treatment

This study was started in 1998 using alemtuzumab manufactured at the Therapeutic Antibody Centre. During June 2000, commercially produced material became available and was subsequently used. No obvious differences were observed in the clinical responses. A total of 542 patient samples were received and tested. Two were mislabeled and are not included here. The overall mean CV was 8%. Assessment of pharmacokinetics in 7 patients was inadequate due to the limited number or inappropriate timing of samples.

Patient no. 8036 (who started with alemtuzumab from the Therapeutic Antibody Centre and was switched to commercial alemtuzumab) never achieved concentrations above the LLOQ. This patient had rapidly made a strong antiglobulin response to the alemtuzumab, which probably neutralized all of the administered antibody ("Antiglobulins following subcutaneous treatment"). The patient never showed any clearance of lymphocytes but showed continued reactions at the injection site. Because of this, the patient was switched to intravenous administration after 6 weeks.

There were some changes from the dose regimen due to clinical reactions. There were 2 patients who received lower doses throughout (10 mg and 20 mg) and 2 (including patient no. 8036, above) who were switched to intravenous dosing. The dose escalation rate was often slower than originally planned, typically taking 1 to 2 weeks to reach 30 mg. After this, the planned dose was usually maintained but only 1 of the 21 patients analyzed received the maximum planned dose of 1573 mg. The mean total dose was 1249 mg and the lowest dose was 712 mg.

The highest concentrations ranged from 0.6 μg/mL to 24.8 μg/mL and the mean (5.4 μg/mL) was the same as in the intravenous study (Table 2). However, the cumulative dose before the concentration reached 1.0 μg/mL ranged from 146 mg to 1106 mg (mean 551 mg), which was substantially more than in the intravenous study. An example of the time course in one patient illustrating this delay is shown in Figure 6.

Inadequate samples were available for analysis of terminal phase pharmacokinetics. No correlation could be demonstrated between alemtuzumab concentrations and clinical responses because nearly all of the patients (18/20) were responders (CR or PR), and levels of MRD were not reported.

Antiglobulins following intravenous treatment

A total of 519 serum samples from 30 patients treated with intravenous alemtuzumab were tested for antiglobulins. These included all samples taken more than 24 hours after the last dose and representative pre-dose samples during treatment. All of them were below the limit of detection (488 U/mL). In 12 patients there were no or very few samples available after the end of treatment, and in the other 18 patients samples were only available between 2 and 6 weeks after the last dose. Therefore, we cannot completely exclude the possibility of delayed responses.

Antiglobulins following subcutaneous treatment

A total of 281 samples were tested from 21 patients (one patient had adequate late samples for antiglobulin measurement, but none suitable for pharmacokinetic analysis). These included all samples taken after the last dose and representative samples taken during treatment. There were 4 patients who had no or few samples after the end of treatment but 17 who had adequate posttreatment samples. All samples except those described below were below the LOD.

One patient (no. 8027) had a single sample taken at day 238 (114 days after the last dose) that gave a weak signal just above the LOD. All previous and subsequent samples were less than the LOD. The patient made a good clinical response to therapy, achieving a CR, and no relevant adverse effects were noted. It is possible that the patient made a very weak, transient response to the alemtuzumab, but we doubt that it could have been clinically significant.

One patient (no. 8036) made a very high-titer response, reaching a peak of approximately 3.7×10^6 U/mL 24 days after the last dose of alemtuzumab (Figure 7). This antiglobulin bound strongly to CAMPATH-1G, the parental rat antibody from which alemtuzumab was humanized, but did not bind other human IgG1

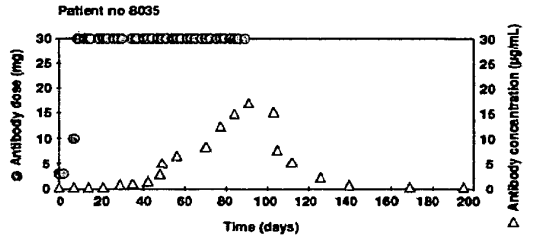


Figure 6. Example of alemtuzumab concentrations in a patient treated with subcutaneous antibody. After an initial dose escalation, 30 mg of alemtuzumab was administered 3 times a week for 12 weeks (○). Serum samples were taken before the dose, once a week (△). This patient made a good clinical response to the therapy and achieved relatively high concentrations of antibody.

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Table 2. Alemtuzumab dose and serum concentrations: subcutaneous study

Patient no.	Cumulative dose, mg	No. of samples	Above LOD	Terminal samples	Dose to reach 1 μg/mL	Max concentration, μg/mL	After last dose, μg/mL
8016	1308	24	2	0	726	1.3	ND
8015	1508	28	1	0	> 156	0.6	ND
8018	1575	29	3	0	673	1.5	ND
8017	1215	20	2	0	> 153	0.9	ND
8021	983	28	12	3	353	5.3	4.1
8026	1378	23	3	0	958	1.4	ND
8027	1268	43	9	1	636	6.6	6.6
8028	1363	37	13	3	403	5.8	5.8
8029	1133	34	6	1	605	7.3	7.3
8030	1553	45	16	1	473	4.3	4.2
8031	712	27	2	1	712	1.4	1.4
8032	723	17	2	0	393	2.5	2.5
8033	1502*	26	10	0	542	3.4	3.4
8034	1412	24	16	4	259	6.3	5.8
8035	1039	24	15	6	426	17.1	17.1
8036	819*	21	0	0	> 819	< 0.5	< 0.5
8038	1489	19	8	0	1106	2.8	2.8
8039	1526	19	10	0	508	4.4	ND
8040	1466	26	19	7	146	24.8	24.8
8041	852	23	6	0	482	3.5	3.5
Minimum	712	17	0	0	146	0.6	< 0.5
Mean	1249	27	8	1.6	551	5.4	6.4
Maximum	1573	45	19	7	1106	24.8	24.8

*Some doses for patients no. 8033 and no. 8036 were given intravenously due to infusion site reactions during the subcutaneous administration.

monoclonal antibodies. Therefore, essentially all of the response was directed against the alemtuzumab idiotype. No free alemtuzumab could be detected throughout treatment and there was no significant depletion of tumor cells during therapy. This patient developed a pronounced injection site reaction including erythema, edema, and slight local pain but otherwise no marked general symptoms. As a result, the patient did not achieve the 30-mg dose level during 6 weeks of subcutaneous dosing and alemtuzumab was subsequently administered intravenously. Due to nonresponsiveness, the patient was subsequently treated with fludarabine and went into a long-lasting partial remission. He was retested 18 months after the end of alemtuzumab treatment (continuing in PR) and still had a high-titer antiglobulin (approx 1.8×10^5 U/mL). There was a very low anti-alemtuzumab signal in one of 2 samples taken before therapy. This patient might have had a natural anti-IgG (rheumatoid factor) prior to therapy and perhaps was predisposed to make an antiglobulin response.

A search was made for samples from other patients who had received subcutaneous alemtuzumab. Samples were collected between 4 to 50 months after the end of treatment from an additional 9 patients of the original subcutaneous study plus 2 others who received subcutaneous alemtuzumab as part of a compassionate program. A substantial response was detected in patient no. 8020 of approximately 4800 U/mL at 32 months after the end of treatment. Like the response in patient no. 8036, this response cross-reacted on the parental rat antibody but not on other human antibodies, showing it to be essentially anti-idiotype. The patient had not responded to the therapy, but was reported to have prolonged skin reactions to the infusions. A very weak response was detected in one other patient of 541 U/mL, barely above the LOD. Unlike the other 2 patients, this serum cross-reacted with other human IgG but not with CAMPATH-1G. We consider that it is a nonspecific rheumatoid-like factor and probably had no clinical significance with respect to alemtuzumab.

In total therefore, of 32 patients treated with subcutaneous alemtuzumab (30 who were previously untreated with any anti-CLL agent), 2 produced a significant antiglobulin response which persisted at potentially neutralizing concentrations for more than a year. Both of these patients had received alemtuzumab manufactured at the Therapeutic Antibody Centre, as did most of the patients in this study, although patient no. 8036 had been switched to the commercial product after 3 weeks.

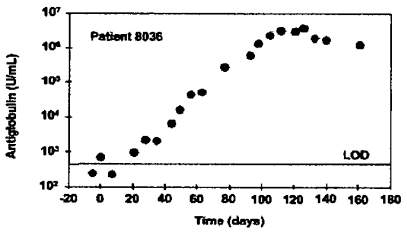


Figure 7. Antiglobulin response to alemtuzumab following subcutaneous treatment. This patient received subcutaneous alemtuzumab at doses up to 10 mg until day 37 (cumulative dose, 89 mg) and subsequently received 30 mg 3 times a week intravenously until day 80, followed by 30 mg 3 times a week subcutaneously until day 102. Samples were taken once a week to measure antiglobulin responses. One U/mL is equivalent to 1 ng/mL of a standard monoclonal anti-idiotype. The horizontal line indicates the limit of detection (LOD).

Discussion

Alemtuzumab is extensively used for patients with B-CLL who have failed treatment with alkylating agents and purine analogues and investigators are exploring its use in other disease settings. To enable safe and effective use it is essential to understand alemtuzumab's pharmacokinetics and biodistribution as well as the potential for anti-idiotype responses, which all may differ according to disease, patient status, and route of administration. We have started to address these issues by comparing blood concentrations of

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alemtuzumab and antiglobulin responses in 2 different studies for treatment of CLL, namely intravenous treatment of chemotherapy-resistant disease and first-line subcutaneous treatment. The studies were initiated independently and had different objectives; nevertheless the pharmacologic results are instructive.

Alemtuzumab may cause substantial "first dose" reactions as a result of cytokine release when it is given intravenously.[3,18,19] Therefore, the standard treatment regimen involves dose escalation and frequent, relatively small doses, though it has not been formally established whether the reactions are strictly dose related. A treatment course for chemotherapy-resistant CLL consists of approximately one gram of the antibody. However, a cumulative dose of 40 mg to 100 mg is highly immunosuppressive and clinically effective in patients without bulky tumors, for example those treated for prevention of transplant rejection,[11,20] multiple sclerosis,[21] and other autoimmune diseases,[22-24] as well as to eradicate detectable minimal residual disease in CLL.[25] The biodistribution of alemtuzumab is likely to depend on the bulk of tumor cells, possibly more so than other monoclonals because CD52 is such a highly expressed antigen.

There are approximately 5×10^5 binding sites for alemtuzumab on a normal lymphocyte.[26,27] There are about 10^{12} lymphocytes in a healthy adult.[28] At least 125 mg of alemtuzumab is therefore required to saturate all of the CD52 sites. CLL cells express a similar amount of CD52 antigen to normal lymphocytes[27] but their total number may be 10 times greater. Therefore, more than 1 g of alemtuzumab could be required to saturate all the receptors in some patients, especially considering that the antibody has a relatively low affinity and that there will be a significant clearance during a 12-week course of treatment. Hence, it is not surprising that the biodistribution and clearance of antibody during the treatment of patients with CLL is dominated by the tumor burden and cannot be fitted to a simple pharmacokinetic model.

There was a strong correlation between the concentrations of antibody throughout intravenous treatment and the ultimate clinical response, or the achievement of minimal residual disease (MRD) negativity in the bone marrow by flow cytometry. It has recently been reported that survival correlates better with MRD than with the standard NCI criteria for clinical responses.[29] Without a good measure for the initial total tumor burden it is difficult to distinguish cause and effect. One possibility is that patients with high tumor burden were treated with inadequate amounts of antibody to achieve sufficient receptor site saturation and see a clinical effect. In any event, it appears that measurements of alemtuzumab concentrations after 2 to 4 weeks of treatment might provide an indicator of the ultimate outcome and guide the physician whether to continue, stop, or adjust the dose of antibody. Most of the antibody was cleared within a few days of the final dose in the nonresponders. However, the half-life at the end of treatment in the responders was comparable with those previously reported for patients without bulky tumors.

Accumulation of alemtuzumab in the blood was significantly slower in the subcutaneous study and it took on average about 6 weeks longer to reach 1.0 μg/mL (an arbitrary threshold known to be potentially lympholytic). This could be a slight overestimate because samples were not always collected every week. Nevertheless, the eventual maximum trough concentrations were very similar in both studies. We cannot tell whether the slower accumulation of antibody in the blood was due to less favorable biodistribution, more effective binding to tumor cells, or the slower dose escalation used in the subcutaneous study compared with that used with intravenously treated patients. It should be possible to resolve this by studies of alemtuzumab given subcutaneously to patients not suffering from malignancies (ie, without excess $CD52^+$ cells).

Because clinical responses were much more frequent (19% CR and 68% PR),[8] we could not detect a significant correlation between blood concentrations and clinical responses in the subcutaneous study. With only 2 nonresponders in the group of patients we analyzed, the sample was too small to discern any relationship.

Clear-cut results were obtained from the measurements of antiglobulin responses (antihuman antibody [HAHA]). There were no detectable responses at all in the intravenous study (heavily pretreated patients) and only one patient in the subcutaneous study (previously untreated patients) made a strong response, reaching a peak of approximately 3.7×10^6 U/mL 3 weeks after the final dose. This was equivalent to 3.7 mg/mL of the monoclonal anti-idiotype standard and represented a substantial proportion of the total Ig in the patient. Similar titers were previously reported during treatment of rheumatoid arthritis patients with subcutaneous alemtuzumab[13] when all of the patients (10/10) made a positive response. After screening 11 other patients we found one with a significant antiglobulin titer of 4800 U/mL. Considering that this sample was taken 32 months after the last treatment with alemtuzumab, it seems likely that this patient also had made a very strong response.

Previous studies on antiglobulin responses to alemtuzumab have given widely different results. In 12 patients treated for kidney transplant rejection no antiglobulins were detected, in contrast to 15 of 17 patients treated with the parental rat antibody CAMPATH-1G.[11] In 167 patients treated for chemotherapy-resistant lymphoid malignancies there were only 3 weak or borderline responses. However, out of 115 patients treated for rheumatoid arthritis, there were 59 positive responses (ILEX Pharmaceuticals, unpublished data on file, July 2003), some of high titer similar to that reported here for patient no. 8036.[13] Many factors could affect the immunogenicity of alemtuzumab, including the dose and length of treatment, the underlying disease, prior exposure to chemotherapy, concomitant immunosuppressive drugs, and possibly the route of administration or the formulation of the drug. Subcutaneous administration might allow more effective antigen presentation, for example, by Langerhans cells and with less "excess" antibody circulating to provide tolerogenic signals, the balance might be tipped toward an immune response. From our present study we cannot say whether subcutaneous administration in CLL is more immunogenic than intravenous administration since there were other differences between the 2 groups, most notably the subcutaneous group had received no prior chemotherapy. Even if the 2 groups had been comparable, the difference in responses (0/30 vs 2/32) did not reach statistical significance. This issue needs to be investigated in larger numbers of patients. Both patients who made an antiglobulin response had been treated with alemtuzumab manufactured at the Therapeutic Antibody Centre. We are aware that the manufacture, formulation, or storage of biologics might affect their immunogenicity, but so far we have no evidence to suggest that there is a major difference between the academic or commercial source since antiglobulin responses have been reported in other patients treated with alemtuzumab from either source. The 2 patients (no. 8020 and no. 8036) who produced anti–alemtuzumab antibody on subcutaneous treatment, unlike the other patients, did not show significant reductions in lymphocyte counts but had marked local skin reactions which did not diminish with continued therapy. An antiglobulin response should be considered when there is no drop in the blood lymphocyte count and/or there are persistent injection site reactions.

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Alemtuzumab provides a useful therapeutic option for patients with CLL. It has excellent activity as first-line treatment and can give good responses in patients who are unresponsive to conventional chemotherapy. The optimal dose route and regimen are not yet known. However, the subcutaneous route can deliver concentrations of antibody comparable to the original intravenous route. The dominant factor influencing biodistribution and pharmacokinetics appears to be the tumor burden. With intravenous dosing, serum concentrations of alemtuzumab can provide an early prediction of the ultimate response and might be useful to guide dose adjustments to improve responses or reduce side effects. Strong anti-idiotype responses may occur in a small minority of patients. Although not associated with serious adverse effects, such responses neutralize the therapeutic benefit and therefore patients with a known antiglobulin response should not be further treated with this monoclonal antibody.

Acknowledgments

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(05 71 2607.0 / 1720571)
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

DRUG NAME: Alemtuzumab

SYNONYM(S): Campath, Ldp 103, Mabcambath, MabCampath-1H

COMMON TRADE NAME(S): MABCAMPATH®

CLASSIFICATION: monoclonal antibody

Special pediatric considerations are noted when applicable, otherwise adult provisions apply.

MECHANISM OF ACTION:

Alemtuzumab is a recombinant human IgG-derived monoclonal antibody that binds to antigen CD52 which is found on the surface of B and T lymphocytes, most monocytes, macrophages and NK cells, and certain granulocytes, but not hematopoietic stem cells.[1] Lysis of CD52-positive cells occurs via complement activation, antibody-dependent cytotoxicity, and apoptosis.[2]

PHARMACOKINETICS:

Table refers to intravenous (IV) dosing. Subcutaneous (SC) administration of alemtuzumab achieves concentrations similar to those with intravenous alemtuzumab, although with a slightly higher cumulative dose.[3,4]

Distribution	cross blood brain barrier?	no information found
	volume of distribution	mean 0.18 L/kg (range 0.1-0.4 L/kg)
	plasma protein binding	no information found
Metabolism	no information found	
	active metabolite(s)	no information found
	inactive metabolite(s)	no information found
Excretion	no information found	
	terminal half life	mean 11 h (range 2-32 h) after the first dose; 6 d (range 1-14 d) after 12 weeks
	clearance	wide variability, affected by tumour burden[5]; clearance decreases with repeated administration due to decreased receptor mediated clearance (loss of CD52 receptors in the periphery)
Elderly	comparable AUC in patients older than 65 years of age	

Adapted from standard reference[1] unless specified otherwise.

USES:

Primary uses:
*B-cell chronic lymphocytic leukemia[1,6]
T-cell prolymphocytic leukemia[6-8]

*Health Canada approved indication

Other uses:
Cutaneous T-cell lymphoma[7]
Peripheral T-cell lymphoma[7]

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SPECIAL PRECAUTIONS:

Contraindicated in patients who have a history of hypersensitivity reaction to alemtuzumab, active infections, underlying immunodeficiency (e.g., seropositive for HIV), or other active malignancies.[1]

Caution:

- in patients with previous hypersensitivity or anaphylactic reactions to other monoclonal antibodies[9]
- gradual dose escalation and appropriate pre-medication is required; for more information, see paragraph following the **Side Effects** table[1]
- single doses of alemtuzumab greater than 30 mg or cumulative doses greater than 90 mg per week should not be administered as they are associated with a higher incidence of pancytopenia[1]
- transfusion-related graft-versus-host-disease can occur in patients with severe lymphopenia and can be life threatening; patients receiving alemtuzumab should receive irradiated blood products, effectively eliminating this risk[1]
- *Pneumocystis* pneumonia (PCP) and herpes virus prophylaxis are recommended upon initiation of therapy[1,6,10,11]
- use alemtuzumab with caution in patients with recent exposure to the chicken pox virus[9]

Hepatitis B (HBV) reactivation: All lymphoma patients should be tested for both HBsAg and HBcAb. If either test is positive, such patients should be treated with lamivudine 100 mg/day orally, for the entire duration of chemotherapy and for six months afterwards.[6,10] Such patients should also be monitored with frequent liver function tests and HBV DNA at least every two months. If the HBV DNA level rises during this monitoring, management should be reviewed with a hepatologist and consideration given to halting chemotherapy.[6]

Cytomegalovirus reactivation: for more information, see paragraph following the **Side Effects** table

Carcinogenicity: no information found[1]

Mutagenicity: no information found[1]

Fertility: Alemtuzumab has been shown to bind to mature sperm.[1] No long term studies have been performed on men or women to determine the effect on infertility. Patients of reproductive potential should use effective contraceptive methods during treatment and for a minimum of 6 months following alemtuzumab therapy.[1]

Pregnancy: FDA Pregnancy Category C.[7] Either studies in animals have revealed adverse effects on the fetus (teratogenic or embryocidal or other), and there are no controlled studies in women, or studies in women and animals are not available. Alemtuzumab should be given only if the potential benefit justifies the potential risk to the fetus. Human IgG is known to cross the placental barrier, therefore alemtuzumab may also cross the placenta and cause fetal B and T lymphocyte depletion.[1]

Breastfeeding should be discontinued during treatment and for at least 3 months following the last dose of alemtuzumab as human IgG is excreted in breast milk.[1]

SIDE EFFECTS:

The table includes adverse events that presented during drug treatment but may not necessarily have a causal relationship with the drug. Because clinical trials are conducted under very specific conditions, the adverse event rates observed may not reflect the rates observed in clinical practice. Adverse events are generally included if they were reported in more than 1% of patients in the product monograph or pivotal trials, and/or determined to be clinically important.[12] When placebo-controlled trials are available, adverse events are included if the incidence is $\geq$ 5% higher in the treatment group.

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Table refers to IV data unless specified. SC hematological and infectious complications are similar to IV.

ORGAN SITE	SIDE EFFECT
	Clinically important side effects are in ***bold, italics***
allergy/immunology	anaphylactic reaction (<1%)[13] first dose reactions, infusion-related reactions with SC use, injection site reactions and fever
	angioedema (<1%)[13]
	antibody development (2%)
	autoimmune diseases; Graves' disease, Guillain-Barre syndrome, hypothyroidism, Goodpasture's syndrome, autoimmune thyroiditis[14]
	mouth edema (1%, severe 1%)
blood/bone marrow/ febrile neutropenia	anemia (77-80%, severe 38%)[13]; autoimmune hemolytic anemia (1%)[13]
	atypical lymphocytes (1%, severe 1%)
	immunosuppression,[1,15] lymphopenia (100%)[15]; onset in 2-4 weeks, may persist for over one year[10]
	neutropenia (85%, severe 64%)[13]; during the first weeks of therapy,[15] nadir 4-6 weeks,[15] median duration 21 days[13]; febrile neutropenia (5-10%, severe 1%)[13]
	pancytopenia (5%, severe 3%),
	thrombocytopenia (72%, severe 50%) nadir 2-4 weeks,[15] median duration 21 d (range 2-165 d); autoimmune idiopathic thrombocytopenia (2%)
cardiovascular (arrhythmia)	arrhythmias (<1%)[13]
	tachycardia (5-11%, severe 1%)[13]
cardiovascular (general)	cardiac ischemia, infarction (<1%)[13,16,17]
	hypotension (30-32%, severe 3%)[13]; with SC use (0%)[3]
	hypertension (9-11%, severe 1%)[13]
coagulation	disseminated intravascular coagulation (<1%)[13,18]
	deep vein thrombosis, pulmonary embolism (<1%)[13]
	thrombocytopenic purpura (2%, severe 0%)
constitutional symptoms	asthenia (9%, severe 0%), malaise (5%, severe 0%), fatigue (27-34%, severe 4%)[13]; with SC use (5%, severe 2%)[3]
	fever (82-85%, severe 14%)[13]; usually occurs 5-6 hours after the start of the infusion[19] with SC use (70%, severe 2%)[3]
	insomnia (1-10%, severe 0%)[13]
	rigors (85%, severe 15%); with SC use (17%, severe 2%)[3]
	sweating (15%, severe 1%)
	temperature change sensation (5%, severe 0%)
	weight loss (5%, severe 0%)
dermatology/skin	*extravasation hazard: none*[20]
	bullous eruptions (1%, severe 1%)
	erythematous rash (4%, severe 1%)
	flushing (4%, severe 0%)

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ORGAN SITE	SIDE EFFECT
Clinically important side effects are in ***bold, italics***	
	injection site reaction (1%, severe 0%) with SC use (90%, severe 2%)[3]; onset approximately 24 h after the first dose, often persists until the fourth administration[21]
	pruritus/urticaria (21-30%, severe 1-5%)[13]; with SC use (0%)[3]
	rash (29-40%, severe 3%)[13]; with SC use (0%)[3]
endocrine	ADH secretion abnormality (<1%)[22]
	hyperthyroidism (<1%)[1]
gastrointestinal	*emetogenic potential: rare*[23]
	anorexia (13-20%, severe 1%)[13]
	ascites (<1%)[13]
	colitis, enteritis (<1%)
	constipation (2%, severe 0%)
	dehydration (2%, severe 2%)
	diarrhea (13-22%, severe 1%)[13]; with SC use (0%)[3]
	dyspepsia (4%, severe 0%); ulcer (<1%)[13]
	mucositis (2%, severe 0%)
	nausea (49-54%, severe 2%)[13]; late nausea is rare[15] with SC use (0%)[3]
	stomatitis (5%, severe 1%); ulcerative stomatitis (3%, severe 0%)
	taste loss (2%, severe 1%)
	vomiting (37-41%, severe 4%)[13]; with SC use (0%)[3]
hemorrhage	GI hemorrhage (<1%)[13]
	hemoptysis (1%, severe 0%)
	CNS hemorrhage (<1%)[13]
	purpura (8%)[13]
hepatobiliary/pancreas	liver dysfunction (1%, severe 0%)
	pancreatitis (<1%)[13]
infection	**bacterial** (34-40%)[10]; usually occurs within 8 weeks, mycobacterial and tuberculosis have occurred months later[10] bacteremia without sepsis (4%)[24] *Listeria monocytogenes* meningitis (1%, severe 1%) *Pneumocystis* pneumonia (PCP)
	fungal (18-21%)[10]; majority occur within 3 months of treatment, late infection have occured[10] *Aspergillosis* (2%, severe 1%) candidiasis (8-10%, severe 1%)[13] *Cryptococcal* pneumonia (1%, severe 1%) mucormycosis (2%, severe 2%) *Torulopsis* pneumonia (1%, severe 1%)

ORGAN SITE	SIDE EFFECT
Clinically important side effects are in ***bold, italics***	
	not otherwise specified (7%, severe 1%)
	pneumonia (7-18%, severe 13%)[24]
	protozoan[7,24]
	sepsis (15-18%, severe 13%)[13]
	upper respiratory tract infection (6%, severe 0%)
	urinary tract infection (<3%, severe 0%)[24]
	viral (43-45%)[10] adenovirus[25] Cytomegalovirus (CMV) reactivation (4-66%)[3,10,14,15,24,26-28]; usually occurs between weeks 3-8 when T-cell numbers are low,[15] symptomatic CMV reactivations and infections (1-6%, severe 3%)[1,24,26,28] herpes simplex (10-13%, severe 1%)[13,24] herpes zoster (5%, severe 1%) Parvovirus B19 induced red cell aplasia (<1%)[29,30]
lymphatics	edema (1-13%, severe 0%)[13]
metabolic/laboratory	acidosis (<1%)[13]
	hypocalcemia (2%, severe 0%)
	hyponatremia (1%, severe 1%)
	increased alkaline phosphatase (<1%)[13]
	positive Coombs test without hemolysis (2%)
musculoskeletal	arthralgia (1%, severe 0%)
	myalgia (9-11%, severe 0%)[13]
neurology	anxiety (3%, severe 0%)
	confusion (2%, severe 1%)
	depression (2-7%, severe 0%)[13]
	dizziness (5-12%, severe 0%)[13]; vertigo (3%, severe 0%)
	leukoencephalopathy (<1%)[13]
	neurological symptoms associated with previous multiple sclerosis lesions; reversible, do not occur if pre-medicated with high dose steroids
	seizure (<1%)[13]
	sensory neuropathy; dysthesia (15%),[13] paresthesia (6%, severe 0%), hypoesthesia (3%, severe 0%)
	somnolence (3-5%, severe 0%)[13]
	tremor (6%, severe 0%)
ocular/visual	conjunctivitis (2%, severe 0%)
	endophthalmitis (1%, severe 1%)
	optic neuropathy (<1%)
pain	abdominal pain (6-11%, severe 2%)[13]

ORGAN SITE	SIDE EFFECT
Clinically important side effects are in ***bold, italics***	
	back pain (5%, severe 2%)
	chest pain (6-10%, severe 1%)[13]
	headache (18-24%, severe 1%)[13]; with SC use (0%)[3]
	SC injection site pain (61%, severe 2%)[3]
	not otherwise specified (7%, severe 1%)
	skeletal pain (3-24%, severe 1%)[13]
pulmonary	bronchitis (9%, severe 1%)
	bronchospasm (6%, severe 2%)
	cough (4-25%, severe 1%)[13]
	dyspnea (18-26%, severe 6%)[13]; with SC use (0%)[3]
	hypoxia (2%, severe 1%)
	pharyngitis (4-12%, severe 0%)[13]
	pneumonitis (3%, severe 3%)
	pulmonary infiltration (1%, severe 1%)
	respiratory depression (<1%)
	rhinitis (1-7%, severe 0%)[13]
	sinusitis (6%, severe 1%)
renal/genitourinary	hematuria (1%, severe 1%)
	decreased renal function/renal failure (<1%)[13,18]
sexual/reproductive function	binds to mature sperm
syndromes	influenza-like symptoms (5%, severe 0%)
	tumour lysis syndrome (<1%)[13]
vascular	phlebitis (<1%)
	vasospasm (1%, severe 0%)

Adapted from standard reference[1] unless specified otherwise.

Infusion reactions: Intravenous administration of alemtuzumab is usually accompanied by transient infusion-related side effects that manifest as flu-like symptoms. These acute cytokine-mediated reactions are generally mild to moderate. The reactions includes nausea and vomiting, hypotension, dizziness, rigors, fever, diarrhea, sudden sweating, shortness of breath, chills, and/or rash.[1,15] Serious and potentially fatal complications including myocardial infarction, cardiomyopathy, cardiac arrest, arrhythmias, bronchospasm, and acute respiratory distress have also been reported.[1,13] Symptoms are most common during the first week of therapy and decrease with each subsequent administration.[2,2,9,31] To prevent infusion related reactions:

- give diphenhydramine 50 mg PO and acetaminophen 650 mg PO 30 minutes prior to alemtuzumab[1,6]
- pre-medicate with prednisone 10 mg PO 15-30 minutes prior to alemtuzumab for the first 1-2 weeks[6,15]; since corticosteroids cause immunosuppression, prophylaxis should be discontinued after 1-2 weeks[15]
- start with low doses of alemtuzumab (3 mg) and escalate as tolerated[1] (for more information, see **Dosage Guidelines**); it is not known if the infusion reactions are strictly dose-related[5]

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- meperidine 25 mg IV may lessen rigors,[6,9] patients experiencing meperidine-induced nausea should receive antiemetics[19]
- for patients experiencing severe rash, premedication with H_2 receptor antagonists (e.g., ranitidine) is recommended[19]
- fever commonly begins 5-6 hours after the infusion starts; a second dose of acetaminophen may be required[19]

During the infusion, the patient's pulse, respiratory rate and blood pressure should be monitored every 15 minutes during the first hour, then every 30 minutes.[6] Patients should be observed for 1 hour after the infusion.[6] To treat severe infusion-related symptoms, stop the alemtuzumab infusion and give hydrocortisone 200 mg IV.[1] Beta-2 agonists for wheezing and volume support for hypotension may be given.[32] If acute infusion reactions persist, the infusion time may be extended to up to 8 hours from the time of dose preparation.[31] Mild first-dose side effects tend to not preclude patients from completing therapy.[32]

Subcutaneous administration of alemtuzumab reduces infusion-related symptoms, while maintaining the same efficacy and improving convenience.[33] The incidence of nausea and vomiting, rigors, rash, hypotension, and dyspnea are significantly reduced with SC dosing, though fever may not be reduced appreciably (see Side Effects Table).[3] Almost all patients receiving SC alemtuzumab will develop a transient grade 1 or 2 local skin reaction.[3] IV corticosteroids do not affect the intensity or duration of these skin reactions.[3] Ice packs applied to the injection site 15 minutes prior to and 15 minutes post injection may decrease the severity of local skin reactions. Cold compresses applied to the injection site for 15-20 minutes, several times a day after treatment may also help alleviate symptoms. Most SC first-dose reactions disappear after 1-2 weeks.[3] Patients should be observed for 1 hour after the dose has been administered until at least three 30 mg doses have been given.[6] After that, monitoring may be discontinued.[6] Data from an ongoing study suggests that dose escalation may not be necessary with SC administration.[15]

Immunosuppression / opportunistic infections: Patients are at risk for opportunistic infections due to the profound lymphopenia induced by alemtuzumab.[1] Alemtuzumab-induced lymphopenia is non-dose dependant; single doses may reduce CD4+ and CD8+ T-cells for months.[15] Serious and sometimes fatal bacterial, fungal, viral and protozoal infections have occurred with infection rates ranging from 23-79% in pre-treated patients.[1,10] Although the majority of infections occur within 3 months of starting treatment, infections have been reported up to 6 months later.[10] If an infection occurs, alemtuzumab therapy should be interrupted but may be reinitiated following the resolution of the infection.[1]

Upon initiation of alemtuzumab patients should receive prophylaxis against PCP and herpes virus infections with:

- trimethoprim/sulfamethoxazole (TMP/SMX) DS PO twice daily three times weekly (TMP/SMX is the preferred prophylaxis; dapsone, pentamidine and atovaquone have also be used[10]
- acyclovir 400 mg PO BID (alternatively, valacyclovir 500 mg PO twice daily)[3]

Prophylaxis should be continued for a minimum of 2 months following the last dose of alemtuzumab or until CD4+ counts are ≥200 cells/μL, whichever occurs later.[1] Full recovery of CD4+ and CD8+ counts may take more than 12 months[1]; optimal length of post-treatment prophylaxis has yet to be determined.[10,15,19] Prophylaxis against PCP and herpes may decrease, but not eliminate, the occurrence of these infections.[1] Routine antifungal prophylaxis is not currently recommended but may be considered for high risk patients.[10,26]

Cytomegalovirus reactivation is the most common infection associated with alemtuzumab. T-cell depletion increases the risk of reactivation of CMV.[15,19] Reactivation usually occurs 3-8 weeks after initiation of therapy and coincides with T-cell nadir. Patients receiving alemtuzumab should have weekly testing for CMV replication by PCR or CMV antigenemia (pp65ag) assays.[6,11,19] Patients should also be monitored for symptoms of CMV infection.[6,19,34] If a positive screening test occurs, alemtuzumab treatment should be interrupted until testing becomes negative. Patients who are symptomatic should be treated immediately with ganciclovir.[10,14,15,19] Alemtuzumab has been restarted after clinical symptoms resolve and PCR testing is negative without further reactivation.[3,14,15,19]

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INTERACTIONS:

AGENT	EFFECT	MECHANISM	MANAGEMENT
diagnostic serum tests that utilize antibodies[1,13]	inaccurate test results[1] and potential for increased risk of allergic reactions[13]	monoclonal antibodies may result in the formation of human antichimeric antibodies (HACA); the presence of HACA may increase the risk of hypersensitivity reactions when other monoclonal antibodies are administered[13]	interpret with caution
monoclonal antibodies[1,35]	increased risk of allergic or hypersensitivity reactions to monoclonal antibodies; especially in patients that experienced previous hypersensitivity reactions to monoclonal antibodies[1]	monoclonal antibodies may result in the formation of human antichimeric antibodies (HACA); the presence of HACA may increase the risk of hypersensitivity reactions, thrombocytopenia, or diminished therapeutic response when other monoclonal antibodies are administered; monoclonal antibodies that also form HACA may be of particular concern	monitor for toxicity/response, patients who developed hypersensitivity to alemtuzumab may develop hypersensitivity reactions to other monoclonal antibodies
killed virus vaccines[1]	ability to respond to vaccines following therapy is unknown; duration of decrease response unknown; estimates vary from 3 months - 1 year[9]	alemtuzumab may decrease the ability to generate a humoral response to vaccines	immunize prior to therapy if possible, potential for decreased benefit of vaccine if administered during or within 1 year after therapy
live virus vaccines[1]	ability to respond to vaccines following therapy is unknown, risk of infection by the live vaccine virus; duration of risk unknown; estimates vary from 3 months - 1 year[9]	alemtuzumab may potentiate replication of the vaccine virus, decrease the ability to generate a humoral response to the vaccine and enhance the adverse effects of live vaccines[9]	avoid during and within 1 year after therapy

SUPPLY AND STORAGE:

For basic information on the current brand used at the BC Cancer Agency, see Chemotherapy Preparation and Stability Chart in Appendix.

SOLUTION PREPARATION AND COMPATIBILITY:

For basic information on the current brand used at the BC Cancer Agency, see Chemotherapy Preparation and Stability Chart in Appendix.

Additional information: *Diluted solution for infusion*: compatible with PVC bags and administration sets[1]

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Compatibility: consult detailed reference

PARENTERAL ADMINISTRATION:

BCCA administration guideline noted in ***bold, italics***

Subcutaneous	***rotate sites on thighs or abdomen****
Intramuscular	no information found
Direct intravenous	contraindicated
Intermittent infusion	***over 2 hours***
Continuous infusion	no information found
Intraperitoneal	no information found
Intrapleural	no information found
Intrathecal	no information found
Intra-arterial	no information found
Intravesical	no information found

*Subcutaneous administration of alemtuzumab significantly reduces flu-like symptoms making it the preferred route of administration.[12]

DOSAGE GUIDELINES:

Refer to protocol by which patient is being treated. Numerous dosing schedules exist and depend on disease, response and concomitant therapy. Guidelines for dosing also include consideration of absolute neutrophil count (ANC). Dosage may be reduced, delayed or discontinued in patients with bone marrow depression due to cytotoxic/radiation therapy or with other toxicities.

Adults:

BCCA usual dose noted in ***bold, italics***

	Cycle Length:	
Intravenous:	***titration 1 week***[1,6]***:***	***3 mg IV for one dose on day 1, then 10 mg IV for one dose on day 3, and 30 mg IV for one dose on day 5*** • **titration may be handled more slowly if required (e.g., infusion-related toxicities > NCI Grade 2)** • gradual dose escalation is required at the initiation of therapy and after treatment interruptions of 7 days or more; dose escalation to the recommended maintenance dose of 30 mg is required and can be accomplished in 3-7 days in most patients • doses greater than 30 mg per day or greater than 90 mg per week are not recommended
	maintenance 1 week[1,6]***:***	***30 mg (range 3-30 mg) IV for one dose on days 1, 3 and 5 (total dose per cycle) 90 mg [range 9-90 mg])*** • consecutive daily dosing may also be used[1] • gradual dose escalation is required after treatment interruptions of 7 days or more

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BCCA usual dose noted in ***bold, italics***

Subcutaneous:

Cycle Length:

titration 1 week[3,6]***:*** ***3 mg SC for one dose on day 1, then 10 mg SC for one dose on day 3, and 30 mg SC for one dose on day 5***

- **titration may be handled more slowly if required (e.g., infusion-related toxicities > NCI Grade 2)**
- gradual dose escalation is required at the initiation of therapy; dose escalation to the recommended maintenance dose of 30 mg is required and can be accomplished in 3-7 days in most patients
- doses greater than 30 mg per day or greater than 90 mg per week are not recommended

maintenance 1 week[3,6]***:*** ***30 mg (range 3-30 mg) SC for one dose on days 1, 3 and 5 (total dose per cycle 90 mg [range 9-90 mg])***

- consecutive daily dosing may also be used[1]

Concurrent radiation: additive bone marrow suppression may occur; dose reduction may be required when used concurrently or consecutively[9]

Dosage in myelosuppression:[1] modify according to protocol by which patient is being treated; if no guidelines available, refer to the table below

ANC ($x10^9$/L)		Platelets ($x10^9$/L)	Dose
< 0.25	or	≤ 25	**first occurrence:** hold treatment and resume at the same dose when ANC ≥ 0.5 and platelets ≥ 50 (restart at 3 mg if delay between doses ≥ 7 days and escalate to 30 mg as tolerated) **second occurrence:** hold treatment and resume at 10 mg when ANC ≥ 0.5 and platelets ≥ 50 (restart at 3 mg if delay between doses ≥ 7 days and escalate to a maximum dose of 10 mg) **third occurrence:** discontinue treatment permanently
For baseline ANC ≤ 0.5 and/or platelets ≤ 25: if ANC or platelets decrease to ≤ 50% of baseline value, hold therapy and resume when ANC and platelet count return to baseline values (restart at 3 mg if delay between doses ≥ 7 days and escalate to 30 mg as tolerated)			
There is no information on the safety of resumption of alemtuzumab in patients with autoimmune cytopenias or marrow aplasia. [1]			

Dosage in renal failure: no information found

Dosage in hepatic failure: no information found

Dosage in dialysis: no information found

Children: has been used[36]

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Opposition by: Pfizer Inc.
Our Ref.: M2367 OPP(EP)

EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

15 July 2010
EMA/HMPC/16633/2009
Committee on Herbal Medicinal Products (HMPC)

Assessment report on *Vitis vinifera* L., folium

Based on Article 10a of Directive 2001/83/EC as amended (well-established use)

Based on Article 16d(1), Article 16f and Article 16h of Directive 2001/83/EC as amended (traditional use)

Final

Herbal substance(s) (binomial scientific name of the plant, including plant part)	*Vitis vinifera* L., folium
Herbal preparation(s)	Herbal substance - Not applicable Herbal preparations: - Comminuted dried leaves as herbal tea (TU) - Powdered herbal substance (TU) - Soft extract (2.5-4:1; water (TU) - Dry extract (4-6:1; water) (WEU)
Pharmaceutical forms	Comminuted herbal substance as herbal tea for oral use (TU). Herbal preparation in solid dosage forms for oral use (WEU and TU). Herbal preparation in semi-solid dosage forms for cutaneous use (TU).
Rapporteur	Dr Ioanna Chinou, Gloria Garcia-Llorente

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1. Introduction

The aim of this report is to assess the preclinical and clinical available data on Vitis viniferae folium for preparing a Community herbal monograph and Community List entry. This report is based on the documentation provided by the European Medicines Agency (EMA) completed by additional searches and information taken from recent international literature on Vitis viniferae folium.

Bibliographic searches have been performed in MEDLINE (1966-2008); EMBASE (1974-2008). The search terms were: Plant extracts; Plant leaves; Red vine leaf extract; Roter Weinlaubextrakt; Extract Red vine / wine Leaf / Leaves *Vitis vinifera*; Folia *Vitis vinifera*.
Only preparations of *Vitis vinifera* as a single ingredient are considered for the monograph, while the studies performed with combinations are not discussed in this report.

1.1. Description of the herbal substance(s), herbal preparation(s) or combinations thereof

The plant originates from North Africa, South Africa or South West Europe. *Vitis vinifera* L., Grape vine, is a perennial, defoliating limber with a wooden often twisted stem which can reach a length of 30 meters, but it is usually cut back to 1-3 meters. The shrub develops climbing branches forking to twigs from where the long-stemmed, alternating arranged leaves protrude. The vine leaf is heart-shaped, thin, long-stemmed, cordate with palmate venation and with 5-7 dentate lobes, divided by more or less deep and open sinuses. It can be up to 15 cm long and 12 cm across at its widest point, its colour is a uniform dark red. The upper surface is glabrous, but the lower surface may be pubescent. The venation is prominent on the lower surface. At the lower tendrils the flower panicles with numerous yellow-greenish flowers are formed. The fruits, arranged in large and long clusters are soft and pulpy berries with yellow-green, reddish or purplish dark-blue skin [Pharm. Franc. X., 1996].
Vitis vinifera belongs to the *Vitaceae* family. Several subspecies and varieties are distinguished among which is the subspecies *sylvestris* (Gmelin) Berger, recognised as the spontaneous form of *V. vinifera* L., the subspecies *caucasica* Vavilov, occurring in both wild and cultivated form. It is supposed that from these two, the cultivated form *Vitis vinifera* ssp. *sativa* DC has been grown [Bombardelli & Morazzoni 1995, Bombardelli et al. 1997].

- Herbal substance

The herbal substance consists of the dried leaves of the black to pulp-red grape and has a faintly perceptible odour. The herbal drug is harvested by hand in the autumn following the grape harvest. Drying takes place under natural conditions in accordance with local climatic conditions.

Latin Name: Vitis viniferae folium (*Vitis vinifera* L., var. *tinctoria*, *Vitaceae*) "Vitis vinifera" or "Vitis folium";
Common Names: vine leaves or vineleaves or vine leaf or vineleaf or grape leaf (English);
Feuilles de vigne (French), Rebenblätter, Weinlaub (German), Fogli della vite (Italian), Hogas de la vid (Spanish), Folhas da videira (Portuguese), Wijnstok bladeren (Dutch), liść winorośli właściwej (Polish), Φύλλο Αμπέλου (Greek).

The crude herbal substance complies with the monograph "Vigne Rouge" French Pharmacopoeia [Pharm. Franc. X., 1996]. The powder is reddish-brown. Examined under the microscope, the powdered red vine leaf shows the following characteristics: more or less dense, unicellular, long, tapering, covering trichomes, thick-walled, with bulbous or truncated base and a lumen divided into loculi; numerous raphides of calcium oxalate are contained in these cells or scattered about; fragments

of parenchyma containing twinned crystals of calcium oxalate; a few fragments of epidermis with polygonal cells and some reticulate venation.
Since the beginning of the last century in many studies the chemical constituents of the different parts of grape vine have been investigated. Fruit acids, tannins and pigments are the substances mainly responsible for taste, odour and colour [PDR for Herbal Medicines, 2004].

- Herbal preparation(s)

Comminuted herbal substance

Powdered herbal substance

Soft extract (DER 2.5-4:1); extraction solvent water

Dry extract (4-6:1; water)

See also market overview in 1.2

- Combinations of herbal substance(s) and/or herbal preparation(s) including a description of vitamin(s) and/or mineral(s) as ingredients of traditional combination herbal medicinal products assessed, where applicable.

Not applicable.

1.2. Information about products on the market in the Member States

Member State	**Regulatory Status**	**Comments**[1]
Member state	products, indications	Legal status
Austria	1) **360 mg-filmcoated tablet** 1 tablet contains: 360 mg dry extract (DER 4-6:1), extraction solvent water 2) **capsules** 1 capsule contains: 180 mg dry extract (DER 4-6:1), extraction solvent water 3) **oral drops** 1 ml contains 73.5 mg extract (corresponding 1.2 mg Aesculin) in ethanolic solution (the DER and the extraction solvent are not declared in the SPC at the moment) Since 1) December 2005 2) January 2000 3) March 1993 1) 1 tablet daily (up to 2 tablets). Duration of use: max. 3 months 2) 2 capsules in the morning (up to 4 capsules). Duration of use: max. 3 months 3) 30 drops in the morning in fasting state **Indications** 1,2) chronic venous insufficiency grade I and II; swelling of feet and lower leg	WEU

[1] Not mandatory field

Member State	Regulatory Status	Comments[1]
	3) phlebitis, edemas in the legs (also during pregnancy), varicose veins	
Belgium	**Hard-capsules** 350 mg powder per capsule powder, corresponding to 8% total polyphenols and minimum 0.20% of anthocyanosides Since 2005 oral administration Adults: 1 to 2 capsules three times daily, during the meals with a large glass of water. Maximal dose is 6 capsules per day. Duration of use should be limited to 3 months. If symptoms do not improve during use, consult a doctor **Indications** used in case of subjective symptoms of venous insufficiency in legs, such as heavy legs, after exclusion of all serious pathologies such as phlebitis, thrombophlebitis and thrombosis	WEU
Bulgaria	Not known	
Cyprus	Not known	
Czech Republic	**(WEU)** Vitis viniferae folii extractum aquosum siccum (4–6:1), 180 mg – standardized to flavonoids content 3-7% **Pharmaceutical form** Capsule **Posology** 2 capsules corresponding to 360 mg of the extract in the morning, during first 3 weeks increase of the dose to 4 capsules is possible to reach faster relief since 2001?? **Indications** Prevention and therapy of symptoms of light or starting moderate forms of chronic venous insufficiency related to varicose veins such as swelling of the calves, heavy legs, tingling	
Denmark	No authorized herbal medicinal products containing *Vitis vinifera* as a single drug preparation are on the market.	
Estonia	Not known	
Finland	Not known	
France	**(WEU)** Soft extract since 1970 **Pharmaceutical form** eye drops **Posology** 1 drop 2 to 8 times daily. 1 g of extract/100 ml **Indications** uninfectious conjonctival irritations **TU** **Preparations** Powder since 1982 dry aqueous extract since 1987, 1990 All for oral use, in adults **Posology**	

Member State	Regulatory Status	Comments[1]
	1) 1 hard capsule 3 times daily (5 if necessary) 350 mg of powdered drug/capsule 2) 1hard capsule 2 times daily 200 mg of extract/capsule 3) 1 hard capsule 1 to 3 times daily containing 169 mg of extract/capsule **Indications** 1); 2); 3) - Traditionally used in the symptomatic treatment of functional disorders of cutaneous capillary fragility, such as ecchymosis, petechias, etc. - Traditionally used:, in subjective signs of venous insufficiency, such as heavy legs - in haemorrhoidal symptoms combination herbal preparations with Mélilot (*Melilotus*) Marron d'inde (*Aesculus hippocastanum*) Hamamélis (Hamamelis)	
Germany	**TU** **Preparations**: soft extract (2.5-4:1), extraction solvent: water at least since 1976 **Pharmaceutical form** cream **Posology** for cutaneous use in adults 10 g cream contain 282 mg soft extract **Indications** Traditionally used to relieve symptoms of tired legs **Risks** (adverse drug effects, literature) Allergic reactions, itching and erythema, worsening of skin irritation. Contact allergy and/or hypersensitivity reactions **WEU** 1) soft extract (4-6:1), extraction solvent: water 2), 3) dry extract (4-6:1), extraction solvent: water In the market 1), 3) at least since 1976 2) 2005 1) oral liquid 2) film-coated tablet 3) capsule, hard all for oral use in adults **Posology** 1) 1 x daily 6 ml liquid if necessary 1 x daily 12 ml liquid 10 ml (= 11.013 g) liquid contain 0.6 g soft extract 2) 1 x daily 1-2 containing 360 mg dry extract each 3) 1 x daily 2 containing 180 mg dry extract each if necessary 1 x daily 4 **Indications** For symptomatic treatment of chronic venous insufficiency, which is characterised by swollen legs, a feeling	

Member State	Regulatory Status	Comments[1]
	of heaviness, pain, itching, cramps in the calves at night **Risks** Common gastrointestinal complaints, nausea. Hypersensitivity reactions of the skin (itching and erythema, urticaria) have been reported	
Greece	No authorized herbal medicinal products containing *Vitis vinifera* as a single drug preparation are on the market	
Hungary	1)180 mg capsules 1 capsule contains: 180 mg dry extract (DER 4-6:1), extraction solvent water (2008) 2) oral drops 81.4 mg Vitis viniferae rubrae folii extr. spissum(4-6:1), corresponding to 1.2 mg aescin/ml (1997) 3) Varixinal capsules 180 mg dry native extract of *Vitis vinifera* L., folium (DER: 4-6:1) extraction solvent: water (2009) 1) 2 capsules once a day 2) 30 drops in the morning in fasting state 3) 2 capsules once a day **Indications** 1) chronic venous insufficiency; at stage of C_2, C_3 and C_{4A} according to CEAP 2); 3) For alleviating the discomfort feeling (fatigability, tension of the legs) of the lower extremities occurring in the course of the mild venous circulation disturbance 1); 3) It is not recommended during pregnancy and lactation. Adverse effects: Gastrointestinal disorders (Common) stomach and intestinal discomfort, nausea Skin and subcutaneous tissue disorders: (Uncommon) rash, itching, hives	
Iceland	Not known	
Ireland	Not known	
Italy		Food supplements only.
Latvia	**Preparations** *Vitis viniferae* folii extractum aquosum siccum (4–6:1) 180 mg – standardized to flavonoids content 3-7% since 2004 **Pharmaceutical form** Capsule **Indications** Prevention and treatment of signs related to chronic venous insufficiency	
Lithuania	Not known	
Luxembourg	Not known	
Malta	Not known	
Netherlands	Not known	

Member State	Regulatory Status	Comments[1]
Norway	Not known	
Poland	Vitis viniferae extractum siccum (5-7:1), extraction solvent: water, capsule hard Product allowed onto the market according to previous polish pharmaceutical low (less than 10 years). **Posology** oral use: adults, 2 capsules once a day (in the morning) **Indications** support of mild symptoms of chronic venous insufficiency	
Portugal	No authorized herbal medicinal products containing *Vitis vinifera* as a single drug preparation are on the market	One authorised combination product.
Romania	Not known	
Slovakia	**Preparations** Vitis viniferae folii extractum aquosum siccum (4–6:1) 180 mg – standardized to flavonoids content 3-7% since 2005 **Pharmaceutical form** Capsule **Posology** 2 capsules (360 mg) once a day, in the morning time. Total daily dose can be increased up to 4 capsules once a day, in the morning time **Indications** 1) Prevention and treatment of signs related to chronic venous insufficiency, which is usually expressed in connection with varicoses and oedema in the lower parts of legs, fatigue, pins and needles and pain in legs **Risks (adverse drug effects)** Allergic reactions including generalized rash and urticaria. Nausea, gastric complaints	
Slovenia	Not known	
Spain	**Preparations** **(WEU)** Vitis viniferae folii extractum aquosum siccum (4–6:1), 180 mg – standardized to flavonoids content 3-7% **Posology** 2 capsules (360 mg) once a day, in the morning time. Since 2001 **Indications** Prevention and treatment of signs related to chronic venous insufficiency, which is usually expressed in connection with varicoses and oedema in the lower parts of legs, fatigue, pins and needles and pain in legs **(TU)** Powdered herb. substance 270 mg/caps	
Sweden	No authorized herbal medicinal products containing *Vitis vinifera* as a single drug preparation are on the market	
United Kingdom	Not known	

Regulatory status overview

Member State	Regulatory Status				Comments (not mandatory field)
Austria	☒ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	(4-6.1, water extr tab, 2005, 180 mg caps, 2003, drops 73.5 mg/ ml, corresp. 1.2 (aesculin), 1993
Belgium	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	WEU Caps 350 mg 1- 6/d
Bulgaria	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Cyprus	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Czech Republic	☒ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Ext aq (4-6:1) standardized 3-7% flavonoids. Caps 180 mg, 2001
Denmark	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	No product registered
Estonia	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Finland	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
France	☒ MA	☒ TRAD	☐ Other TRAD	☐ Other Specify:	WEU, eye drops Aqueous soft extract (6:1) since 1970 TU, powder(1989) or water extr. for oral use (1987)
Germany	☒ MA	☐ TRAD	☒ Other TRAD	☐ Other Specify:	dry water extr. (4-6:1), 180mg 1976, (360 mg) 2005 oral liquid, 10 ml contain 0.6 g soft extr. **TU** soft water extr. (2.5-4:1), 1976, cream, 10 g contain 282 mg soft extr.
Greece	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	No product registered
Hungary	☒ MA	☐ TRAD	☒ Other TRAD	☐ Other Specify:	(4-6.1, water extr tab, 2005, 180 mg caps, 2008 & 2009, drops 81.4 mg/ ml, corresponding. 1.2 (aesculin), 1997
Iceland	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Ireland	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known

Member State	Regulatory Status				Comments (not mandatory field)
Italy	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Food Suppl. No product registered
Latvia	☒ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	WEU Ext aq (4-6:1) standardized 3-7% flavonoids caps 180 mg, 2004
Liechtenstein	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Lithuania	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Luxemburg	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Malta	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
The Netherlands	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Norway	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Poland	☒ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	
Portugal	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	No product registered
Romania	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Slovak Republic	☒ MA	☐ TRAD	☒ Other TRAD	☐ Other Specify:	WEU Ext aq (4-6:1) standardized 3-7% flavonoids. Caps 180 mg, 2005
Slovenia	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known
Spain	☒ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	WEU Ext aq (4-6:1) standard. 3-7% flavon. caps 180 mg Powdered herb. substance 270 mg/caps
Sweden	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	No product registered
United Kingdom	☐ MA	☐ TRAD	☐ Other TRAD	☐ Other Specify:	Not known

MA: Marketing Authorisation
TRAD: Traditional Use Registration
Other TRAD: Other national Traditional systems of registration
Other: If known, it should be specified or otherwise add 'Not Known'
This regulatory overview is not legally binding and does not necessarily reflect the legal status of the products in the MSs concerned.

2. Historical data on medicinal use

2.1. Information on period of medicinal use in the Community

Red grapevine leaves, from *Vitis vinifera* L. [Fam. *Vitaceae*], are rich in flavonoids including anthocyanins, oligomeric proanthocyanidins (OPCs) etc.
The knowledge of the medicinal potential of grape vine *(Vitis vinifera)* can be traced back far in history:

- In Europe, the leaves of Vitis vinifera are documented in the literature of traditional medicine for their astringent and homeostatic properties where they are utilized in the treatment of diarrhoea, bleeding, haemorrhoids, varicose veins and other circulatory and venous diseases [Anonymous 2004; 2005; 2006; Bombardelli & Morazzoni 1995].
- In Turkish folk medicine, vine leaves are known to have a diuretic effect, while the juice of leaves have been used as an eye bath [Kosar et al. 2007].
- Native North American indigenous peoples used the leaf tea of related fox grape (*Vitis labrusca* L.) for treating diarrhoea, as well as for hepatitis, stomach aches and thrush and externally poulticed the wilted leaves for sore breasts, rheumatism, headaches and fevers. Other closely related Vitis species have been used similarly.
- In Indian Medicine: Grape is used for headache, dysuria, scabies, skin disease, gonorrhoea, haemorrhoids and vomiting [PDR for herbal Medicines 2004].

Since ancient times, beneficial effects on health have been ascribed to wine and vine leaves, as confirmed by numerous "recipes" reported in Egyptian papyri, the Sumerian tablets, the writings of Hippocrates of Cos (5th-4th century BC), Celsus (1st century AD), Galen (130-201 AD) and Paracelsus (1493-1541). The use of vine leaves has been documented. Retracing therapeutic literature from France showed the origin of using red leaves shortly after first incidence of the respective grape vine varieties called "teinturiers" [Schneider, 2007i]. The herbal substance and herbal preparations have a long history of use in folk medicine where French winegrowers collected the red vine leaves at the time of the grape harvest to make infusions and paste-like poultices from them. The infusions were filled into bottles and regularly ingested in small quantities. The paste from vine leaves was used for the treatment of swollen, painful legs. Nowadays, the extracts of grapevine leaves were developed into modern herbal medicaments mainly used against venous diseases and for its effects on microcirculation [Rabe et al. 2005; Volonté M, Petrini, 2004; Volonté et al. 2003]. The vine, fresh and brined or fermented are used as food (mixtures of rice and spices with or without meat are wrapped with vine leaves) and have been widely consumed as traditional foods (Dolmas) around the Mediterranean countries [Kosar et al. 2007]. They are also used for diarrhoea, vomiting and varicose treatment [Gharib Naseri & Heirari 2006].

There is evidence of the use of grapevine leaf outside France, in Italy in 1957, where the Biosedra preparation was also tested in vascular disorders in gynaecology, producing a positive effect on capillary fragility.

As early as 1960, a clinical study was published in Germany on the venous efficacy of a product, which contained a fluid extract of grapevine leaf, with a drug/extract ratio of 1:1. The content of anthocyanin was set at 600 µg/ml,which was subsequently changed to 1.2 mg bioflavonoids per ml (German *Rote Liste* 1974). In 1969, a company registered the preparation as a medicinal product, with the indications of varices, phlebitis, thrombophlebitis, calf cramps and leg oedema. The successor product, has been marketed since 1971. The capsules and tablets contain 180 mg and 360 mg of dried extract of grapevine leaves.

An overview of grapevine leaf components and its supposed pharmacological action as well as the polyphenol composition of *Vitis vinifera* can be found in the literature [Anonymous 2003; 2004; Petrini et al. 2003; Schneider, 2007i; 2007ii; Schneider et al. 2008; Schaefer & Red 2003].
Nonclinical *in vitro* and *in vivo* studies suggested protective effects of components from extracts of grapevine leaves on the venous system; e.g. procyanidines [Maffei et al. 1994; Constantini et al. 1999], and flavonoids [Nees et al. 2003]. For the extract preparation *(Extractum Vitis viniferae foliae aquosum siccum, 4-6:1)*, dried leaves of red varieties of *Vitis vinifera* L. (Vitis viniferae folium) which comply with the monograph described in Pharmacopée Français (10ème edition) for "vigne rouge" are

used. Thus, the herbal substance consists of the dried leaves of the black to pulp-red grape which finally undergo a specific production process resulting in a defined flavonol content in the dry extract preparation. While the whole dry extract preparation as such is considered as active agent, it is particularly characterized by its content of flavonol glycosides and glucuronides, i.e. quercetin-3-O-β-D-glucuronide, quercetin-3-O-β-glucoside, and kaempferol-3-glucoside. These flavonoids are considered to contribute predominantly to pharmacological effects. The extract AS 195 contains a total of 4-7% of flavonol glycosides, quantified as quercetin-3-O-β-D-glucuronide.

Grapevine leaves and extracts thereof have been traditionally used for the treatment of symptoms associated with venous insufficiency for more than 70 years, in France. Extracts have been introduced in a number of European countries, e.g. Austria, Belgium, Czech Republic, Spain, Switzerland, Italy, and the United Kingdom. It is indicated for the prevention and treatment of Chronic venous insufficiency (CVI), associated with varicose veins including oedema of the lower leg, heavy or tired legs, sensation of tension, tingling and pain.

Based on the survey of products available in the Member states, the traditional use of red leaves preparations has been demonstrated especially in France, Germany, Spain and Austria, for at least 30 years.

Red vine leaf has been included in the French official list entitled "Avis aux fabricants concernant les demandes d'Autorisation de mise sur le marché" by virtue of which, the products containing it were subjected to a simplified registration procedure before the implementation of the Directive 2004/24/EC.
In France: "Vigne Rouge" (= red vine leaf) is regarded as one of the herbal drugs whose efficacy and safety has been proven by thorough literature studies and long-term traditional use. The Pharmacopèe Française X ed. includes the monographs of "Vigne rouge" and "Extrait de vigne rouge (sec)" and the traditional use of red vine leaves is discussed in "Rotes Weinlaub" [Schneider, 2007i; 2007ii].

Herbal medicinal products with red vine leaf have a long tradition in France. The official compendium "Médicaments à base de plantes", issued by the French department "Ministère des Affaires sociales et de la solidarité", released 1990, and comprised the long tradition of diverse herbals in France. There are listed many herbals that have been used in France for a long time, at least for about 15–20 years. [Les Cahiers de l'Agence 1998] state the use of red vine leaf for symptomatic relief of chronic venous disease ("No 015+016: " Traditionellement utilise dans le traitement symtomatique des troubles fonctionnels de la fragilité capillaire cutanée, tels que ecchymoses, pétéchies, etc."; No. 017+018: "Traditionnellement utilisée: - dans les manifestations subjectives de l'insuffisance veineuse telles que jambes lourdes; - dans la symptomatologie hémorroïdaire").
Different products prepared of water extracts of "Vigne Rouge" (5:1) have been available in oral forms, in France to treat blood vessels fragility in order to reduce the feeling of heavy legs and haemorrhoids related disorders. The posology range from 169 mg to 200 mg, two to three times a day, for four weeks:

Vigne rouge, capsules: 1 capsule contains 200 mg of grapevine leaves dry aqueous extract is used, a DER of about (4-6:1). The product is registered since 1987. The manufacturer recommends the use of 2 capsules per day (1 capsule mornings and one evening, with a glass of water). The product is traditionally used to reduce fine blood vessels fragility, in order to reduce the feeling of heavy legs and haemorrhoids related disorders.
OR
1 capsule contains 169 mg of grapevine leaves dry extract (3:1). The manufacturer recommends the use of up to 3 capsules per day. The product is traditionally used to reduce the feeling of heavy legs, haemorrhoids related disorder and fine blood vessels fragility.

An article from 'Deutsches Medizinisches Journal' published in 1960 by K. Güthenke, "Wesen und Behandlung der Bindegewebsschwäche, insbesondere der Veneninsuffizienz mit Weinblattextrakt" (Nature and Treatment of weak connective tissues, in particular venous insufficiency, ref.) describes the long traditional use of grapevine leaves for treatment of diseases of the leg veins, and demonstrates that vine leaf extracts have been already traditionally used before 1960, in Germany. Registrations for the active ingredient *Extractum vitis viniferae foliae aquosum siccum* exist since 1969. Since 1974 a solution for oral use has been available. 20 ml contained 1-2 g of extract from leaves of *Vitis vinifera* (1:4-5) and 0.02 g of aesculin. In 1999 aesculin was removed from the formulation. The recommended dosage is 2-3 x 30 drops per day. The product is traditionally used to treat diseases of the leg veins (f.e. varicose veins, chronic venous insufficiency), to reduce pains and feeling of heavy legs, night time cramps in the calf, itching and swollen legs. The use of this product can be proven for over 30 years.

In 1991 the galenical form of hard gelatine capsules was registered and marketed for the first time. One capsule contained a combination of 180 mg of *Extractum vitis viniferae foliae aquosum siccum* and 3 mg aesculin (a coumarine glucoside that naturally occurs in the horse chestnut *Aesculus hippocastanum*). This herbal medicinal product was indicated for the treatment of venous insufficiency, varices, haemorrhoids, heavy tired legs and feet, essential oedema and calf cramps. The daily posology was 2-3 capsules a day. In 1999, aesculin was removed from the product and the only active substance declared was the dry extract of grapevine leaves. The capsules contain 180 mg of *Extractum vitis viniferae* (extraction solvent: water, DER: 4–6:1), with a recommended use of 2-4 capsules per day.
A cream (ointment) -containing soft extract (4-6:1; water) in a cream base
(10 g cream contain 282 mg soft extract)- has been traditionally used since 1976 to relieve symptoms of tired legs applying it several times daily.

Conclusions on traditional use
Based on information obtained from Member states and data retrieved from handbooks it can be concluded that the following extracts and uses of grapevine leaf extracts fulfil the criteria for traditional use:

Herbal substance

Not applicable.

Herbal preparations:

- Comminuted dried leaves
- Powdered dried leaf
- Soft extract (2.5-4:1; water) in a cream base

Concerning the preparation: dry extract (3:1; water), the product has been on the market since 1990 (France) and does not comply with the requirements of Directive 2004/24/EC for THMP. There is no complete information on the manufacturing process and DER of this preparation, and it is not possible to evaluate the similarity with other dry extracts under WEU.

2.2. Information on traditional/current indications and specified substances/preparations

Indication:

Traditional herbal medicinal product:

a) To relieve symptoms of discomfort and heaviness of legs related to minor venous circulatory disturbances.

b) For symptomatic relief of itching and burning associated with haemorrhoids.

c) For symptomatic treatment of cutaneous capillary fragility.

The product is a traditional herbal medicinal product for use in the specified indication exclusively based upon long-standing use.

2.3. Specified strength/posology/route of administration/duration of use for relevant preparations and indications

Posology:

-Dried leaves in herbal teas, 40 g/l. One cup (10g/250 ml) 2-4 times/day [ESCOP 2009]
-Comminuted total powder: 270-350 mg, 3 times/day (before the meals), up to 2100 mg/day
-Soft extract (2.5-4:1; water), in a cream base, (10g cream contain 282 mg soft extract), a thin layer should be spread on the affected area 1-3 times/day

-Dry extract (DER 4-6:1; water), single dose: 360-720 mg, daily dose: 360-720 mg

3. Non-Clinical Data

Non-clinical *in vitro* and *in vivo* studies suggested protective effects of components from extracts of grapevine leaves on the venous system; e.g. procyanidines [Maffei et al. 1994; Constantini et al. 1999], and flavonoids [Nees et al. 2003].

3.1. Overview of available pharmacological data regarding the herbal substance(s), herbal preparation(s) and relevant constituents thereof

Constituents

Vine leaves contain a wide range of polyphenol flavonoids including flavon(ol)-glycosides and glucuronides, quercetin-3-O-beta-D-glucuronide (most abundant of flavonoids), isoquercitrin, anthocyanins, oligomeric proanthocyanidins [Hmamouchi et al. 1997], catechin, epicatechin monomers and dimmers and gallic acid. The phytoalexin trans-resveratrol, another polyphenolic substance belonging to the stilbene group, can also be found in grape vine [Langcake et al. 1979]. It has been referred in the international literature that $AlCl_3$ is a potent elicitor of resveratrol production in vine leaves [Andrian et al. 1996]. In vine leaves, also organic acids appear, mainly malic and oxalic acid but also tartaric acid. Citric, fumaric and succinic acid can be detected in the leaves only in traces. Compared to the grape berries, grape leaves are richer in the content of carotenoids and vitamin C. According to the French Pharmacopoeia, the dried leaves of grapevine should contain at least 4% of total polyphenols and 0.2% of anthocyanins [Pharm. Franc. X., 1996; Jonadet 1983; Lardos & Kreuter 2000; Laparra et al. 1989].

The leaves of the red varieties are very rich in tannins from the catechin group. The composition in tannins of leaves depends on the phase of development and on their position on the plant. In autumn, catechin, gallocatechin and epicatechingallate can be found in the leaves. From catechins and/or leukoanthocyanidines so called oligomeric proanthocyanidins, colourless substances, are formed. The greatest part of anthocyans consists of malvidin glucosides but also delphinidin, cyanidin and petunidin glucosides occur [Laparra et al. 1989]. The highest content of anthocyans can be detected in the red leaf especially in autumn, in the time between the vintage and the shedding of leaves [Raynaud, 2006,

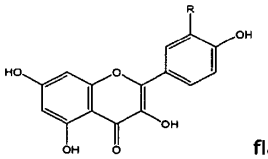
Darné & Glories 1988]. From a pharmacological point of view, the polyphenols, for example flavonoids, are the most important substance group [Bruneton 1999; 2002; Raynaud, 2005; Diaz Lanza et al. 1995].

Analytically the following chemical compounds have been determined in the leaves of *Vitis vinifera*:
- *Flavonoids* (up to 3.5% for red vine leaf, the content is higher in green leaves 4-5%): including kampferol-3-0-glucosides, quercetin-3-O-glucosides
- *Tannins*: procyanidolic oligomers (proanthocyanidins, about 4%) including constituents monomers of cathechin epicatechin
- *Stilbenes*: resveratrol and viniferins [Chung et al. 2003]
- *Fruit acids*: including tartaric acid, malic acid, succinic acid, citric acid, oxalic acid
- *Phenylacrylic acid derivatives*: p-coumaroyl acid, caffeoyl acid, feruloylsuccinic acid

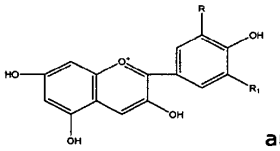
In a recent comparative study of 135 samples of grapevine leaves of different origin, the flavonol, anthocyanin and polyphenol contents have been determined. Total flavonol content was found to be between 0.6% and 3.5%, anthocyanin content between 0.2% and 1.45% and polyphenol content between 4.6% and 18.9%. HPLC methods used for determining anthocyanins and flavonols in grapevine leaves were validated and findings were compared to results produced by assays described in the French Pharmacopoeia. Whereas the correlation between conventional photometric and HPLC methods was satisfactory for anthocyanins, the correlation between the pharmacopoeia assay for total polyphenols and the HPLC analysis for flavonols was poor. As flavonol compounds are considered relevant for the vasoprotective effect of grapevine leaves, their content in starting material used in the production of herbal medicines needs to be quantified [Schneider et al. 2008].

Overview on main polyphenolic compounds in Grape Vine

Flavonoids	Flavones (Quercetin Kaempferol), Flavanes
Anthocyans	Leucoanthocyanidins, Anthocyanidins -responsible for the blue and red coloring of leaves, flowers and fruits -The concentration of anthocyans in the red colored leaf is high.
Catechins	• Grapevine leaf is rich in cathechins • Concentration of cathechins is dependent on: ->the leaf's position on the plant the phase of development of the leaf • In autumn cathechin, gallocathechin, epicathechingallat are present in the leaf.
Stilbenes	Resveratrol, *trans*-Resveratrol belong to the stilbenes (phenolic sub group) is a phytoalexin= stress-induced plant metabolite resveratrol can be found only in stressed leaves stress factors : i.e. fungal infection, UV-irritation, injury ->resveratrol is present in different forms depending on the stage of the plant's stress answer

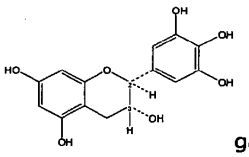


flavonol

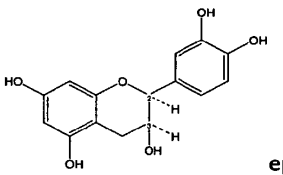


anthocyan

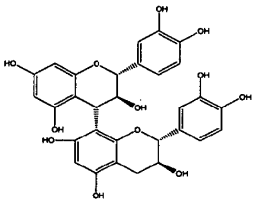
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gallocatechin



epicatechin



procyanidol

Primary pharmacodynamics

In Europe, the leaves of *Vitis vinifera* are documented in the literature of traditional medicine through several comprehensive reviews for their astringent and homeostatic properties where they are utilized in the treatment of diarrhoea, bleeding, haemorrhoids, varicose veins and other circulatory and venous diseases [Bombardelli 1997; Anonymous 2003; 2004; Petrini et al. 2003; Schneider, 2007i; 2007ii; Schneider et al. 2008; Schaefer & Red 2003; Volonté & Petrini 2004; Volonté et al. 2003; Weber, 2000].

Anti-inflammatory and anti-oedematous effect

In an *in vitro* study, venular endothelial cells were isolated from Wistar rats and cultivated on porous filters to confluent monolayers. These preparations respond to certain release products from simultaneously activated blood platelets and polymorphonuclear granulocytes (PMN) with a rise in hydraulic conductivity that, *in-situ*, would lead rapidly to local oedema, arteriolar constriction and venular thrombosis. In this model, selectively activated PMN alone induced only a modest increase in endothelial hydraulic conductivity that could be prevented by uric acid, an antioxidant. ASA prevented the activation of the blood cells. A standardized extract from grapevine leaves (AS 195), containing in particular the flavonoids quercetin-3-O-ß-D-glucuronide and isoquercitrin (quercetin-3O-ß-D-glucoside), not only prevented the deleterious effect of the release products on the venular endothelial monolayers but, applied promptly to an endothehium damaged by prior exposure to these release products, resulted in the repair of the endothehium [Nees et al. 2003]. In another study, the scavenge by procyanidines (polyphenol oligomers from *Vitis vinifera* seeds CAS 85594-37-2) of reactive oxygen species (ROS) involved in the onset and the maintenance af microvascular injury has been studied in phosphatidylcholine liposomes (PCL) using two different models of free radical generation. In an iron-promoted (Fenton-driven) model, procyanidines had a remarkable, dose-dependent antilipoperoxidant activity (IC50 = 2.5 µmol/l), more than one order of magnitude greater than that of the monomeric unit catechin (IC50 = 50 µmol/l). In the second model, procyanidines were highly effective in preventing conjugated diene formation in both the induction (IC50 = 0.1 µmol/l) and propagation (IC50 = 0.05 µmol/l) phases. The scavenging effect of tocopherol was weaker with IC50 of 1.5 and 1.25 µmol/l [Maffei Facino et al. 1994; Wollina et al. 2006].

The grapevine leaf extract is characterized by its content in flavonol glycosides and glucuronides, in particular quercetin-3-O-β-D-glucuronide, isoquercitrin (quercetin-3-O-β-glucoside), and kaempferol-3-O-glucoside. The most informative investigations were performed using confluent venular endothelial cells from animal and from human origin [Nees, 2003]. An attack by release products from simultaneously activated blood platelets (P) and polymorphonuclear granulocytes (PMN) leads to a breakdown of the venular endothelial barrier. Clinically this would result in formation of oedema and

constriction of microvessels. Red vine leaf RVLE extract was able to support the repair of the venular barrier after an attack by said release products. These effects could be demonstrated on cells of from animal origin as well as on cells isolated from the human heart [Nees 2003i, 2003ii]. Quercetin-3-O-ß-D-glucuronide that had been isolated from the extract and was shown to be the major metabolite being present in human plasma after ingestion of RVLE acted in the same way. The extent to which cellular gaps opened and allowed the supernatant to flow through could be measured as "hydraulic conductivity". 0.7 mg dry RVLE/ml incubation medium nearly prevented the opening of the barrier in the presence of activated PMN/P. Preincubation of the venular cell layer with 50 µM quercetin-3-O-ß-D-glucuronide for 7 days markedly reduced the hydraulic conductivity compared to untreated cells.

In a publication by [Jonadet et al. 1983], the authors describe studies conducted with anthocyanosides extracted from *Vitis vinifera* (a), *Vaccinium myrtillus* (b) and *Pinus maritimus* (c). The results obtained *in vitro* indicated that these compounds inhibit elastase, a proteolytic enzyme which plays a role in the deterioration of conjunctive tissue and elastic fibers and is involved in certain pathological vascular conditions. The IC50 values are 0.13 mg/ml for (a), 0.20 mg/ml for (b) and 0.31 for (c). Lineweaver-Burk curves revealed that the inhibition was not competitive. Results obtained *in vivo* show that the angioprotective activities of these compounds can be classified in decreasing order as follows: (a), (b) and (c).

Nees developed a measurable and reproducible *in vitro* experimental model to investigate the effect of substances capable of modifying the hydraulic conductivity of the endothelial barrier of the venules. In *in vitro* experiments, this extract has been shown to have a "sealing" effect on the endothelium of the venules and a protective action against fluid extravasation induced by incubation with chemical mediators of inflammation [Nees S et al. 2003i].

Red vine leaf extract (RVLE) prevented the deleterious effect of the release products of P and PMN on venular endothelial cells. In addition, the extract was able to support the repair of the venular barrier after an attack by said release products [Smith, 1999].

The anti-inflammatory activity of the oligomeric stilbene a-viniferin from *Vitis* has been also tested as well as its mode of action though inhibition of cyclooxygenase-2 and inducible nitric oxide synthase [Chung et al. 2003].

Hepatoprotective activity

The hepatoprotective effect of ethanolic extract and its four different fractions ($CHCl_3$, EtOAc, n-BuOH, and remaining water fraction) of *Vitis vinifera* L. leaves was investigated against carbon tetrachloride (CCl_4)-induced acute hepatotoxicity in rats. The ethanolic extract was found active at 125 mg/kg dose (per os). The ethanolic extract was fractionated through successive solvent-solvent extractions and the n-BuOH fraction in 83 mg/kg dose possessed remarkable antioxidant and hepatoprotective activities. Liver damage was assessed by using biochemical parameters (plasma and liver tissue MDA [malondialdehyde], transaminase enzyme levels in plasma [AST-aspartate transaminase, ALT-alanine transferase] and liver GSH [glutathione] levels). Additionally, the pathological changes in liver were evaluated by histopathological studies. Legalon 70® Protect was used AS standard natural originated drug [Orhan et al. 2007].

Antimicrobial activity

Ethanol extracts of *Vitis vinifera* (leaves, raw fruits, young branches; 2:1:1, v/v/v), were investigated for their antimicrobial activity against 14 pathogenic bacterial species and a yeast, *Candida albicans*, using the agar well diffusion method, 19 Turkish traditionally used medicinal plants. *Vitis* leaves showed broad-spectrum antimicrobial activity [Oskay & Sari 2007].

Antioxidative activity

The effect of grape leaf extract (GLEt) on antioxidant and lipid peroxidation states in liver and kidney alcohol induced toxicity has been evaluated. *In vitro* studies with DPPH* and ABTS* (cation radical) showed that GLEt possesses antioxidant activity. *In vivo* administration of ethanol (7.9 g/kg bw/day) for 45 days resulted an activity of liver marker enzymes (AST, ALT, ALP and GGT), lipid peroxidation markers (TBARS, lipid hydroperoxides) in liver and kidney with significantly lower activity of SOD, CAT, GPx, GST and non-enzymatic antioxidants (vitamin E, vitamin C and GSH) in liver and kidney AS compared with control rats. Administration of ethanol along with GLEt significantly decreased the activities of liver markers enzyme in serum towards near normal level. GLEt at a dose of 100 mg/kg was highly effective than 25 and 50 mg/kg body weight. In addition GLEt also significantly reduced the levels of lipid peroxidation and addition, significantly restored the enzymic and non-enzymatic antioxidants level in liver and kidney of alcohol administration rats. This observation was supplemented by histopathological examination in liver and kidney. These data suggest that GLEt exerts its protective effect by decreased the lipid peroxidation and improving antioxidants status, thus proving itself AS an effective antioxidant in alcohol induced oxidative damage in rats [Pari & Suresh 2008].
The antioxidative activity of the ethanolic extract of *Vitis vinifera* L. leaves was investigated. The ethanolic extract of *V. vinifera* leaves at 250 mg/kg dose was found to have the highest antioxidant activity [Sendogdu et al. 2006]. Comparable results have been published by [Kosar et al. 2007], from extracts from fresh, dried and brined leaves of *Vitis* [Monagas M et al. 2006].

Bronchodilatory activity

It has been investigated the effect of grape leaf hydroalcoholic extract on isolated rat tracheal contractions induced by KCl and acetylcholine. The trachea was removed from male adult Sprague-Dawley rat and placed in an organ bath containing Krebs-Henseleit solution and contractions were recorded isometrically. The results demonstrate that the grape leaf extract at 0.5, 1, 2, 4 and 8 mg/ml significantly reduces the tracheal contractions induced by KCl (60 mM) dose-dependently ($P<0.0001$). Acetylcholine (55 µM)-induced tracheal contractions were also attenuated at the same concentration of the extract ($P<0.0001$). The grape leaf extract induced relaxation in the KCl-induced contraction in trachea was unaffected neither by nitric oxide (NO) synthase inhibitor (L-NAME, 100 µM) nor by beta-adrenoceptor antagonist (propranolol 1 µM). Our results suggest that the bronchodilatory effect of grape leaf extract is mediated via the voltage dependent calcium channels on the smooth muscle cells membrane. Furthermore, the beta-adrenergic and NO are not involved. The extract was prepared from dried grape leaves 50 g were wixed with 230 ml of ethanol 70% for 72 hours at room temperature and stirred four times a day. The mixture was filtered and the solvent evaporated. The obtained extract was 9.5 g [Gharib Naseri & Heidari, 2006].

Vasorelaxant effect on isolated rat aorta

The relaxant effect of *Vitis vinifera* leaf hydroalcoholic extract (VLHE) on isolated rat thoracic aorta contractions induced by phenylephrine and KCl, and the role of aorta endothelium on this action has been investigated. Rat aorta was removed and placed in an organ bath containing Krebs-Henseleit solution and aorta contractions were recorded isometrically. The results demonstrate that the VLHE at 0.125, 0.25, 0.5, 1 and 2 mg/ml reduces the endothelial intact aorta contracted by phenylephrine (1 mu M) significantly and dose-dependently. Endothelial denuded aorta showed the same relaxation but in much less extent. The IC_{50} of these two groups were 0.454±0.08 and 1.73±0.23 mg/ml respectively. VLHE also reduced the aorta contractions induced by KCl (80mM). The relaxatory effect of VHLE on KCl-induced contractions were less than those evoked by phenylephrine. Soluble guanylate cyclase inhibitor (methylene blue, 10 mu M) and nitric oxide synthase inhibitor (L-NAME, 100 mu M) reduced the VHLE-induced relaxation in the intact aorta significantly but, atropine (1 mu M) was unable to decrease this vasorelaxant effect. These results suggest that the most vasorelaxant effect of VHLE

on rat aorta is endothelium-dependent and also nitric oxide (NO) and cGMP are involved in this action [Gharib Naseri et al. 2004].

Spasmolytic effect

The effect of grape leaf hydroalcoholic extract (GLHE) on rat colon contractions induced by some spasmogens has been investigated. A piece of distal colon from male adult Wistar rats were dissected and mounted in an organ bath containing Tyrode solution and colon contractions recorded by an isotonic transducer under 1g resting tension. The GLHE (0.5- 4 mg/ml) reduced the contractions induced by KCl (60 mM), $BaCl_2$ (4 mM), acetylcholine (1 mu M) dose-dependently ($P<0.001$). The spasmolytic effect of GLHE on ACh-induced contraction was unaffected by propranolol (1 mu M), phentolamine (1 mu M), L-NAME (300 mu M), and naloxone (1 mu M). In Ca^{2+}-free but rich in KCl (120 mM) Tyrode solution, cumulative concentrations of $CaCl_2$ induced colon contractions which, were inhibited by the extract. Glibenclamide (3 mu M) had no effect on the extract spasmolytic activity, but tetraethylammonium (5 mM) contracted the pre-relaxed colon induced by the extract. Results suggest that the grape leaf hydroalcoholic extract spasmolytic effect is due to the blockade of the voltage dependent calcium channels and activation of Ca^{2+}-operated potassium channels [Gharib Naseri et al. 2006].
The effect of *Vitis vinifera* leaf hydroalcoholic extract (VLHE) on isolated rat tracheal contractions induced by KCl and acetylcholine has been also studied. The trachea was removed from male adult Sprague Dalwey rat and placed in an organ bath containing Krebs-Henseleit solution. The tracheal contractions were recorded isometrically under 1.5 g initial tension.

Results: The results demonstrate that the VLHE at 0.5, 1, 2, 4 and 8 mg/ml reduces the tracheal contractions induced by KCl (60 mM) significantly and dose-dependently ($P<0.0001$). Acetylchline (55 mu M)- induced tracheal contractions were also attenuated by the same extract doses significantly ($P<0.0001$). The VHLE-induced relaxation in the KCl-induced contraction in trachea was not affected neither by nitric oxide synthase inhibitor (L-NAME, 100 mu M) or beta-adrenoceptor antagonist (propranolol 1 mu M) and by muscarinic receptors antagonist (atropine 30 mu M).

Conclusion: These results suggest that the relaxant effect of VHLE on rat trachea is evoked via voltage dependent calcium channel blockage and beta-adrenoceptors, NO and cholinergic receptors are not involved in this relaxant effect of VHLE [Gharib Naseri & Heidari, 2006].

Diuretic activity

Aqueous and alcoholic extracts of *Vitis vinifera* leaves were tested for diuretic activity in rats. The parameters studied on individual rat were body weight before and after test period, total urine volume, urine concentration of Na^+, K^+ and Cl^-. In the present study alcoholic and aqueous extracts of *Vitis vinifera* leaves (100 mg/kg of body weight) showed increase in urine volume, cation and anion excretion. Furosemide was used as a reference diuretic [Shastry et al. 2002].

Secondary pharmacodynamics

Flavonoid-containing herbal preparations and isolated flavonoids have been reported to exhibit a wide range of biological effects, including antioxidant and enzyme-modulating actions and anti-allergic, anti-atherosclerotic, antithrombotic, antiviral, antibacterial, anti-inflammatory, antiproliferative, anticarcinogenic, antispasmodic and diuretic effects [Middleton et al. 1992, 1996; Hertog et al. 1996; Hollmann and Katan 1999; Pietta 2000].

In vitro tests
In the context with Chronic venous insufficiency (CVI) it would be of interest to evaluate whether grapevine leaf extract exhibits any effects on platelet aggregation. The effect of flavonoids on platelet aggregation was studied *in vitro* using platelet-rich plasma from four healthy male volunteers aged 24,

29, 35 and 47 years. The flavonol glycoside quercetin 3-O-β-D-glucoside and various flavonoid aglycones including quercetin, apigenin and (+)-catechin were added to both platelet-rich plasma and washed platelets at concentrations of 0, 0.25, 2.5, 25, 250 and 2500 µmol/l. Indomethacin was used as a positive control. The results showed that the flavonoid aglycones inhibited platelet aggregation but that quercetin-3-O-β-D-glucoside did not significantly affect aggregation at any of the concentrations tested [Janssen et al. 1998].

In vivo tests

A randomised, placebo-controlled, multiple crossover study was conducted, in which 18 healthy volunteers (9 men and 9 women; mean age 25 ± 8 years; BMI 22 ± 1 kg/m^2) were given a preparation of onions equivalent to 114 ± 3 mg quercetin every day for 2 weeks in accordance with a fixed schedule. Blood samples were taken from the participants at defined intervals and the relationship between platelet aggregation and flavonoid concentration was plotted. The results showed that a daily dose of 114 mg quercetin did not have any significant effect on platelet aggregation. The mean plasma concentration of quercetin was 447 ± 117 ng/ml during the study period [Janssen et al. 1998].
Besides the flavonoids, grapevine leaves contain the following types of compound that are likely to be carried over to an aqueous extract [Bombardelli & Morrazoni 1995]:

- anthocyanins
- phenolic acids
- catechins and proanthocyanidins (flavanols)
- anorganic acids, sugars and mineral salts

The anthocyanins present in grapevine leaves are mainly glucosides of cyanidine, peonidine and malvidine [Boucheny A, Brum-Bousquet 1990]. Anthocyanins have been reported to possess antioxidant and vasoprotective properties. There are some doubts whether these effects measured in *in vitro* experiments or in animal studies using high doses are relevant for humans [Prior, 2004]. Absorption of anthocyanins appears to be low [Lapidot et al. 1998]. The amount of anthocyanins administered with 360 or 720 mg grapevine leaf extract is considered negligible in respect of the pharmacodynamic action of the substance.
The phenolic acids present in grapevine leaves are mainly derivatives of cinnamic acid, vanillic acid and caffeic acid [Boucheny A, Brum-Bousquet 1990]. These compounds are ubiquitous in vegetables and preparations of herbal origin and therefore not expected to contribute to the specific pharmacological effects of grapevine leaf extract.
The catechins found in grapevine leaves are mainly catechol and epicatechol [Boucheny A, Brum-Bousquet 1990]. Of the condensed catechins (proanthocyanidins) procyanidins B1 and B2 are the prevailing compounds, followed by procyanidins B3 and B4. Procyanidins have been reported to possess antioxidant and anti-oedematous activity [Bombardelli & Morrazoni 1995]. The study did not investigate the form in which the flavonoids were resent prior to administration. Onions are known to be rich in quercetin glucosides, especially quercetin-4′-glucoside and quercetin-3, 4′-O-diglucoside. After administration of fried onions quercetin glucuronides can be detected in plasma [Aziz et al. 1998; Graefe et al. 2001]. It can be supposed that quercetin glucuronides do not influence platelet aggregation *in vivo*.

Hypocholesterolemic effect

Oral administration of procyanidins from grape seed produced a hypocholesterolemic effect in a high-cholesterol animal feed model (rats). Specifically it prevented an increase in total and LDL plasma cholesterol and a decrease in HDL [Tebib, 1994].
In normal and hypercholesterolemic rabbit aortas, OPCs significantly lowered the amounts of cholesterol bound to aortic elastin compared to controls [Wegrowski, 1984].

Antiproliferative activity

Procyanidin-rich fractions from grape *Vitis vinifera* extract showing different mean degrees of polymerization, percentage of galloylation (percentage of gallate esters) and reactive oxygen species-scavenging capacity were tested on HT29 human colon cancer cells. It has been observed that the most efficient fractions in inhibiting cell proliferation, arresting the cell cycle in G_2 phase and inducing apoptosis were the grape fractions with the highest percentage of galloylation and mean degree of polymerization. Additionally, the antiproliferative effects of grape fractions were consistent with their oxygen radical-scavenging capacity and their ability to trigger DNA condensation-fragmentation [Lizarraga et al. 2007].

Antidiabetic activity

The acute and subacute antidiabetic activities of the ethanolic extract of *Vitis vinifera* L., leaves were investigated. The acute effect was studied on the normoglycaemic, glucose hyperglycaemic and streptozotocin-induced diabetic rats; and the subacute effect was studied on same diabetic rats for 15 days. The blood glucose levels were measured by using blood glucose measuring strips based on glucose-oxidase method. The ethanolic extract of *V. vinifera* leaves at 250 mg/kg dose was found to possess a high antidiabetic activity. Mainly condensed tannins and flavonoids were suggested to contribute in the activity [Sendogdu et al. 2006].
The acute and subacute (15 days) hypoglycaemic and antihyperglycaemic effect of the two different doses (250, 500 mg/kg) of the aqueous extract from the leaves of *Vitis vinifera* L. were evaluated in this study. The aqueous extract was further fractionated through successive solvent extractions and the acute effect of different doses of its subfractions, 25 mg/kg for ethylacetate fraction, 80 mg/kg for *n*-butanol fraction and 375 mg/kg for remaining aqueous fraction were investigated using normal, glucose-hyperglycaemic and streptozotocin-induced diabetic rats. Blood glucose levels were measured according to the glucose oxidase method. Tolbutamide was used as a reference drug at a dose of 100 mg/kg. The antioxidant activity of the test samples was studied in the liver, kidney, heart tissues of diabetic rats by measuring malondialdehyde (MDA) and glutathion (GSH) levels. All results were compared to the diabetic control groups. The results showed that the EtOAc fraction was rich in polyphenolics and possessed a significant antihyperglycaemic and antioxidant activity equipotent with the reference hypoglycaemic agent (tolbutamide), when evaluated in diabetic rats [Orhan et al. 2006].

Other activities

The anti-herpes as well as the anti-mutagenic activities from the leaves and procyanidins from *Vitis vinifera* have been also assayed and published in the international literature [Girre et al. 1990; Liviero et al. 1993].

Pharmacodynamic interactions

Pharmacodynamic drug interactions of whole extracts or isolated constituents have not been reported. Various sources in the literature have suggested that the desired therapeutic effect of many flavonoids is usually obtained only after repeated oral intake of the respective preparations. This implies that the flavonoids and their metabolites accumulate in the body, and do not exert the pharmacodynamic effects seen in *in vitro* studies until a pharmacological threshold dose and relevant plasma levels has been reached. This hypothesis was confirmed by the results of a clinical study by [Kiesewetter et al. 2002]. A significant improvement in key CVI symptoms in the active treatment groups versus placebo could not be observed after 6 weeks, but became evident after 12 weeks of treatment. This could also explain why earlier studies reported a "small" or "undetectable" effect following single oral administration.

3.1.1. Assessor's overall conclusions on pharmacology

The aqueous and ethanolic extracts of *Vitis viniferae* folium have been used as adjunctive therapy for Chronic venous insufficiency (CVI), and have shown a potential benefit [Abascal & Yarnell 2007]. Based on available preclinical data, it can be concluded that the mechanism of action of orally administered extract in CVI is not well known.

3.2. Overview of available pharmacokinetic data regarding the herbal substance(s), herbal preparation(s) and relevant constituents thereof

Data on the pharmacokinetics of the whole extract or relevant components are not available.

Flavonoids are the substances which are regarded as being responsible for the pharmacological properties of grapevine leaf extract. In the plant, flavonoids are generally present in genuine form as glycosides. Grapevine leaf extract as it has been referred previously contains as major flavonoid components quercetin-3-O-glucuronide, isoquercitrin (quercetin-3-O-glucoside) and kaempferol-3-O-glucoside. The substances present in blood after ingestion of plant extracts containing polyphenols usually differ from the native compound, as they are metabolites resulting from digestive and hepatic activity. The examination of pharmacokinetics and metabolism of the major and active constituents of such plant extracts in humans is therefore strongly recommended [Manach et al. 2005].
Various sources in the literature have suggested that the desired therapeutic effect of many flavonoids is usually obtained only after repeated oral intake of the respective preparations. This implies that the flavonoids and their metabolites accumulate in the body and do not exert the pharmacodynamic effects seen in *in vitro* studies until a pharmacological threshold dose and relevant plasma levels has been reached. This hypothesis was confirmed by the results of a clinical study by [Kiesewetter et al. 2000]. A significant improvement in key CVI symptoms in the active treatment groups versus placebo could not be observed after 6 weeks, but became evident after 12 weeks of treatment.

The grapevine leaf extract represents a complex mixture of different classes of compounds, having each their own pharmacokinetic characteristics. A significant portion of the components of grapevine leaf extract belongs to the group of polyphenols, such as anthocyanins, proanthocyanidins, catechins and phenolic acids [Bombardelli & Morazzoni 1995; Boucheny & Brum-Bousquet 1990]. Recently, [Manach et al. 2005] presented a review of various bioavailability studies in humans concerning polyphenols. They came to the conclusion that anthocyanins and proanthocyanidins are absorbed to a very low extent only. As an exception, the major circulating forms of anthocyanins are the intact glycosides [Manach et al. 2004]. The polymeric nature and therefore high molecular weight of proanthocyanidins should limit their absorption through the gut barrier. Hydroxycinnamic acids such as caffeic acid are rapidly absorbed from the small intestine and are conjugated, in particular glucuronidated in the same way as flavonoids. However these compounds are naturally esterified in plant products (as chlorogenic acid) and this impairs their absorption. Only the colonic microflora possesses esterases which are capable of hydrolyzing these bindings. Consequently, the efficacy of absorption of phenolic acids is markedly reduced for their esterified forms compared with the free form [Manach et al. 2004].

3.2.1. Assessor's overall conclusions on pharmacokinetics

No information is available on pharmacokinetic interactions. No information is available on metabolites.

3.3. Overview of available toxicological data regarding the herbal substance(s)/herbal preparation(s) and constituents thereof

Single-dose toxicity

The toxicity of vine leaf aqueous extract (4-6:1) following a single oral dose was determined in SPF Wistar rats (3 males and 3 females per group). The doses used were 3000, 4000 and 10.000 mg/kg. There were no signs of toxicity and no mortalities over the 14-day observation period, and no pathological or histological changes were seen at autopsy.

The toxicity of vine leaf aqueous extract (4-6:1) following a single oral dose was also determined in CFI mice (3 males and 3 females per group). The doses used were 2000, 4000 and 10.000 mg/kg. There were no signs of toxicity and no mortalities over the 14-day observation period, and no pathological or histological changes were seen in autopsy [Rabe 2005; ESCOP, 2009].

Repeat-dose toxicity

The toxicity of vine leaf soft extract when given over a 90-day period was determined in SPF Mol-Wistar rats (12 males and 12 females per group) in a study which was subjected to QAU inspection and carried out in accordance with GLP standards. The animals were given oral doses of 25, 125 or 250 mg/kg/day, or a placebo. A satellite group given 250 mg/kg/day was subjected to follow-up observation. No mortalities occurred during the study period. Observations made during the study period on bodyweight, food intake etc. revealed no evidence of any systemic toxic effects due to the extract. The values found during haematological and clinical chemistry testing were within biological limits. The autopsy findings and the results of histological tests did not reveal any evidence of changes due to the extract. The doses of extract used were therefore judged to be non-toxic in the rat.

The maximum daily dose of extract is 720 mg, which represents a dose of 10.3 mg/kg for a person weighing 70 kg. This is about one-thousandth of the maximum dose of extract used in the single-dose toxicity studies. As the maximum dose used in these studies did not give rise to any signs of toxicity, it is extremely unlikely that any toxic effects will occur in man. Preparations containing vine leaf extract have been used in European Union countries for many years and there have been no reports of any harmful effects in man to date. On this basis, it is reasonable to assume that the products reviewed in this report will not pose any safety risk on therapeutic administration [ESCOP, 2009].

Genotoxicity

The mutagenic potential of vine leaf extract was assessed in a range of established *in vitro* and *in vivo* test models (Ames test, point mutation assay and chromosomal aberration assay). It was assessed in an Ames Salmonella/microsome plate assay using Salmonella typhimurium TA-100, TA-1535, TA-98, TA-1537 and TA-1538 with and without metabolic activation; S9 mix prepared from rat liver was used as the activation system. The assay was carried out in accordance with EU recommendations and OECD Guideline 471. Five concentrations of the vine leaf extract ranging from 8 to 5000 µg/plate were used, and positive and negative controls were also included. The results obtained did not provide any evidence to suggest that vine leaf extract has any mutagenic potential [ESCOP, 2009].

Mutagenic potential of grapevine leaf extract was also assessed in two independent point mutation assays carried out on the HGPRT locus of Chinese hamster V79 cells with and without metabolic activation; S9 mix prepared from rat liver was used as the activation system in each case. The assays were designed and conducted in accordance with the guidelines and recommendations on genotoxicity testing that were current at the time. On the basis of preliminary toxicity tests, a concentration range of 3.0 - 5000 µg/ml was selected for use in the assays. Positive EMS and DMBA controls as well as a

vehicle control were included. Neither of the assays provided any evidence to suggest that the extract used had any mutagenic potential in the concentration range tested [ESCOP, 2009].

A micronucleus assay was carried out on NMRI mice (5 males and 5 females per group) to establish the potential of vine leaf soft extract for causing chromosomal aberrations. The assay, which complied with the relevant EU and OECD guidelines, was carried out using oral doses of the extract of 1.0, 3.0 and 10.0 ml/kg. Positive controls (cyclophosphamide 40 mg/kg) and vehicle controls (sodium chloride 0.9%) were included. Under the conditions employed, there was no significant increase in micronuclei frequencies in polychromatic erythrocytes.

The methanolic and aqueous extracts from Greek varieties of *Vitis vinifera* demonstrated *in vitro* antimutagenic effects against mutagenicity induced by bleomycin and hydrogen peroxide in Salmonella typhimurium strain TA102 [Stagos et al. 2006].
Oligomericproanthocyanidins from grape seed demonstrated in vitro antimutagenic activity [Liviero et al. 1993].
In addition, among the pharmacologically active flavonoids (quercetin, quercetin 3-O-β-D-glucuronide and isoquercitrin) in the vine leaf extract used is known that quercetin has given positive results in the Ames test. However, the mutagenic potential of quercetin has not been confirmed in *in vivo* tests carried out in rats and mice [Hertog & Hollman 1996].

Carcinogenicity

No data is available. However, it was demonstrated that anthocyanin-rich extracts from bilberry (*Vaccinium myrtillus* L.), chokeberry (*Aronia meloncarpa* E.), and grape *(Vitis vinifera*) inhibited multiple biomarkers of colon cancer in rats [Lala et al. 2006].
There has also been no evidence in the literature so far to indicate that flavonoids as a whole have any carcinogenic properties. Indeed, a large number of *in vitro* studies have been carried out which suggest that flavonoids have anticarcinogenic effects. However, the results obtained *in vitro* have yet to be confirmed in *in vivo* test models. Depending on the type and quantity of food consumed, the human intake of flavonoids is between 100 and 1000 mg per day [Middleton 1996].

In toxicity and carcinogenicity studies carried out on quercetin, promoted by the FDA, four groups of 50 male and female F344 rats were given 40, 400 or 1900 mg/kg of quercetin, or a placebo, in feed over a period of two years. There was no evidence of carcinogenic activity in the female rats and only slight evidence of carcinogenic activity in male rats. The carcinogenic effects, which took the form of renal tubule hyperplasia and nephropathy, were observed only in the high-dose group. No evidence of chronic toxicity was found [NTP: Toxicology and carcinogenesis studies of quercetin (CAS No. 117-39-5) in F344 rats (feedstudies) 1992].

Reproduction toxicity

The maternal and embryofoetal toxicity of an orally administered aqueous preparation of vine leaf soft extract was investigated in pregnant Himalayan rabbits in a study which was carried out in accordance with GLP standards and subjected to QAU inspection. Four groups of animals (12 per group) were used. One group received a placebo whilst the other three groups were given doses of 300, 1000 or 3000 mg/kg/day on days 6-18 of gestation. All doses were administered in a volume of 10 ml/kg. No signs of maternal toxicity were seen during the observation period and there was no visible evidence of foetal retardation. Slight, non-significant skeletal retardations and variations were seen in the foetuses of animals receiving the highest dose (3000 mg/kg of the extract per day). These variations were within biological limits in all cases and did not occur in any of the other groups. There was no evidence of changes due to the extract in any of the other findings, and no teratogenic effects were seen. On the basis of the results obtained, the NOEL for the extract was defined as > 3 g.

In the reproduction study conducted with rabbits no evidence of any teratogenic effects was found. The recommended maximum dose is equivalent to a daily intake of 10.3 mg/kg of vine leaf extract. In the Leuschner study up to 3000 mg/kg/day of the extract, were applied without resulting in any teratogenic effect. The recommended maximum human dose of vine leaf extract is about 300 times lower than the highest dose used in the Leuschner study [Leuschner F, Mitterer KE, 1993]. Examination of the influence of *Extractum vitis viniferae* on the pregnant rabbit and the fetus by oral administration [LPT Report No. 7441/92. 1993; ESCOP, 2009].

Daily doses of 3000 mg/kg extract from grapevine leaves administered to female rabbits during organogenesis (6th to 18th day of pregnancy) did not exhibit teratogenic effects [Antistax® SPC, 2005].

3.3.1. Assessor's overall conclusions on toxicology

Several recent data on oral single-dose and repeat-dose toxicity, as well as on genotoxicity and reproduction toxicity of vine leaf have been reported.
In vitro and *in vivo* studies on the mutagenic potential of vine leaf extracts have not shown any evidence of mutagenic activity. Carcinogenic effects are not expected to occur, especially as vine leaf extract has been used in medicinal products for many years without any evidence of such effects being reported.

4. Clinical Data

4.1. Clinical Pharmacology

Pathophysiology of CVI

The term Chronic venous insufficiency (CVI) describes a clinical picture in which chronic venous diseases of diverse aetiology are manifested by similar or related symptoms and complaints. CVI is mostly caused by venous and capillary hypertension, which causes a persistent, chronic, venous stasis in the skin of the lower leg, manifested most characteristically upon postural challenge [Alguire & Mathes 1997]. The impairment of venous return that is responsible for the venous capillary hypertension can be caused by degenerative, dilating venous conditions of the superficial (primary varices), the transfascial (insufficiency of the perforating veins), or the deep system ("deep" varicosis, insufficiency of the main veins) [Jünger et al. 1994]. Chronically disturbed haemodynamics of deep or superficial veins due to obstructed venous segments or valvular incompetence lead usually to trophic changes in the inner ankle area of the lower limbs and disturbances in the microcirculation of the skin have been considered to be major contributors for skin changes associated with chronic venous hypervolaemia and venous hypertension. Cutaneous microangiopathy of clinical relevance such as enlarged, tortuous capillaries surrounded by micro-oedema contributes to the skin alterations in the lower limbs and determines the course of CVI [Fagrell 1995; Jünger et al. 1996]. CVI can be classified according to its haemodynamic, morphological, and clinical aspects. The clinical grading system, based on Widmer's classification, as well as other international classification systems, e.g. CEAP (Clinical Class, Etiology, Anatomy and Pathophysiology), [Widmer LK, 1981; Partsch H, 1994; Porter JM et al. 1995]. The most important therapeutic approaches include compression therapy, physiotherapy, surgical treatment and sclerotherapy.

Drug therapy

Drug therapy is a treatment option in incipient and mild to moderate Chronic venous insufficiency (CVI) (Widmer grades I and II, CEAP Clinical classes 2, 3, or 4a).

Topical drug treatment

Antioedema drugs are mostly plant derived substances for which an antiexudative and antioedematous action and efficacy have been confirmed in experimental studies and clinical trials. Experimentally, most of them have a membrane stabilising action that leads to a decrease in capillary permeability. Some of these substances also have venotonic properties.
Long-term treatment with antioedema drugs can also be considered in primary and secondary CVI or if invasive measures are not applicable [Gallenkemper et al. 1998]. The majority of these products contain plant extracts or semisynthetic derivatives of plant-derived substances. The most commonly used are coumarins (α-benzopyrone derivatives); saponins/horse chestnut seeds extracts; flavonoids (γ-benzopyrone derivatives); rutin, diosmin, and hesperidin; anthocyanins and procyanidins.
Procyanidins, like the anthocyanins, are flavonoids that are not available as monosubstances. Procyanidins are present in relevant amounts in standardised extracts such as grapevine leaf extracts (*Vitis vinifera*) and are formed through condensation reactions of flavonols [Hostettmann K et al. 1994].
The complex syndrome of symptoms involved in CVI led to investigate the activity of vine leaf preparations on two distinct parameters affecting CVI. On the one hand the effects of the product on microcirculation, on the other on the typical objective and subjective symptoms of CVI such as the presence of oedema and the typical CVI complaints "tired, heavy, and swollen legs", or tension and pain in the legs.

4.1.1. Overview of pharmacodynamic data regarding the herbal substance(s)/preparation(s) including data on relevant constituents

No data available.

4.1.1.1. Assessor's overall conclusions on pharmacodynamics

At present, the mechanism of action of vine leaf water extract in chronic venous insufficiency cannot be considered clarified.

4.1.2. Overview of pharmacokinetic data regarding the herbal substance(s)/preparation(s) including data on relevant constituents

The vine leaf extract is a complex extract containing more than 200 different identified substances. The clinical effects of the grapevine leaf extract cannot be attributed to a specific individual constituent but can be ascribed with reasonable plausibility to the extract as a whole. It is therefore virtually impossible to perform classical pharmacokinetic studies with the complete product and pharmacokinetic studies of individual constituents such as flavonoids will only provide limited information with regard to clinical efficacy.

Flavonoids (quercetin glucuronide, kaempferol glucosides) are quantitatively important constituents of the whole extract. The systemic bioavailability of flavonoids is probably relatively low and variable. Orally administered flavonoids are susceptible to pre-systemic metabolism by the intestinal flora. It is likely that the absorbed metabolites formed by the microflora in the gut contribute to the biological action of orally administered flavonoids. Systemic flavonoids are metabolised in the liver primarily by conjugation (methylation or sulfuronidation) and/or glucuronidation. The resulting metabolites are excreted either with the urine or with the bile into the intestine, where they undergo further metabolism. The pharmacokinetic input function of systemic flavonoids may well be further extended by this enterohepatic recycling process [Manach & Donovan 2004].

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4.1.2.1. Assessor's overall conclusions on pharmacokinetics

Data on pharmacokinetics of vine leaf extract or relevant components are not available in humans. No reliable methods exist to determine simultaneously the plasma levels of all active ingredients present in the whole extract.

4.2. Clinical Efficacy

Several publications referring to human clinical studies with medicinal products containing *Vitis vinifera* were found in the literature.
Three of the published studies investigated the safety and efficacy of a dry extract of grapevine leaves (4-6:1) extraction solvent water (AS 195).
The safety and efficacy specific information of these studies is presented below.

The clinical information available for aqueous extracts of grape vine leaf investigates the activity on two distinct parameters affecting Chronic venous insufficiency (CVI). On the one hand the effects of the product on microcirculation, on the other on the typical objective and subjective symptoms of CVI such as the presence of oedema and the typical CVI complaints "tired, heavy, and swollen legs", or tension and pain in the legs.

Clinical parameters measured

In all clinical studies, changes in leg volume and/or calf and ankle circumference were included in the endpoints studied. All confirmatory studies planned to show efficacy on subjective symptoms of CVI used the lower leg volume determined by water displacement plethysmography as the primary efficacy endpoint. Other endpoints included the lower leg diameter (at calf height and at the ankle), visual analogue scales (VAS: 0-10 cm) for the main symptoms ("tired, heavy legs", "sensation of tension", "prickling sensation", and "pain in the legs"), and the global assessment of efficacy and tolerability by the patient and by the doctor according to a 4-point verbal rating scale (VRS: "good", "satisfactory", "not satisfactory", and "poor"). These measurements are consistent with the guidelines of the German Society for Phlebology.
In the double-blind, placebo-controlled cross-over study with two treatment sequences of 6 weeks each separated by a 4-week placebo washout period, microcirculation in the most affected CVI leg was measured by laser doppler fluxmetry (685 nm, penetration depth approximately 1 mm) and transcutaneous oxygen monitoring.
Patients were usually questioned about their subjective well-being. They were also instructed to spontaneously report any adverse events experienced. Laboratory tests (haematology, clinical chemistry, and urinalysis) and blood pressure and pulse measurements were carried out in most studies to record safety.

4.2.1. Dose response studies

From the results of [Kiesewetter et al. 2000], a single daily dose of 360 mg of grapevine leaf extract is as effective and well tolerated as a daily dose of 720 mg. On average, the higher dose regimen offers greater efficacy in terms both of the extent and duration of action, but is roughly equivalent with regard to subjective symptoms reduction. The lower dose, however, is considered to be sufficient in the majority of patients and has been chosen to reduce the intake of extract, thus most likely decreasing the likelihood of observing adverse events. Consequently, this is the dosage that has been approved in some marketing authorisations.
No pharmacokinetic or pharmacodynamic studies, however, were performed to support the posology and daily dose proposed.

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4.2.2. Clinical studies (case studies and clinical trials)

Several publications referring to human clinical studies investigating the safety and efficacy of a dry extract of red *Vitis vinifera* leaves (4-6:1) extraction solvent water (AS 195) were found in the literature. Some others studies have been assessed in this AR, that originate from confidential in house files of Boehringer Ingelheim. The company allowed using those data for assessment and monograph/list entry establishment.

Double-blind, Placebo-controlled studies

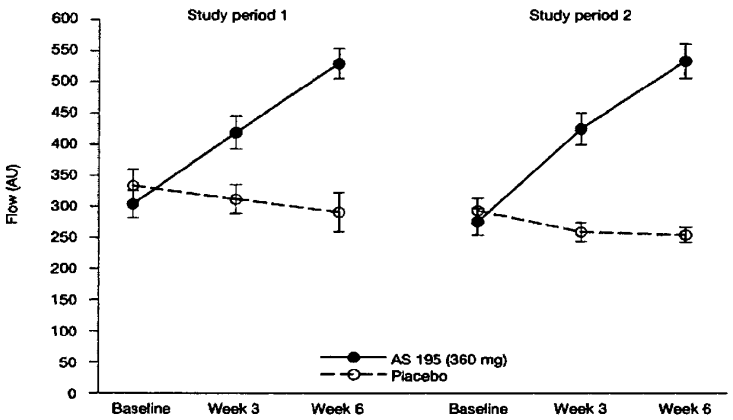
[Kiesewetter et al. 2000]: In a 12-week, randomized, double-blind, placebo-controlled, parallel-group, multicenter study conducted in accordance with the principles of Good Clinical Practice (GCP), the efficacy and safety of once daily doses of 360 and 720 mg RVLE (AS 195) were compared to placebo in male and female outpatients aged between 21 and 80 years old (with 52% ≥ 60 years) with body weights ranging from 46 to 120 kg (Broca index: median 7.3, range: -30 to +82%; this correspond to a BMI of approx. 25 kg/m^2). And stage I and incipient stage II Chronic Venous Insufficiency (CVI) with no major dystrophy of the skin. The majority (72%) of the patients were women. Compression therapy and the administration of diuretics were not permitted during the study period.
Patients were randomly assigned to a double-blind treatment with placebo, 360 mg RVLE or 720 mg RVLE once daily for 12 weeks, preceded and followed by a single-blind 2-week placebo treatment for baseline run-in and end-of-trial washout, respectively. Study criteria were evaluated at baseline, after 6 and 12 weeks of treatment and 2 weeks after discontinuation of treatment. Of the **260** patients enrolled and randomized, 219 completed the study in accordance with the protocol, 86 test subjects took a single daily dose of 360 mg AS 195, 84 took 720 mg AS 195/day and the remaining 87 test subjects received a placebo. During weeks 13 and 14 none of the participants received any further medication.

Subjectively, there was an improvement in CVI symptoms at 6 weeks with all treatments, but a further improvement at week 12 was seen only in the active treatment groups: at 12 weeks, the changes compared to baseline were significantly greater ($p < 0.001$) in both active treatment groups than in the placebo group. The study demonstrated that once daily doses of 360 and 720 mg RVLE were effective in the treatment of mild CVI, reducing significantly lower leg edema and improved CVI-related symptoms to a clinically relevant extent.

The treatments were well tolerated. All 260 patients who were enrolled and randomized were included in the safety analysis. This included three patients who withdrew from the study before visit 2 (i.e. before entering the treatment phase). 257 patients received at least one dose of the randomized study medication. Most patients completed the study up to 84 days of treatment. The mean duration of treatment for the 360-mg dose of AS 195 was 81.9 days (n = 86) whilst the mean duration of treatment for the 720-mg dose of AS 195 was 79.7 days (n = 84). Placebo was taken by 87 patients during the randomized treatment period (mean duration of treatment: 79.3 days) and by all patients during the 14-day run-in period (n = 260) and the 14-day follow-up period (n = 246). A total of 34 adverse events (AEs) were reported in 31/260 patients: 3 AEs in 3/260 subjects treated with placebo during the run-in phase; 3 AEs in 3/246 patients treated with placebo during the follow-up period; 10 AEs in 10/87 patients treated with 360 mg AS 195; 2 AEs in 2/85 patients treated with 720 mg AS 195; and 16 AEs in 13/88 patients treated with placebo. Most AEs were considered to be mild (n = 21) or moderate (n = 9). Four AEs were classified as severe, three in patients taking the placebo (phlebitis of the large saphenous vein; knee surgery; abdominal discomfort) and one in a patient taking 360 mg AS 195 (leg hematoma following minor trauma). Six AEs were rated as likely to be causally related to the study medication by the investigator; of these, two (mild constipation and mild hair thinning) were

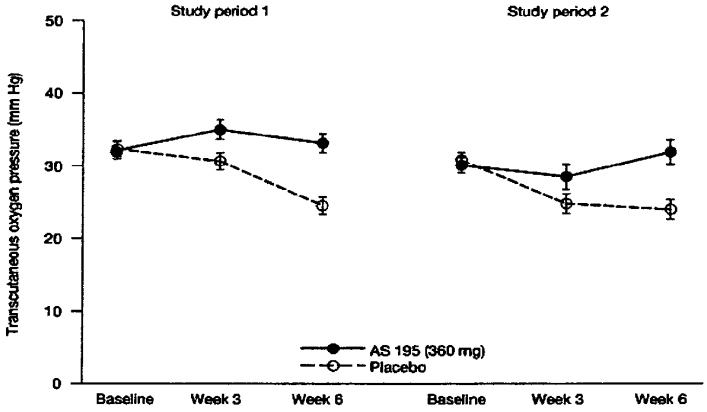
reported in patients treated with 360 mg AS 195, whilst the other four occurred in patients treated with placebo. Gastrointestinal disorders (abdominal discomfort, diarrhea, dyspepsia, dry mouth or retching) were the most frequently cited AEs (n = 11), followed by infections (n = 6), headache (n = 4) and disorders of the musculoskeletal system (n = 4). Two AEs which occurred during treatment with placebo required hospitalization and were labelled as „serious"; there was one incident of vaginal hemorrhage during the run-in phase and one incident of severe acute osteoarthritic complaints in the knee of the affected leg during the randomized treatment period. Three other patients were withdrawn following an AE: 1 patient was withdrawn during the placebo run-in period due to moderate abdominal discomfort; 2 patients were withdrawn from randomized treatment with the placebo, one because of phlebitis of the greater saphenous vein and one because of severe abdominal discomfort. The clinical laboratory data, blood pressure and pulse rate data and 12-lead ECG recordings did not indicate any significant mean or individual changes which were likely to have been due to the study medications. Overall tolerability was rated as follows for the 3 treatments: 360 mg AS 195: good 81%, satisfactory 19%; 720 mg AS 195: good 94%, satisfactory 6%; placebo: good 68%, satisfactory 30%, not satisfactory 2%. The study was conducted in multicentric settings, but most measurements were carried out by only two investigators, thus this trial can be considered basically a monocentric one

[Kalus et al. 2004]: The effect of AS 195 on cutaneous microvascular blood flow, transcutaneous oxygen pressure (tcpO2), and leg oedema was investigated in a randomised, double-blind, placebo-controlled, crossover study in male and female patients, aged ≥ 18 years with an average age of 66 years, with confirmed CVI stage I or II. In this efficacy study, **71** patients (70 during the second phase) were treated with either placebo or 360 mg AS 195 film coated tablets. The first group (n=36) received AS 195 360mg once daily during a first 6-week treatment period, followed by a 4-week placebo washout period and then placebo during the second 6week treatment period. The second group (n = 35) started with placebo and received AS 195 360mg after the placebo wash-out. After 6 weeks, patients in the AS 195 group had increased microvascular blood flow values and transcutaneous oxygen pressure (tcpO2). The active substance group showed a statistically significant increase of 241.8±18.7 Arbitrary Units (AU), while a reduction of 41.0±18.7 AU was recorded in the placebo group ($p<0.0001$).



Microcirculation, measured in arbitrary units (AU) (laser Doppler flow measurement at Doppler frequencies of 10.1–37.2 kHz, mean ± SEM, after 10 minutes of standing) (from [Kalus et al. 2004])

The $TcpO_2$ oxygen reading also rose significantly from baseline levels in the AS 195 group, by 1.35±0.97 mmHg, contrasting with a reduction in the placebo group of 7.27±0.97 mmHg.



Transcutaneous oxygen pressure (mean ± SEM, after 10 minutes of standing) (from [Kalus et al. 2004])

These changes in the measured parameters were associated with corresponding volume changes in the lower leg. After just three weeks of treatment, statistically significant differences were also observed in ankle and calf circumference. After six weeks, these circumferences had fallen by 0.39 and 0.54 cm respectively, in the active group, compared to increases in the placebo group of 0.29 and 0.14 cm respectively. The authors of the study concluded that the administration of AS 195 could have a positive effect on the course of CVI. The minimum number of days of exposure was 38 in the AS 195 and 24 in the placebo group, the maximum number 46 in the AS 195 and 45 in the placebo group. Thirteen (18.3%) subjects out of 71 experienced 16 adverse events (AEs) during the 16 week trial. Ten AEs (14.1%) were of mild, 4 (5.6%) of moderate and 1 (1.4%) of severe intensity. None of the AEs were assessed as related to the trial medication. One patient died from a cardiac arrest. He had been treated with placebo and never received AS 195 in this trial. All patients assessed the overall tolerability as good or satisfactory. Laboratory parameters did not change during the study [Kalus et al. 2004].

[Limoni C, 1996]: A four-week, multicentre, randomised, double-blind versus placebo, parallel-group safety trial to evaluate the tolerability profile of certain capsules (water extr. *Vitis vinifera* siccum 4-6:1) in male and female patients suffering from chronic venous insufficiency. The study population consisted of **105** patients, male and female, aged between 20 and 70 years with chronic venous insufficiency of grade I, II, and III according to Widmer. The patients received 3 capsules of that extract or placebo per day during 28 days. Seventy-two patients were randomised into the active treatment group and 33 into the placebo treatment group. In the absence of a strictly diagnosed CVI, patients with a subjective feeling of heavy and tired legs were admitted as well. Primary endpoints were the incidence of adverse events and the rating of the overall tolerability. Secondary endpoints were laboratory tests and physical examination. There were no differences in tolerability between the two treatment groups. The proportion of volunteers reporting adverse events was similar in both treatment groups: 40.2% in the active treatment group and 36.4% in the placebo group. The most frequent adverse events were mild gastrointestinal disorders, sleep disturbances, and tiredness. Laboratory values did not change in either treatment group during the trial period. The capsules were well tolerated by 84.9% of the volunteers. No severe adverse events were reported.

[Vix et al. 2007]: This 12-week, double-blind, randomised, placebo-controlled, multicentre trial was carried out to evaluate the efficacy and tolerability of film-coated tablets, extr. *Vitis vinifera* (***4-6:1 water***) 360 mg/day orally, in male and female patients suffering from chronic venous insufficiency. The design and methodology used closely reflected those used in BI trial by Schaefer 2004).
The time course of the change from baseline in limb volume was similar for both treatment groups with a more marked improvement of leg oedema over time in patients treated with these tablets. After 84 days of treatment, limb volume was reduced by -21.38 (±11.30) g of displaced water in the verum group. The adjusted mean reduction in limb volume in the placebo group was less pronounced with -10.71 (±11.56) g. However, the difference between treatment groups (-10.67 ±14.35 g) was not statistically significant.

For the secondary endpoint "change from baseline in calf circumference", the adjusted mean difference to placebo after 42 days of treatment with these tablets was -0.42±0.22 cm in the FAS (p=0.0596) vs. -0.31±0.24 cm on study day 84 (p=0.1851). In the Per-Protocol Set (PPS), the difference to placebo was -0.47 (±0.23) cm on study day 42, with a p-value just reaching significance at 0.0445. The evaluation of the four subjective symptoms of CVI, as assessed by VAS, yielded similar results: after 42 days of treatment, the adjusted mean differences between active treatment and placebo were -0.44 cm for "tired, heavy legs", -0.73 cm for "sensation of tension in the legs", and -0.48 cm for "pain in the legs" (vs. -0.38 cm, -0.04 cm, and 0.18 cm on study day 84). There was no difference between treatment groups regarding the symptom "tingling sensation in the legs". Global efficacy was rated as good or satisfactory by 70.9% of all patients treated with these tablets and 67.7% of all patients allocated to placebo whereas the investigators' ratings were 69.0% and 62.6%, respectively. The assessment of quality of life by the Tuebingen QoL questionnaire resulted in a better mean score for verum group compared with placebo regarding the subscale "leg complaints", the subscale most specifically related to CVI. The differences, however, were not statistically significant.

[Schaefer & Petrini 2004]: This placebo controlled, randomised, parallel group study was carried out in 2004. **247** CVI patients (CEAP-Scores 3 or 4a) were entered in the study: 121 were treated with the grapevine leaf extract AS 195 360 mg as a single tablet and 126 with placebo for 12 weeks. For the primary endpoint, the difference related to placebo, adjusted for centre effect and baseline values, in changes of displaced water from baseline until Day 84 was -10.42 cm (SE: 11.84; 95% CI: -33.76, 12.92). The difference in changes between the two treatment groups was not statistically significant. Subjective CVI symptoms diminished in both treatment arms similarly during the first three weeks. A statistically significant difference between the two treatment groups in favour of verum, was found for the symptoms "sensation of tension in the legs" at Day 42 (p=0.0211, ANCOVA) and "pain in the legs " at Day 84 (p=0.0222, ANCOVA). At all other time points no statistically significant difference was detected between the two treatment regimens for the symptoms "tired heavy legs" as well as "tingling sensation" in the legs. After 84 days of treatment, the limb volume as measured by the water displacement method was statistically not significantly more reduced in subjects allocated to verum than in those randomised to placebo. Careful inspection of the results showed that the unexpected high intra- as well as inter-individual variability of limb volume determinations contributed significantly to the outcome of the study. The variability between 2 or 3 replicates of the limb volume determination, for example, exceeded 20 g of displaced water in more than 20% of patients (range 0% to 44.1%) in 8 out of 23 study sites (35%). This study was negative in terms of a primary endpoint, although there was evidence of symptomatic benefit from the active treatment.

[Schaefer E & Petrini LE, 2003]: A 17 week, randomised, double-blind, placebo controlled cross-over trial to evaluate the efficacy of water extr. *Vitis vinifera* ***4-6:1*** film coated tablets (AS 195) given as 2 capsules of 180 mg of AS 195 daily 360 mg/day p.o., in **179** male and female Japanese patients with swellings of calf and ankle due to disorders of venous reflux belonging to Class 1 according to Porter's classification, in improving microcirculation of the skin in the leg of male and female patients

suffering from chronic venous insufficiency The microcirculation study has demonstrated that objective signs associated with CVI (microvascular blood flow, $tcpO_2$, ankle circumference and calf circumference) can be improved significantly after just 3 weeks of oral treatment with a single daily dose of 360mg of the grapevine leaf extract AS 195. These results build on those from Harrison trial (1998) showed that AS 195 reduces lower leg oedema and calf circumference in patients suffering from CVI treated once daily for 12 weeks. Out of the 179 evaluable subjects 38.5% experienced a marked and 42.5% a moderate global improvement of CVI symptoms. Improvement was rated as "moderate" by 59.8% of the patients with regards to heaviness/tiredness, 69.9% with regards to tension, 79.2% to tingling, 74.0% to pain, and 74.4% to itching. Circumference of calf (mean±SD) was 33.5±2.99 cm prior to treatment and 33.0±2.94 cm at 12 weeks, of the ankle (mean±SD) 22.3±1.84 cm prior to treatment and 21.9±1.82 cm at 12 weeks after treatment ($p<0.05$). These differences were not statistically significantly different from baseline.

Open-label, Observational studies

[Schaefer et al. 2003]: The 6-week, open, uncontrolled multicentre safety trial was conducted to specifically evaluate the tolerability and safety of AS195 film-coated tablets, 360 mg/day *per os*, in **65** male and female patients, aged 25 - 82 years, suffering from chronic venous insufficiency grade I or II (Widmer classification). The verum group received two film-coated tablets once daily for 42 days (360 mg/day). The primary objective of the study was to assess the tolerability and safety of AS 195 extr. *Vitis vinifera* siccum ***4-6:1***) film-coated tablets. The observational study was conducted in accordance with the requirements of the Swiss licensing authority (Swissmedic: application document) in a total of 11 study centres (general practitioners) between June and October 2002. Three visits (study enrolment, baseline examination at the start of the study, final examination at the end of the study, with a follow-up examination if necessary) were planned. The study was carried out in accordance with GCP. At the end of the study, all subjective symptoms of CVI (tired, heavy legs, sensation of tension in the legs, tingling sensations in the legs, pain in the legs) were statistically significantly improved. The global assessment of efficacy by the patients and by the investigators was rated as good or satisfactory in most of the patients. The primary variable was the number and intensity of adverse events (AEs), with particular emphasis placed on AEs regarded by the investigator to be treatment-related. Secondary safety variables included the global assessment of tolerability by both the patients and investigators as well as vital sign assessments. AS 195 film coated tablets were well tolerated during the whole study. Overall, six patients (9.2%) experienced a total of 7 AEs of mild or moderate intensity judged by the investigator as potentially causally related to study medication. They included gastrointestinal problems (4 patients), headache (1 patient), anorexia (1 patient), and erythematous rash (1 patient). Four patients of whom experienced a significant AE necessitated study discontinuation in three cases and dose reduction in one case. Most patients rated the global tolerability of AS 195 film-coated tablets as good (49 patients, 75.4%) or satisfactory (12 patients, 18.5%). Three patients (4.6%) rated tolerability as bad. One patient (1.5%) gave no rating. The global assessment of tolerability by the investigators was in good agreement with those of the patients. The investigators rated the tolerability as good in 50 patients (76.9%), as satisfactory in 10 patients (15.4%), as not satisfactory in one patient (1.5%), and as bad in two patients (3.1%). No relevant changes of vital signs (systolic and diastolic blood pressure, pulse rate) were measured. Patient´s compliance was rated as good [Schaefer et al. 2003].

Assessment The present open-label, multicentre observational study demonstrated that AS 195 film-coated tablets were effective and well tolerated in outpatients with chronic venous insufficiency grade I or II supervised in a setting of private practices. No change of the safety profile has been noted.

[Monsieur & Van Snick 2006]: A small, open observational clinical study was conducted in **39** patients suffering from Chronic Venous Insufficiency (CVI), grade I or II according to the Widmer

classification, (grade 2 to 4 of the international CEAP classification). Patients were treated with 180 mg of RVLE (AS 195) twice daily (360 mg in total) for six weeks. The parameters investigated were objective measurements of lower leg volume and the circumference of the leg as well as subjective criteria such as heaviness and pain in the leg. A clear and significant improvement of all parameters was observed after two weeks of treatment. This effect was still present and increased slightly after four and six weeks in all objective and subjective parameters tested in this study.

Assessment This study has shown a fast onset of action and an efficacy of the AS 195 extract in chronic venous insufficiency. Adverse events/side effects: not specified. In general, the treatments were well tolerated. Three patients prematurely stopped the trial for reasons not linked to the treatment or to the disease. No change of the safety profile.

Clinical studies with grapevine leaf extract (4-6:1, extract solvent: water) (AS 195)

Study ID	**Study Dates** **Persons**	**Design Control type Study objective**	**Study &, Ctrl Drugs Dose, Route, & Regimen**	**Subjects per Arm treated / completed**	**Duration**	**Gender M/F Median Age (Range)**	**Diagnosis Inclusion Criteria**	**Primary Endpoint**	**Outcome**
Kiesewetter, 2000	April Sept-1998 **260**	randomized, double-blind, parallel, dose response Placebo Efficacy, tolerability, safety	AS 195 caps 180mg, *per os* 2-0-0 AS 195 capsules 360 mg, *per os* 2-0-0 Placebo	360 mg : 86/84, 720 mg :84/79 87/80	12 wks	24/62 56.2±12.4 years (mean±SD) 27/57 55.7±13.8 years (mean±SD) 20/67 59.2±11.5 years (mean±SD)	CVI Grade I, II according to Widmer	Baseline adjusted changes of lower limb volume assessed by water displacement plethysmography	Positive
Schaefer & Petrini 2004	2004 **247**	randomized, double-blind, parallel Placebo Efficacy, tolerability	AS 195 tablets 360 mg, *per os* 1-0-0 Placebo 1-0-0	360 mg : 121/117 126/123	12 wks, 2 wks single blind placebo	20/99 53.0 years (20–83 years) 15/111 53.0 years (23–79 years)	CVI CEAP-Scores 3 or 4a	Baseline adjusted changes of lower limb volume assessed by water displacement plethysmography	Negative
Schaefer & Petrini 2003	2003 **179**	randomized, double-blind, controlled cross over trial Placebo Efficacy	AS 195 tablets 360 mg, *per os* 1-0-0 Placebo 1-0-0	360 mg	17 weeks	No data	CVI Grade I, II according to Porter	Baseline adjusted changes of lower limb volume Changes in CVI symptoms	Negative
Vix et al. 2006	2006 **211**	randomized, double-blind, parallel Placebo Efficacy, tolerability	AS 195 tablets 360 mg, *per os* 1-0-0 Placebo 1-0-0	360 mg : 103/98 99/95	12 wks	32/71 61.0 years (29–86 years) 26/73 61.0 years (30–87 years)	CVI CEAP Scores 3 or 4a	Baseline adjusted changes of lower limb volume assessed by water displacement plethysmo-graphy	Negative
Kalus 2004	2002, completed **71**	randomized, double-blind, cross-over Placebo	AS 195 tablets 360 mg, *per os* 1-0-0	360 mg : 70/70	6 wks	16/55 65.2±7.7 years (mean±SD) (32–76)	CVI Grade I, II according to Widmer	resting flux, change from baseline measured by Laser Doppler fluxmetry	Increase of microvascular blood flow

Study ID	Study Dates Persons	Design Control type Study objective	Study &, Ctrl Drugs Dose, Route, & Regimen	Subjects per Arm treated / completed	Duration	Gender M/F Median Age (Range)	Diagnosis Inclusion Criteria	Primary Endpoint	Outcome
		Efficacy, tolerability	Placebo 1-0-0						
Schaefer et al. 2003	June 2001 Oct-2001 **65**	Open/ Observational Uncontrolled Tolerability, safety, Efficacy,	AS 195 tablets 180 mg, *per os* 2-0-0	360 mg : 65/59	6 wks	7/58 M: 65.9 years (mean) (56-82 years) F: 56.6 years (mean) (25–80 years)	CVI Grade I, II according to Widmer	Incidence and intensity of adverse events; Changes in CVI symptoms as secondary endpoint	Safety study Positive
Monsieur 2006	2006 **39**	Open Tolerability, safety, Efficacy	AS 195 tablets 180 mg, *per os* 2-0-0	360 mg	6 wks		CVI Grade I, II according to Widmer	Baseline adjusted changes of lower limb volume Changes in CVI symptoms	Positive
Limoni 1996	Dec-1996 May 1997 **105**	randomized, double-blind, parallel, dose response Placebo Multicentre Tolerability, Safety	AS 195 caps. 180 mg, *per os* 1-0-2 Placebo 1-0-2	540 mg : 72/64 33/27	4 wks	6/66 45.2 years (18.4–67.9 years) 4/29 36.7 years (25.6–70.6 years)	CVI Grade I, II. III according to Widmer	Safety Global improvement	Safety

4.2.3. Clinical studies in special populations (e.g. elderly and children)

No information available.

4.3. Overall conclusions on clinical pharmacology and efficacy

The clinical efficacy of an extract from red vine leaves (RVLE; AS 195) was investigated in double blind, placebo-controlled studies [Kiesewetter et al. 2000; Kalus et al. 2004] and in open observational studies [Schaefer et al. 2003; Monsieur & Van Snick, 2006] during treatment of chronic venous insufficiency. In these studies, the RVLE was showed an acceptable clinical efficacy and tolerability, while there were some others with negative results [Schaefer & Petrini 2003; 2004; Vix et al. 2006]. Treatment with RVLE has been shown to lead to a reduction of leg volume, measured using both water plethysmography and calf and ankle circumference, and of the subjective symptoms investigated in the studies reviewed.

The extract AS 195 was consistently more effective than placebo in reducing leg oedema and improving symptoms of Chronic Venous Insufficiency (CVI) across most efficacy parameters evaluated, even though not always a statistically significant superiority could be shown.

5. Clinical Safety/Pharmacovigilance

5.1. Overview of toxicological/safety data from clinical trials in humans

The safety profile of vine leaf extracts can be described as acceptable from all clinical studies in Chronic Venous Insufficiency (CVI) patients and from its use from products on the market. The safety results obtained from the clinical studies conducted so far show that the oral use of vine leaf extracts are well tolerated by most patients. No drug-related serious adverse events were reported during the clinical trials, and when reported that were mild and transient. Seven non-serious adverse events (constipation, dermatitis, headache, hair thinning, menometrorrhagia, urticaria) or moderate (erythematous rash) of mild intensity leading to patient withdrawals, were suspected to be related to the active trial medication.

5.2. Patient exposure

The controlled studies evaluated 1073 patients in total [Kiesewetter et al. 2000; Kalus et al. 2004; Schaefer & Petrini 2004; Vix 2006, Limoni 1996]. The open studies, evaluated by [Schaefer et al. 2003] and by [Monsieur and Van Snick, 2006] comprised 104 patients.

5.3. Adverse events

No health hazards or side effects are known in conjunction with the proper administration of designated therapeutic dosages. A reversible inhibition of intestinal enzyme activity (alkaline phosphatase, sucrose and dipeptidyl peptidase) was demonstrated in animal models [Tebib, 1994; PDR for Herbal Medicines, Thomson, 2004].

Brito et al. [2008]; Mur et al. [2006]: Vine pollen allergies have been reported with *Vitis vinifera* pollen and an extract thereof In Castilla-La Mancha, Spain, the area with the highest density of vineyards in the world. Two cases of allegic reactions to *Vitis vinifera* pollen and grape have been previously reported. The aim of a prospective study by Brito et al. [2008] was to determine the clinical relevance and biochemical characteristics of vine pollen in the Spanish province of Ciudad Real. The authors designed a prospective study of patients treated in the allergy units from Puertollano and Ciudad Real for respiratory symptoms of 4 months' duration in the year 2000. Skin prick tests with a standard aeroallergen battery and *V. vinifera* pollen extract were performed on all patients. The authors also performed conjunctival and bronchial provocation tests and serum specific IgE and sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblotting on the patients who tested positive for *Vitis vinifera* pollen.

The results of this prospective study show that *V. vinifera* pollen has a moderate clinical relevance from an allergic point of view, particularly affecting those subjects who are exposed to it during the working hours and those who perform leisure activities close to vine fields. During a period of four months, the authors performed skin prick tests to vine pollen in patients who attended the outpatient clinic with suspected respiratory allergy, finding sensitisation to *Vitis vinifera* pollen in 9 of the 200 patients seen (98 patients had allergy to other pollen). In conclusion, in areas with a high density of vineyards, vine pollen can reach midrange air concentrations and could be the cause of hay fever in those individuals with the highest level of exposure [Brito et al. 2008; Mur et al. 2006].

Assessment: In summary, there is no new safety specific information provided. No change of the safety profile is suggested.

Reports on immunoglobulin E (IgE)-mediated allergic reactions to grapes and wine are limited in the literature. Nevertheless, grapes are widely grown and consumed in Mediterranean countries. The

object of a prospective study by Kalogeromitros et al. [2005] was to present clinical features, *in vivo* and *in vitro* allergy testing, and human leukocyte antigen (HLA) serotyping in patients with recurring reactions to grapes and grape products. Eleven unrelated Greek patients, six men and five women (aged 16-44 years; mean, 26.9 years) were enrolled based on a documented history of IgE-mediated reactions to grapes, wine, or other grape products. Their evaluation included full history, reaction severity, clinical examination, skin-prick tests with food allergens and molds, serum IgE, specific IgEs to the same allergen battery, and HLA typing. Patients reported 35 grape-induced episodes of anaphylaxis ranging from moderate to severe. The authors described several cases of repeated IgE-mediated reactions to grapes, vine and other grape products. Similar clinical manifestations in different courses of grape allergen, almost identical prevalent co-sensitisations to other already known allergenic fruits, and the first determination of HLA antigens in grape-allergic patients are the core of this study.
The authors suggest that allergy to grapes may not be as uncommon as generally believed in the literature and should be considered as a possible offending cause in certain episodes of food-induced anaphylaxis probably in genetically at-risk individuals. However, allergic reactions to *Vitis vinifera* are known and reported in rare cases [Kalogeromitros et al. 2005].

Assessment: If hypersensitivity reactions are labelled correctly in the PIL and SPC, there is no change of the safety profile of medicinal products.

[Crowell et al. 2004]: Resveratrol-Associated Renal Toxicity. Resveratrol, (3, 5, 4'-trihydoxystilbene) a compound found in grapes, mulberries, and peanuts, has antimycotic, antiviral, and beneficial cardiovascular and cancer preventive activities. To evaluate the potential toxicity of resveratrol, rats were administered by gavage 0, 300, 1000, and 3000 mg trans-resveratrol per kilogram body weight per day for 4 weeks. Most of the adverse events occurred in the rats administered 3000 mg per kilogram body weight per day. These included increased clinical signs of toxicity; reduced final body weights and food consumption; elevated BUN (blood urea nitrogen), creatinine, alkaline phosphatase, alanine aminotransferase, total bilirubin, and albumin; reduced hemoglobin, hematocrit, and red cell counts; and increased white cell counts. Increases in kidney weights and clinically significant renal lesions, including an increased incidence and severity of nephropathy, were observed. Diffuse epithelial hyperplasia in the bladder was considered, equivocal and of limited biological significance. No histological effects on the liver were observed, despite the clinical chemistry changes and increased liver weights in the females. Effects seen in the group administered 1000 mg resveratrol per kilogram body weight per day included reduced body weight gain (females only) and elevated white blood cell count (males only). Plasma resveratrol concentrations in blood collected 1 h after dose administration during week 4 were dose related but were relatively low given the high dosage levels; conjugates were not measured. Under the conditions of this study, the no observed adverse effect level was 300 mg resveratrol per kilogram body weight per day in rats (corresponding to 21 g in a 70 kg human) [Crowell et al. 2004].

Assessment: These results cannot be transferred to medical products containing Vitis vinifera or extracts thereof.

In summary, there is no safety specific information that is relevant for medical products. The reported adverse events / side effects were mild to moderate. The majority of the above mentioned events are considered to be related to underlying diseases, incidental concomitant disorders or other coincidences but not causally related to *Vitis vinifera*. From these published adverse events, no change of the safety profile of *Vitis vinifera* can be concluded. According to current knowledge, gastrointestinal disorders and hypersensitivity reactions should be labelled in the PIL and SPC.

The Periodic Safety Update Report for *Vitis vinifera* has been compiled within the joint BAH PSUR project. It summarizes safety data from 01/Nov/2003 to 01/Sep/2008 [BAH 2008].

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In the report period no new safety relevant issues were found in the literature. There were no reports, clinical or experimental studies identified that give reason for a change in the assessment of the safety and benefit/risk ratio of *Vitis vinifera*.

5.4. Serious adverse events and deaths

The case of a 51 years old female patient is reported. She was hospitalized due to an acute icterus, which had developed over 15 days and was associated with asthenia, nausea and anorexia. Beyond the mucocutaneous icterus, the clinical examination did not show any abnormality, especially no chronic hepatopathy. The laboratory parameters suggested a cytolysis due to an ALAT value that was twelve times higher than normal, γ-GT and alcalic phosphatase plasma concentrations two times higher than normal and total bilirubine of 87 µmol/L. Normal findings in the abdominal ultrasonic examination lead to the diagnosis of an acute hepatitis. The patient has a history of euthyroidism. The patient did not consume alcohol and was in a good nutritional condition. The etiologic examination of this hepatitis was negative for infections, immunological and metabolic causes. A hepatic punction-biopsy, revealed a discrete cholangitis with focal ductal hyperplasia, a major cholestasis with numerous biliary thromboses and intrahepatocytic biliary pigments, a focal hepatocellular necrosis, a lymphohistiocytic infiltrate of weak intensity without significant fibrosis. Altogether this suggested an acute cholestatic hepatitis with either viral or toxic origin. The review of the medical questionnaire revealed sporadic intake of 1 g of acetaminophen and two repeated intakes in an interval of one week, when the first symptoms had appeared. Additionally the patient took *Vitis vinifera tinctoria* (extract of red wine leaves) and Fumitory (extract of *Fumaria officinalis*) daily for two months, as "summer cure" for the treatment of heavy legs and as a stimulant. She stopped the intake of these two products three weeks prior to hospitalization, when the first general symptoms appeared. The patient had already taken *Vitis vinifera tinctoria* at the same time one year before. Fumitory was used for the first time. According to the official method used at the pharmacovigilance centres in France, this case is classified as plausible with a chronological C2 and a semiotic S3 score of two products concomitantly used with paracetamol [Bonnet et al. 2007].

Assessor's comment

This case is classified as serious because of a significant medical reaction including hospitalisation. A causal relationship with *Vitis vinifera* cannot be excluded (plausible temporal relationship). However, the used herbal products are insufficiently described; additionally relevant information on the dosage are lacking. Moreover, the concomitant use of acetaminophen must be considered. Acetaminophen is well known for its hepatotoxicity.

5.5. Laboratory findings

None reported.

5.6. Safety in special populations and situations

The product is not suitable for patients with known hypersensitivity against the herbal substance, the plant family, the herbal preparation or to the excipients of the final product.

5.7. Intrinsic (including elderly and children) /extrinsic factors

Grapevine leaf is not intended for use in children, while no restrictions are known for its use in elderly.

5.8. Drug interactions

No drug interactions have been reported.

5.9. Use in pregnancy and lactation

Grapevine leaf should not be used during pregnancy and lactation as there are not data available.

5.10. Overdose

No information.

5.11. Drug abuse

No information.

5.12. Withdrawal and rebound

No information.

5.13. Effects on ability to drive or operate machinery or impairment of mental ability

No information.

5.14. Overall conclusions on clinical safety

The safety profile of grapevine leaf extracts can be judged as good from clinical studies and from its long term use and market availability. The available literature, on pharmacological and toxicological studies, does not give rise to safety concerns. A special focus was set on the polyphenolic constituents of the extract (proanthocyanidins, flavonoids) that can be linked with the pharmacological effects. The proof of an acceptable safety is not only given by the period of time over which this extract has been used and by the quantitative aspects of their use but also by PSURs data and the safety data obtained during the clinical trials.
Overall, *Vitis vinifera,* leaves can be considered as safe in herbal medicinal products with a positive benefit/risk ratio.

6. Overall conclusions

Well-established use

The clinical efficacy and safety of the dry aqueous extract of grapevine leaves (4-6:1) has been demonstrated in appropriately designed clinical trials and is supported by extensive use of the product in the market since 1999.

The above referred extract showed an influence to the microcirculation and transcutaneous oxygen pressure at the predominantly affected perimalleolar area of the leg in Chronic venous insufficiency (CVI) patients who were treated for six weeks. Moreover, the assayed grapevine leaves extract has shown to lead to a reduction of the volume of oedema and to improvement of the typical subjective symptoms of CVI such as tired, heavy and swollen legs or pain and tension in the legs.

Three double-blind, placebo controlled trials investigated the change in leg volume as measured by water plethysmography as the primary endpoint, another also the changes in ankle and calf

circumference in the secondary endpoints investigated. In several of the clinical trials objective and subjective symptoms of CVI appeared to be effectively reduced, while there were some others giving negative results such as the ones of Schaefer & Petrini 2004 and Vix et al. 2006. The trials were performed according to ICH-GCP.

An additional placebo-controlled and open safety and tolerability trial provided supportive evidence for the efficacy.

This extract can therefore be regarded as an active substance with a well-established medicinal use.

The use of herbal medicinal products prepared with this extract is not recommended during pregnancy and lactation and should not be taken in children and adolescents under 18 years of age, due to the lack of adequate data.

The proposed indication is:

- Herbal medicinal product for treatment of chronic venous insufficiency, which is characterised by swollen legs, varicose veins, a feeling of heaviness, pain, tiredness, itching, tension and cramps in the calves.

Traditional use

Other extracts and herbal preparations from grapevine leaves have been widely used. The safe use of the extract can be stated on the basis of the well-known, long-lasting and traditional use of preparations of grapevine leaves in the folk medicine and as registered medicaments.. Based on the evaluation and assessment of the present documentation of the clinical efficacy and safety, the herbal substance for use as herbal tea or in other oral dosage forms, and the aqueous extracts of grapevine leaf are acceptable as traditional herbal medicinal products.

Sufficient data are available to develop a Community monograph on the well established and traditional use of *Vitis vinifera* L., leaves. The indications are suitable for self-medication. The proposed indications are:

- Traditional herbal medicinal product to relieve symptoms of discomfort and heaviness of legs related to minor venous circulatory disturbances.
- Traditional herbal medicinal product for symptomatic relief of itching and burning associated with haemorrhoids.
- Traditional herbal medicinal product for symptomatic treatment of cutaneous capillary fragility.

The use of the traditional herbal medicinal products is not recommended during pregnancy and lactation and should not be taken in children and adolescents under 18 years of age.

The minimum required data on mutagenicity (Ames test) are available for herbal preparations of grapevine leaves. **The inclusion in the Community list of traditional herbal substances and preparations is recommended.**

Annex

EP 05 71 2607.0
Alexion Pharmaceuticals, Inc.
Our Ref.: M2367 OPP(EP)

AUXILIARY REQUEST

1. Eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), 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), and
   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).

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

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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.

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 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), and

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).

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,

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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%;

(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.

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MAIN REQUEST

1. Eculizumab for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), 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).

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%;

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(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 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).

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.

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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%;

(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.

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Appeal number: T1897/14-3.3.04

Order

1. In accordance with the business distribution scheme of the Technical Boards of Appeal, the following shall hear the above appeal:

Chairman: G. Alt

technically qualified member: R. Morawetz

legally qualified member: K. Garnett

In the case of an extended Board:

technically qualified member: _____

legally qualified member: _____

2. The rapporteur shall be: R. Morawetz

3. The additional rapporteur shall be: _____

4. Back to the Registry for further action.

Munich, 12.11.14

The Chairwoman G. Alt

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Patentanwälte Rechtsanwälte

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Europäisches Patentamt
Herr Eric Bihl
Abteilung CDR

80295 München

Epoline - Mailbox und MyFiles:
Umstellung aller Akten auf Zusammenschluss Nr. 31

München, 07. Januar 2015
BE/TWI

Sehr geehrter Herr Bihl,

wir möchten Sie hiermit beauftragen, eine durchgängige Umstellung aller Akten auf unseren Zusammenschluss Nr. 31 durchzuführen. Außerdem bitten wir Sie, zukünftige Anmeldungen diesbezüglich zu überprüfen und auf unseren Zusammenschluss umzustellen, falls dies nicht der Fall ist.

Unsere Adresse soll bitte zukünftig einheitlich wie folgt in Ihrer Datenbank angegeben werden:

VOSSIUS & PARTNER
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81675 München

Wir bitten Sie um eine Bestätigung nach erfolgter Aktenumstellung per Email an twi@vossiusandpartner.com.

Mit freundlichen Grüßen

Dr. Natalia Berryman

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Partnerschaftsregister Amtsgericht München PR 80

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482
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Page 3663

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EPO - Munich
58
12. März 2015

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Mitteilung neue Gesellschaftsform
VOSSIUS & PARTNER
Patentanwälte Rechtsanwälte mbB
Unser Zeichen: GF

München, 12. März 2015
BE/SKA

Sehr geehrte Damen und Herren,

hiermit teilen wir mit, dass sich die Gesellschaftsform unserer Kanzlei geändert hat in eine Partnerschaft mit beschränkter Berufshaftung. Der neue Name der Kanzlei lautet nun:

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Es wird gebeten, eine entsprechende Umschreibung der Vertreterdaten (Zusammenschluss 31) durchzuführen.

Dr. Natalia Berryman
European Patent Attorney

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EUROPEAN TRADEMARK ATTORNEYS
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4) European Patent Attorney
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Partnerschaftsregister Amtsgericht München PR 89

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Page 3664

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European Patent Office
80298 MUNICH
GERMANY
Tel. +49 (0)89 2399-0
Fax +49 (0)89 2399-4465

Appeal number: T1897/14-3.3.04

Order

Change of the composition of the Board

compare to order of 12.11.14

1.

Chairman: G. Alt

technically qualified member: R. Morawetz

technically qualified member: _____

legally qualified member: L. Bühler

legally qualified member: _____

2. The rapporteur shall be: R. Morawetz

3. The additional rapporteur shall be: _____

4. Reasons:
Change in the business distribution scheme of the board for the current year.

5. Back to the Registry for further action.

Munich, 20.07.15

The Chairwoman G. Alt

EPO Form 3303.16

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Appeal number: **T1897/14-3.3.04**

Order

Oral proceedings are to be held

on 03.05.18

at 09:30 hours

Room N° 0124

Munich 24.07.17

The Chairwoman
G. Alt

Samsung Bioepis Exhibit No. 1002
Page 3666

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81675 München
ALLEMAGNE

Date
27.07.17

Zeichen/Reference/Référence	Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°
M2367 EP APPR	05712607.0 / 1720571
Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire	
ALEXION PHARMACEUTICALS, INC.	

Appeal number: **T1897/14-3.3.04**

Summons to oral proceedings pursuant to Rule 115(1) EPC

You are hereby summoned to the oral proceedings concerning the above appeal.
The proceedings are scheduled to take place

on 03.05.18 at 09:30 hrs in Room 0124

Bob-van-Benthem-Platz 1, 80469 Munich (DE)

The proceedings will be public.

Room 115 is available as a waiting-room.

You are requested to attend outside the appointed room **10 minutes** before the hearing.

Registered letter with advice of delivery

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Appeal number: **T1897/14-3.3.04**

You are reminded that

- if a party who has been duly summoned to oral proceedings does not appear, the proceedings may continue without that party (Rule 115(2) EPC).
- oral proceedings will only be postponed at the request of a party for serious reasons (see Notice in Special edition No. 3 OJ EPO 2007, 115).
- as regards filing authorisations for representatives or company employees, see Decision of the President of the EPO dated 12.7.2007 on the filing of authorisations in Special edition No. 3 OJ EPO 2007, 128.
- concerning the language of the proceedings, attention is drawn to Rule 4 EPC. Notice given pursuant to Rule 4(1) EPC before the first instance is not valid in proceedings before the Boards of Appeal.
- According to Rule 103(2) EPC it is possible to obtain a 50% reimbursement of the appeal fee if the appeal is withdrawn under certain circumstances (OJ EPO 2014, A3).

Composition of the Board

Chairman: G. Alt

Member: R. Morawetz
Member: L. Bühler

For all urgent communications in connection with the oral proceedings please use only EPO Online Services (preferably) or fax No. + 49 (0) 89 2399 3014.

If you are planning not to attend the oral proceedings or if you are aware of any matter that could have a bearing on the appointment of interpreters, you are requested to inform the Board of this, preferably in writing, at the earliest possible opportunity.

The Registrar P. Cremona
Tel.: 089 / 2399 - 3341

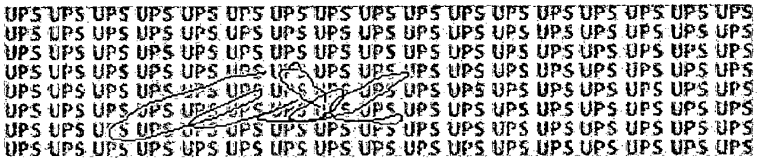
Annex(es): ☑ Confirmation of receipt Form 3936
☐ Communication pursuant to Article 15(1) of the Rules of Procedure of the Boards of Appeal
☐

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Tracking Information

EP/ECT/PCT No.:	**05712607**
Name:	Vossius & Partner
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Country:	DE
Formtype:	T1897/14-3.3.04 F.3011.1
Send Date:	Thursday, July 27, 2017

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Boards of Appeal

The Registry EPO - Munich 100
Name: P. Cremona
Tel.: 089 / 2399 - 3441 31. Juli 2017

Date: 27.07.17

Zeichen/Reference/Référence		Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°
M2367 OPP(EP)	APPR	05712607.0 / 1720571
Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire		
ALEXION PHARMACEUTICALS, INC.		

Appeal number: T1897/14-3.3.04

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Empfangsbescheinigung über den Zugang des vorstehend bezeichneten Schriftstücks
Acknowledgement of receipt of the document specified above
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Unter Bezugnahme auf die Mitteilung im ABl. EPA 7/2010, 377 wird gebeten, die Empfangsbescheinigung mit Empfangsdatum und Unterschrift zu versehen und **umgehend** an das EPA zurückzusenden:

With reference to the Notice in OJ EPO 7/2010, 377, you are requested to date and sign the acknowledgement of receipt and return it to the EPO **immediately:**

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Appeal number: T1897/14-3.3.04

Order

Oral proceedings to be held on 03.05.18

☐ are cancelled.

☑ are rescheduled for 03.05.18 at 09:30 hours

Room N° OP02

Munich, 13.09.17

The Chairwoman G. Alt

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Page 3671

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Datum / Date
18.09.2017

Zeichen/Reference/Référence		Anmeldung Nr. / Application No. / Demande n°. // Patent Nr. / Patent No. / Brevet n°.
M2367 EP	APPR	05712607.0 // 1720571
Anmelder / Applicant / Demandeur // Patentinhaber / Proprietor / Titulaire		
ALEXION PHARMACEUTICALS, INC.		

Appeal number: **T1897/14-3.3.04**

Communication of the Registry

The Registrar P. Cremona
Tel.: 089 / 2399 - 3341

Registered letter with advice of delivery

This document: 2 page(s) including this page

Annex(es): EPO Form 3936

Communication text

Move of the Boards of Appeal to Haar/ Important information for oral proceedings

The oral proceedings appointed for 3 May 2018 will now take place in:

Room OP02
Richard-Reitzner-Allee 8
85540 HAAR

A main waiting area is located at the entrance, directly after the security check. Further waiting rooms are located near to the oral proceedings rooms.

The other details (date, time etc.) in the original summons remain unchanged.

Reason: The Boards of Appeal will by then have moved to a new building in Haar. Oral proceedings will therefore be conducted there.

Please also consult the EPO website for information on travel to the Haar building, parking facilities, hotel accommodation, location map and on-site security arrangements.

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EPO - Munich
101
20. Sep. 2017

Boards of Appeal

The Registry
Name: P. Cremona
Tel.: 089 / 2399 - 3341

Date: 18.09.17

Zeichen/Reference/Référence	Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°
M2367 EP APPR	05712607.0 / 1720571
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ALEXION PHARMACEUTICALS, INC.	

Appeal number: T1897/14-3.3.04

EPA/EPO/OEB Formblatt/Form/Formulaire: 3004

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- **per Fax / by fax / par téléfax (+49 (0) 89 2399-4465 or +31 (0)70 340-3016)**
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19. SEP. 2017

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EUROPEAN PATENT ATTORNEY

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18. SEP. 2017

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09. Nov. 2017

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European Patent Office

80298 M U N I C H

T1897/14-3.3.04
(05 71 2607.0 / 1720571)
Patentee: Alexion Pharmaceuticals, Inc.
Opposition by: Pfizer Inc.
Vossius Ref.: M2367 OPP(EP)

Appeal Number T1551/12-3.3.01
EP 07 75 3249.7
Alexion Pharmaceuticals, Inc.
Vossius Ref.: P2913 EP

EP 16 17 2335.8 – 1466
Alexion Pharmaceuticals, Inc.
Vossius Ref.: P2913 EP/2

EP 16 19 5355.9 - 1466
Alexion Pharmaceuticals, Inc.
Vossius Ref.: P2913 EP/3

EP 16 20 1244.7 - 1410
Alexion Pharmaceuticals, Inc.
Vossius Ref.: R1249 EP/3

München, November 9, 2017
WA/FLG/MUA

We herewith resign from representation of the five above-identified matters.

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European Patent Attorneys
European Trademark Attorneys
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Josef Schmidt, Dipl.-Ing. 1) 9)
Dr. Hans-Rainer Jaenichen, Dipl.-Biol. 1)
Dr. Alexa v. Uexküll, M.Sc. 1)
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Dr. Joachim Wachenfeld, Biol. 1)
Dr. Friederike Stolzenburg, Dipl.-Biol. 1)
Rainer Viktor, Dipl.-Ing. 1)
Dr. Natalia Berryman, Dipl.-Chem. 1)
Dr. Jürgen Meier, Dipl.-Biol. 1)
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Arnold Asmussen, Dipl.-Ing. (FH) 1)
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Switzerland: HR Firmennummer CH-270.9.001.271-9

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Samsung Bioepis Exhibit No. 1002
Page 3677

Please direct all future correspondence to

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It is respectfully requested that this change is registered in the official Register.

Dr. Joachim Wachenfeld
European Patent Attorney

Samsung Bioepis Exhibit No. 1002
Page 3678

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European Patent Office
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European Patent Office
80298 MUNICH
GERMANY

Questions about this communication ?
Contact Customer Services at www.epo.org/contact

J A Kemp
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ROYAUME UNI

Date
20.11.17

Reference	Application No./Patent No.
	05712607.0 - 1412 / 1720571
Applicant/Proprietor	
ALEXION PHARMACEUTICALS, INC.	

Communication of amended entries concerning the representative (R. 143(1)(h) EPC)

As requested, for the above-mentioned European patent application / European patent the entries concerning the representative have been amended as follows:

J A Kemp
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The amendment will be recorded in the Register of European Patents.

For the Opposition Division

Samsung Bioepis Exhibit No. 1002
Page 3679

J A • K E M P

BY ONLINE FILING

The European Patent Office
Bayerstrasse 34
(entrance via Zollstrasse 3)
80335 Munich
Germany

29 March 2018

Dear Sirs

Appeal No. T1897/14-3.3.04
Opposition to European Patent No. 1720571
(Application No. 05712607.0)
ALEXION PHARMACEUTICALS, INC.
Our Ref: C412290EP PXT

I hereby withdraw the Appeal filed by Alexion Pharmaceuticals Inc.

Yours faithfully

Electronically Signed
DR PAMELA M TUXWORTH
ASSOCIATION NO. 811

PATENT ATTORNEYS • TRADE MARK ATTORNEYS
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A list of our partners is available at our principal place of business at the address above. Regulated by IPREG

Europäisches Patentamt
European Patent Office
Office européen des brevets

Submission in opposition proceedings

Representative:

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- representing the proprietor(s):	Alexion Pharmaceuticals, Inc.
Proprietor/representative's reference	C412290EP

The information given below is pertaining to the following patent in opposition proceedings:

Patent No.	EP1720571
Application No.	EP05712607.0
Date of mention of the grant in the European Patent Bulletin (Art. 97(3), Art. 99(1) EPC)	13 June 2012
Title of the invention	METHOD OF TREATING HEMOLYTIC DISEASE
Proprietor of the patent	Alexion Pharmaceuticals, Inc.

Documents attached:

	Description of document	Original file name	Assigned file name
1	Any annexes (other than citation) to an opposition letter - Request to withdraw appeal	Withdraw the Appeal C412290EP PXT.PDF	OTHER-1.PDF

Signatures

Place:	
Date:	**29 March 2018**
Signed by:	**Elizabeth Davenport 55598**
Association:	**J A Kemp**
Representative name:	**DAVENPORT, Elizabeth**
Capacity:	**(Representative)**

Samsung Bioepis Exhibit No. 1002
Page 3682

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Acknowledgement of receipt

We hereby acknowledge receipt of the following submission by the proprietor:

Submission number	6191091	
Application number	EP05712607.0	
Patent number	EP1720571	
Date of receipt	29 March 2018	
Your reference	C412290EP	
Proprietor	Alexion Pharmaceuticals, Inc.	
Title	METHOD OF TREATING HEMOLYTIC DISEASE	
Documents submitted	package-data.xml ep-oppo.pdf (2 p.)	ep-opposition-data.xml OTHER-1.PDF\Withdraw the Appeal C412290EP PXT.PDF (1 p.)
Submitted by	CN=Elizabeth Davenport 55598	
Method of submission	Online	
Date and time receipt generated	29 March 2018, 16:49 (CEST)	
Message Digest	5D:0B:34:04:C2:8E:7F:73:6F:82:BB:60:1A:18:5E:B6:61:51:2C:B6	

Correction by the EPO of errors in debit instructions filed by eOLF
Errors in debit instructions filed by eOLF that are caused by the editing of Form 1038E entries or the continued use of outdated software (all forms) may be corrected automatically by the EPO, leaving the payment date unchanged (see decision T 152/82, OJ EPO 1984, 301 and point 6.3 ff ADA, Supplement to OJ EPO 10/2007).

/European Patent Office/

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Appeal number: T1897/14-3.3.04

Order

Oral proceedings to be held on 03.05.18

☑ are cancelled.

☐ are rescheduled for at hours

Room N°

Munich, 03.04.18

The Chairwoman G. Alt

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Datum / Date
05.04.2018

Zeichen/Reference/Référence	Anmeldung Nr. / Application No. / Demande n°. // Patent Nr. / Patent No. / Brevet n°.
APPR	05712607.0 // 1720571
Anmelder / Applicant / Demandeur // Patentinhaber / Proprietor / Titulaire	
ALEXION PHARMACEUTICALS, INC.	

Appeal number: **T1897/14-3.3.04**

Communication of the Board of Appeal

The Rapporteur R. Morawetz
The Registrar P. Cremona
Tel.: 089 / 2399 - 3341

Registered letter

This document: 7 page(s) including this page

Annex(es):

Communication text

FAX: 089 413 04 111 (29 March 2018)

1. This communication is sent pursuant to Article 15(1) of the Rules of Procedure of the Boards of Appeal (RPBA). Any opinions expressed are provisional and not binding on the board in arriving at its decision (Article 17(2) RPBA).

2. The purpose of this communication is to set out some of the issues to be considered during the oral proceedings.

3. An opposition was filed against the patent. The patent was opposed under Article 100(a) EPC on the grounds of lack of novelty (Article 54 EPC) and lack of inventive step (Article 56 EPC) and under Article 100(b) and 100 (c) EPC. The opponent withdrew its opposition with letter dated 7 October 2013 and thus ceased to be a party to the proceedings.

4. The opposition division continued the opposition proceedings of its own volition. It decided that neither the main request nor the auxiliary request before it met the requirements of Article 56 EPC and revoked the patent.

5. The appeal of the patent proprietor (hereinafter "appellant") lies against this decision.

6. The board adopts the numbering of the documents as set out in point 1 of the decision under appeal (documents D1 to D15). Documents D16 to D19 were submitted by the appellant with its statement of grounds of appeal.

7. The appellant requests that the decision under appeal be set aside and that a patent be granted on the basis of the main request or, alternatively, on the basis of the auxiliary request, both as submitted with the statement of grounds of appeal. These claim requests correspond to the claim requests underlying the decision under appeal.

8. At present the board has the following comments on the claim requests on file.

Main request

Article 56 EPC - claim 1

9. The subject-matter of claim 1 relates to a four-stage dosing schedule for treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH) with the anti-C5 antibody eculizumab.

10. The opposition division held that the subject-matter of claim 1 of the main request lacked an inventive step over the teaching of document D5 as closest prior art when combined with the disclosure of document D6 (see decision under appeal, points 2.10 to 2.27).

11. The appellant did not dispute that document D5 was the closest prior art. According to the appellant the differences between the teaching of document D5 and the claimed subject-matter was twofold: (i) document D5 was silent about the dosage schedule applied prior to the bi-weekly administration of 900 mg and (ii) document D5 was silent about the feature of administering 900 mg every 12 days (see statement of grounds of appeal, point 1.3).

According to the appellant the problem to be solved was the provision of a complete eculizumab dosage regimen which is capable of preventing complement breakthroughs in all PNH patients (ibid.).

12. The appellant submitted that the opposition division had applied an "ex post facto" analysis in selecting document D6 as secondary reference (see statement of grounds of appeal, point 1.4.2), that optimising the eculizumab dosing schedule was not a matter of routine (ibid., point 1.5.1), and that document D5 failed to render decreasing the dosing interval from 14 days to 12 days obvious (ibid., point 1.5.3).

13. At present, the board is not convinced by the arguments submitted by the appellant.

14. Document D5 reports the results obtained in an extension trial, after the patients had completed a pilot trial. Thus, document 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 900 mg eculizumab by i.v. infusion over 30 minutes every other week for one year. To date all patients remain on eculizumab, completing between 11 and 15 months of therapy*" (see abstract, page 509, lines 5 to 8).

15. In the board's view, the skilled person faced with the problem of having to provide a complete eculizumab dosage regimen and knowing from document D5 that the patients receiving 900 mg every other week during the extension study were on a pilot study before would be motivated to look for the dosage regimen used in the pilot study.

16. Documents D2, D3 and D6 all relate to initial studies carried out in PNH patients which patients are said to take part in an extension study. Thus, document D2 reports that "*patients received 600 mg of eculizumab each week for four weeks, after which the dose was switched to 900 mg every two weeks. Ten patients have completed the 12-week treatment, and all choose to enter a 12-month extension trial*" (see second and first paragraph from bottom). Document D3 reports that "*eleven transfusion-dependent PNH patients have 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 (...) all patients have continued on eculizumab in an*

extension study" (see page 87, abstract, lines 5 to 8 and lines 28 to 30). Document D6 discloses "*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. All 11 patients completed the study and are currently participating in an open-label extension study*" (see page 197, abstract, lines 8 to 15).

17. Documents D2 and D3 thus disclose the same dosage regimen (600 mg eculizumab weekly for four doses and then 900 mg every other week) while the dosage regimen disclosed in document D6 contains an extra step compared to the dosage regimen disclosed in documents D2 and D3 (600 mg eculizumab weekly for four doses, followed by 900 mg 1 week later, and then 900 mg every other week) and is thus more detailed.

18. In the board's opinion, the skilled person faced with the problem of having to provide a complete dosing regimen and faced with the very similar disclosures in documents D2, D3 and D6 which apparently relate to one and the same pilot study would choose the most comprehensive dosage regimen, i.e. the one disclosed in document D6. No ex post facto considerations are involved in this choice.

19. The appellant relied on documents D13, D15, D7, D16, D17, D14 to support its argument that the skilled person would have proceeded very cautiously when either increasing the dose or reducing the dose of eculizumab (see statement of grounds of appeal, point 1.5.1).

20. The board notes that documents D14, D15, D16, D17 were not available before the priority date of the patent and could thus not have influenced the skilled person's

Samsung Bioepis Exhibit No. 1002
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attitude.

21. Appellant's arguments based on documents D7 and D13 fail because document D6 discloses that the dosage regimen is well tolerated "*with no patients reporting a serious adverse event*" (see page 197, abstract, lines 15 to 18).

22. As regards the second difference (see point 11), the board notes that document D5 already 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*" (see abstract, page 510a, lines 8 to 12).

23. Document D5 thus discloses that a slight decrease of the dosing interval solves the problem of complement breakthrough. All that the skilled person thus needed to do was to maintain the dose (900 mg) and decrease the dosing interval from "every other week" to slightly less than that until complement breakthrough was prevented. Since the skilled person already knew from document D5 that this would give the desired result, the actual determination of the appropriate dosing interval, i.e. every 12 days, would appear to be a matter of routine devoid of inventive activity.

Auxiliary request

Article 56 EPC - claim 1

24. The opposition division held that the feature "*wherein cell lysis is reduced below 20% of normal serum complement activity when treated under a dosing schedule according to items (i) to (iv)*" was at best

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Page 3690

another read-out to confirm the same resolution of breakthrough already recorded in document D5.

25. The appellant submitted that the effective inhibition of haemolytic activity was a key advantage being associated with the subject-matter claimed in the main and auxiliary requests and that this advantage was expressly incorporated in the main claim of the auxiliary request (ibid., point II).

26. At present, the board fails to see how this line of reasoning addresses the reasons given in the decision under appeal, it appears rather to confirm the point made by the opposition division. In any case, since the subject-matter claimed in the main request is at present considered to be obvious, the board fails to see how the inclusion of an effect associated with a dosage regimen which is considered to be obvious can render the claimed subject-matter inventive.

Concluding remarks

27. Should the appellant choose to file further written submissions, then attention is drawn to Article 114(2) EPC and Articles 13(1) and 13(3) RPBA.

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Date
06.04.18

Zeichen/Reference/Référence	Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°
APPR	05712607.0 / 1720571
Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire	
ALEXION PHARMACEUTICALS, INC.	

Appeal number: T1897/14-3.3.04

Cancellation

1. ☑ The oral proceedings appointed for 03.05.18 have been cancelled.

2. ☐ The oral proceedings appointed for

have been rescheduled to: at .

will now be in Room No.

The other provisions in the summons of 27.07.17 remain unchanged.

The Registrar P. Cremona
Tel.: 089 / 2399 - 3341

Annex(es): ☑ Acknowledgement of receipt - EPO Form 3936

Registered letter with advice of delivery

Europäisches Patentamt
European Patent Office
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Termination of opposition proceedings of patent no. 05712607.0 - 1116/ 1720571 with revocation of the patent 13.04.18

I. **Findings**

1. The opposition proceedings are terminated for the following reason:
The patent was revoked

REVO 01.07.14

The time limit pursuant to Article 108 EPC has expired.
No appeal or application pursuant to Article 122 EPC has been filed.

REVO 2 and DEAD coded and Form 2363 despatched.

2. Fees situation:

OPPO04	010	00625522	12.03.13	EUR	745,00
NOAP04	011	00116739	08.09.14	EUR	1 860,00
CLMS(2)	015	00571629	01.09.06	EUR	765,00

Checked for correct fee payment. Refund(s) made on ...../... .

3. Any models still to hand were returned on ....n/a..... .

4. Enter "DEAD" and dispose of paper file

13.04.2018 ..... Date

Bertoni, Sara ..... Formalities officer

SB51929

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Boards of Appeal

The Registry

Name: P. Cremona
Tel.: 089 / 2399 - 3341

Date: 06.04.18

Zeichen/Reference/Référence	Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°
APPR	05712607.0 / 1720571
Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire	
ALEXION PHARMACEUTICALS, INC.	

Appeal number: T1897/14-3.3.04

EPA/EPO/OEB Formblatt/Form/Formulaire: 3017

Empfangsbescheinigung über den Zugang des vorstehend bezeichneten Schriftstücks
Acknowledgement of receipt of the document specified above
Récépissé du document spécifié ci-dessus

Unter Bezugnahme auf die Mitteilung im ABl. EPA 7/2010, 377 wird gebeten, die Empfangsbescheinigung mit Empfangsdatum und Unterschrift zu versehen und **umgehend** an das EPA zurückzusenden:

With reference to the Notice in OJ EPO 7/2010, 377, you are requested to date and sign the acknowledgement of receipt and return it to the EPO **immediately:**

Conformément au communiqué paru au JO OEB 7/2010, 377, vous êtes prié d'indiquer sur le récépissé la date de réception du document, de signer le récépissé et de le renvoyer **sans délai** à l' OEB:

- **über die Online-Dienste des EPA** (als Anlage zu EPA Form 1038) / **through EPO Online Services** (as annex to EPO Form 1038) / **par les services en ligne de l'OEB** (en tant que pièce jointe au formulaire OEB 1038),
- **per Fax / by fax / par téléfax (+49 (0) 89 2399-4465 or +31 (0)70 340-3016)**
- oder per Post / or by post / ou par courrier

Empfangen am / Received on / Reçu le:

13. 4. 18

Unterschrift / Signature:

Empfangsberechtigter/authorised recipient/
le destinataire ou la personne dûment mandatée

Rücksende-Adresse / Return address / Adresse de retour
(Umschlag / envelope / enveloppe ISO C4 / DL / C6/C5 / C6)

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Received at the EPO on Apr 13, 2018 10:48:30. Page 1 of 1 513
Duration: 04.13.2018 10:47:05 AM - 04.13.2018 10:48:30 AM. This page 1 of 1 was completed at 04.13.2018 10:48:30 AM

Samsung Bioepis Exhibit No. 1002
Page 3694

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Datum/Date
18.04.18

Zeichen/Reference/Référence	Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°
C412290EP APPR	05712607.0 / 1720571
Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire	
ALEXION PHARMACEUTICALS, INC.	

Appeal number: T1897/14-3.3.04

Closure of the appeal proceedings
Withdrawal of the appeal/application/opposition by the sole appellant

The

☑ appeal(s) ☐ opposition(s)

☐ application

of the appellant has been withdrawn by letter dated 29.03.18 and received on 29.03.18 .

The Board has instructed the Registrar to notify all concerned that the appeal proceedings are accordingly closed without a substantive decision.

The appeal fee will be reimbursed pursuant to Rule 103(2)(a) EPC.

By order of the Board of Appeal
The Registrar Danielle Hampe
Tel.: 089 / 2399 - 3341

Annex(es):/

Registered letter

EPO Form 3324

Samsung Bioepis Exhibit No. 1002
Page 3695

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Date
20.04.18

Zeichen/Reference/Référence	Anmeldung Nr./Application No./Demande n°//Patent Nr./Patent No./Brevet n°
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Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire	
ALEXION PHARMACEUTICALS, INC.	

Appeal number: T1897/14-3.3.04

Refund of fees

The following fees was/were paid in respect of the application 05712607.0 :

Fee	Reference	Voucher No	Date	Currency	Amount
appeal-fee	011	00116739	08.09.14	EUR	1.860,00

According to the present state of the file the refund will be made by:

CREDITING THE DEPOSIT ACCOUNT 28050038 (J.A. Kemp)

Amount refundable:	Reference	Currency	Amount
930,00	011	EUR	930,00

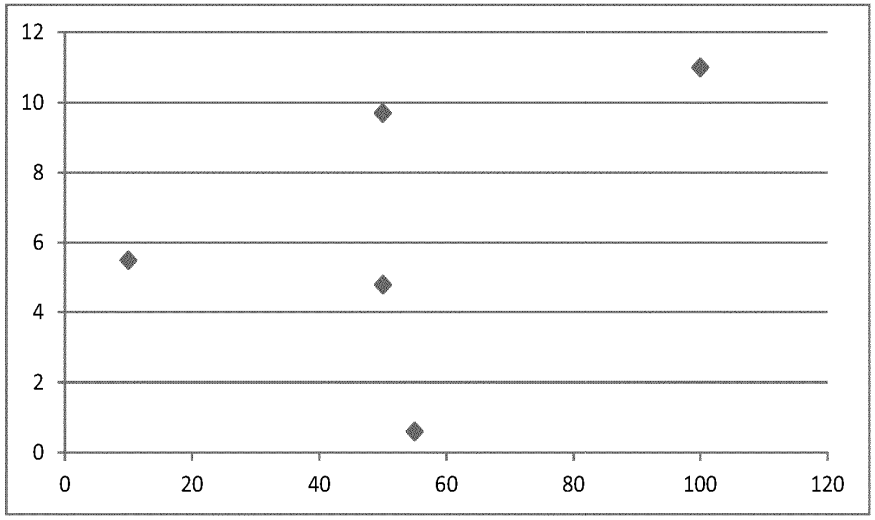
Reason: pursuant to R.103(2)(a) EPC, following the withdrawal of the appeal filed on 28.03.2018.

The Registrar D. Hampe
Tel.: 089 / 2399 - 3341

Annex(es): ☐

% Aggregation Versus Antibody Concentration
From Table 2 of D20 where concentration and % aggregates known)

Antibody	Concentration in Formulation (mg/mL)	% Aggregates
rhyMAbE25	10	5.5
Human anti-IL-8 (IgG2)	50	4.8
RhuMAb HER2	50	9.7
Human Anti-IL-8 (IgG2)	55	0.6
Anti-IL-8 (IgG2)	100	11



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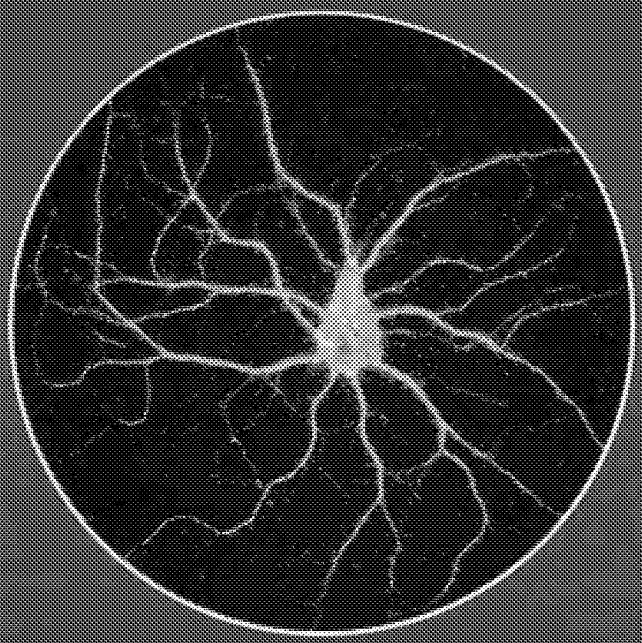
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MOLECULAR BIOLOGY OF THE CELL

THIRD EDITION



Bruce Alberts • Dennis Bray
Julian Lewis • Martin Raff
Keith Roberts • James D. Watson

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MOLECULAR BIOLOGY OF
THE CELL
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Front cover: The photograph shows a rat nerve cell in culture. It is labeled (*yellow*) with a fluorescent antibody that stains its cell body and dendritic processes. Nerve terminals (*green*) from other neurons (not visible), which have made synapses on the cell, are labeled with a different antibody. (Courtesy of Olaf Mundigl and Pietro de Camilli.)

Dedication page: Gavin Borden, late president of Garland Publishing, weathered in during his mid-1980s climb near Mount McKinley with MBoC author Bruce Alberts and famous mountaineer guide Mugs Stump (1940–1992).

Back cover: The authors, in alphabetical order, crossing Abbey Road in London on their way to lunch. Much of this third edition was written in a house just around the corner. (Photograph by Richard Olivier.)

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Intracellular Compartments and Protein Sorting

12

- The Compartmentalization of Higher Cells
- The Transport of Molecules into and out of the Nucleus
- The Transport of Proteins into Mitochondria and Chloroplasts
- Peroxisomes
- The Endoplasmic Reticulum

Unlike a bacterium, which generally consists of a single intracellular compartment surrounded by a plasma membrane, a eucaryotic cell is elaborately subdivided into functionally distinct, membrane-bounded compartments. Each compartment, or **organelle,** contains its own characteristic set of enzymes and other specialized molecules, and complex distribution systems transport specific products from one compartment to another. To understand the eucaryotic cell, it is essential to know what occurs in each of these compartments, how molecules move between them, and how the compartments themselves are created and maintained.

Proteins play a central part in the compartmentalization of a eucaryotic cell. They catalyze the reactions that occur in each organelle and selectively transport small molecules into and out of its interior, or *lumen*. Proteins also serve as organelle-specific surface markers that direct new deliveries of proteins and lipids to the appropriate organelle. A mammalian cell contains about 10 billion (10^{10}) protein molecules of perhaps 10,000 kinds, and the synthesis of almost all of them begins in the cytosol. Each newly synthesized protein is then delivered specifically to the cell compartment that requires it. We shall make the intracellular transport of proteins the central theme of this chapter as well as of the next. By tracing the protein traffic from one compartment to another, one can begin to make sense of the otherwise bewildering maze of intracellular membranes.

The Compartmentalization of Higher Cells

In this introductory section we give a brief overview of the compartments of the cell and of the relationships between them. In doing so, we organize the organelles conceptually into a small number of discrete families, discussing how proteins are directed to specific organelles and how they cross organelle membranes.

All Eucaryotic Cells Have the Same Basic Set of Membrane-bounded Organelles [1]

Many vital biochemical processes take place in or on membrane surfaces. Lipid metabolism, for example, is catalyzed mostly by membrane-bound enzymes, and oxidative phosphorylation and photosynthesis both require a membrane in or-

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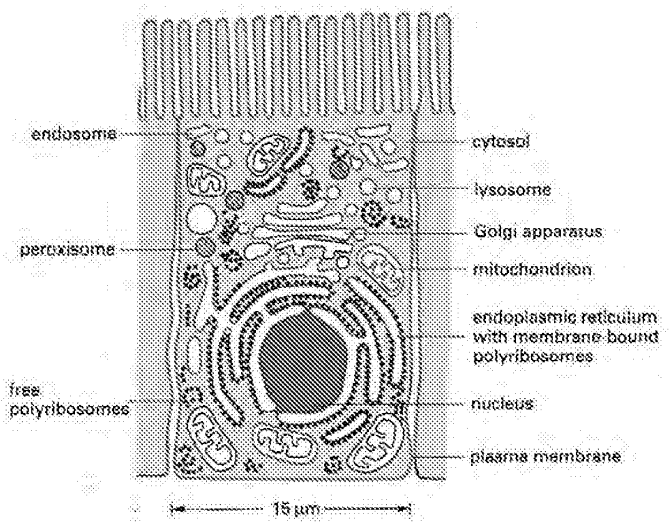


Figure 12–1 The major intracellular compartments of an animal cell. The cytosol (*gray*), endoplasmic reticulum, Golgi apparatus, nucleus, mitochondrion, endosome, lysosome, and peroxisome are distinct compartments isolated from the rest of the cell by at least one selectively permeable membrane.

der to couple the transport of H^+ to the synthesis of ATP. Intracellular membrane systems, however, do more for the cell than just provide increased membrane area: they create enclosed compartments that are separate from the cytosol, thus providing the cell with functionally specialized aqueous spaces. Because the lipid bilayer of organelle membranes is impermeable to most hydrophilic molecules, the membrane of each organelle must contain transport proteins that are responsible for the import and export of specific metabolites. Each organelle membrane must also have a mechanism for importing, and incorporating into the organelle, the specific proteins that make the organelle unique.

The major intracellular compartments common to eucaryotic cells are illustrated in Figure 12–1. The *nucleus* contains the main genome and is the principal site of DNA and RNA synthesis. The surrounding *cytoplasm* consists of the *cytosol* and the cytoplasmic organelles suspended in it. The cytosol constitutes a little more than half the total volume of the cell and is the site of protein synthesis and of most of the cell's intermediary metabolism—that is, the many reactions by which some small molecules are degraded and others are synthesized to provide the building blocks of macromolecules (discussed in Chapter 2).

About half the total area of membrane in a cell encloses the labyrinthine spaces of the *endoplasmic reticulum (ER)*. The ER has many ribosomes bound to its cytosolic surface; these are engaged in the synthesis of integral membrane proteins and soluble proteins, most of which are destined for secretion or for other organelles. We shall see that this reflects an important difference between how proteins are directed to the ER and how they are directed to other cytoplasmic organelles: whereas proteins are translocated into other organelles only after their synthesis is complete, they are translocated into the ER during their synthesis, and hence the ribosomes on which they are made are tethered to the ER membrane. The ER also produces the lipid for the rest of the cell and functions as a store for Ca^{2+} ions. The *Golgi apparatus* consists of organized stacks of disclike compartments called Golgi *cisternae*; it receives lipids and proteins from the ER and dispatches them to a variety of destinations, usually covalently modifying them en route.

Mitochondria and (in plants) *chloroplasts* generate most of the ATP used to drive cellular reactions that require an input of free energy. *Lysosomes* contain digestive enzymes that degrade defunct intracellular organelles, as well as macromolecules and particles taken in from outside the cell by endocytosis. On their way to lysosomes, endocytosed material must first pass through a series of compartments called *endosomes*. *Peroxisomes* (also known as *microbodies*) are small vesicular compartments that contain enzymes utilized in a variety of oxidative

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Table 12–1 **The Relative Volumes Occupied by the Major Intracellular Compartments in a Liver Cell (Hepatocyte)**

Intracellular Compartment	Percent of Total Cell Volume	Approximate Number per Cell*
Cytosol	54	1
Mitochondria	22	1700
Rough ER cisternae	9	1
Smooth ER cisternae plus Golgi cisternae	6	
Nucleus	6	1
Peroxisomes	1	400
Lysosomes	1	300
Endosomes	1	200

*All the cisternae of the rough and smooth endoplasmic reticulum are thought to be joined to form a single large compartment. The Golgi apparatus, in contrast, is organized into a number of discrete sets of stacked cisternae in each cell, and the extent of interconnection between these sets has not been clearly established.

reactions. In general, each membrane-bounded organelle carries out the same set of basic functions in all cell types but varies in abundance and can have additional properties that differ from cell type to cell type according to the specialized functions of differentiated cells.

On average, the membrane-bounded compartments together occupy nearly half the volume of a cell (Table 12–1), and a large amount of intracellular membrane is required to make them all. In the two mammalian cells analyzed in Table 12–2, for example, the endoplasmic reticulum has a total membrane surface area that is, respectively, 25 times and 12 times that of the plasma membrane. In terms of its area and mass the plasma membrane is only a minor membrane in most eucaryotic cells (Figure 12–2).

Table 12–2 **Relative Amounts of Membrane Types in Two Types of Eucaryotic Cells**

	Percent of Total Cell Membrane	
Membrane Type	Liver Hepatocyte*	Pancreatic Exocrine Cell*
Plasma membrane	2	5
Rough ER membrane	35	60
Smooth ER membrane	16	<1
Golgi apparatus membrane	7	10
Mitochondria		
Outer membrane	7	4
Inner membrane	32	17
Nucleus		
Inner membrane	0.2	0.7
Secretory vesicle membrane	not determined	3
Lysosome membrane	0.4	not determined
Peroxisome membrane	0.4	not determined
Endosome membrane	0.4	not determined

*These two cells are of very different sizes, since the average hepatocyte has a volume of about 3000 μm^3 compared with about 1000 μm^3 for the pancreatic exocrine cell. Total cell membrane areas are estimated at about 110,000 μm^2 and 13,000 μm^2, respectively.

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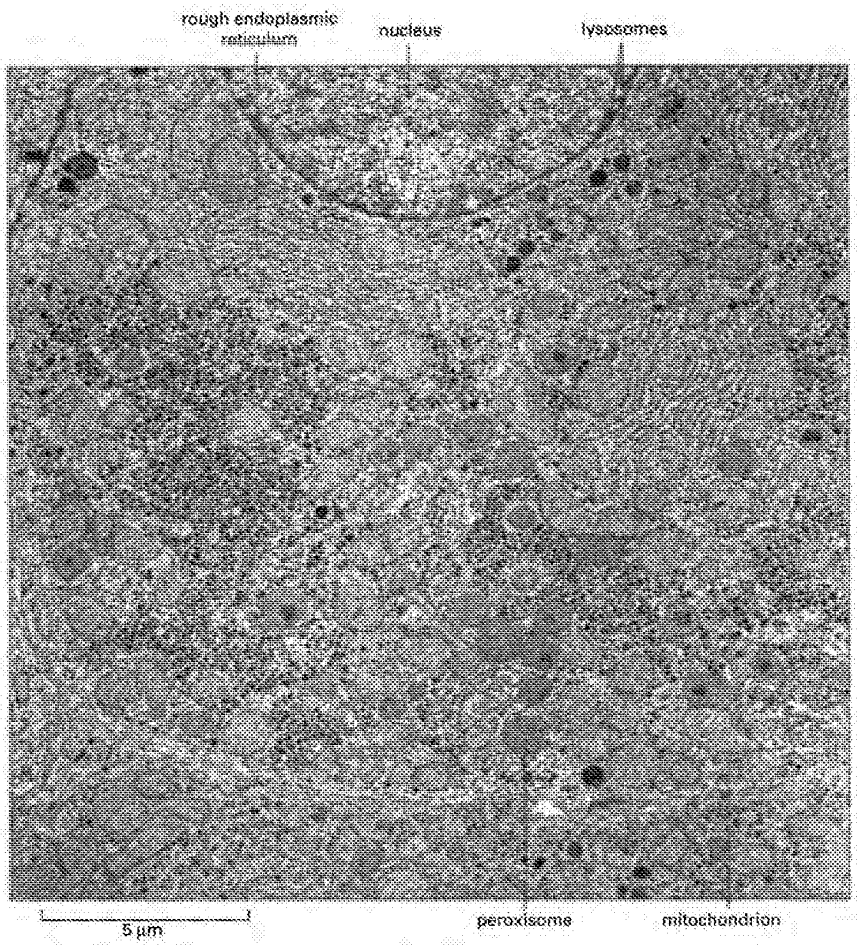


Figure 12–2 Electron micrograph of part of a liver cell seen in cross-section. Examples of most of the major intracellular compartments are indicated. (Courtesy of Daniel S. Friend.)

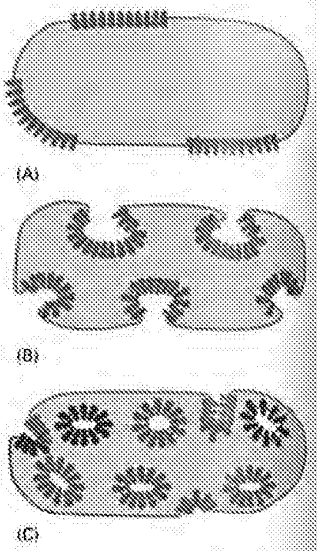


Figure 12–3 Organization of specialized membranes in bacteria. (A) Membrane patches on the cell surface consisting of clusters of specialized membrane proteins. (B) Invaginated patches of plasma membrane that increase the amount of membrane available for a specialized function such as photosynthesis. (C) Internalization of the specialized invaginated membrane to form vesicles, whose interior surface is topologically equivalent to the exterior surface of the cell. Membrane-bounded vesicles of this type are present in some types of photosynthetic bacteria; their topological relationship to the cell surface is similar to that of the ER, Golgi apparatus, endosomes, and lysosomes in eucaryotic cells.

Membrane-bounded organelles are not randomly distributed in the cytosol; instead they often have characteristic positions. In most cells, for example, the Golgi apparatus is located close to the nucleus, whereas the network of ER tubules extends from the nucleus throughout the entire cytosol. These characteristic distributions seem to depend on interactions of the organelles with the cytoskeleton: the localization of both the ER and the Golgi apparatus, for example, is dependent on an intact microtubule array; if the microtubules are experimentally depolymerized with a drug, the Golgi apparatus fragments and disperses throughout the cell and the ER network collapses toward the cell center, or *centrosome*, from which the microtubule array emanates (discussed in Chapter 16).

The Topological Relationships of Membrane-bounded Organelles Can Be Interpreted in Terms of Their Evolutionary Origins [2]

To understand the relationships between the compartments of the cell, it is helpful to consider how they might have evolved. The precursors of the first eucaryotic cells are thought to have been simple organisms that resembled bacteria, which generally have a plasma membrane but no internal membranes. The plasma membrane in such cells therefore provides all membrane-dependent functions, including the pumping of ions, ATP synthesis, protein secretion, and lipid synthesis. Typical present-day eucaryotic cells are 10 to 30 times larger in linear dimension and 1000 to 10,000 times greater in volume than a typical bacterium such as *E. coli*. The profusion of internal membranes can be seen in part as an

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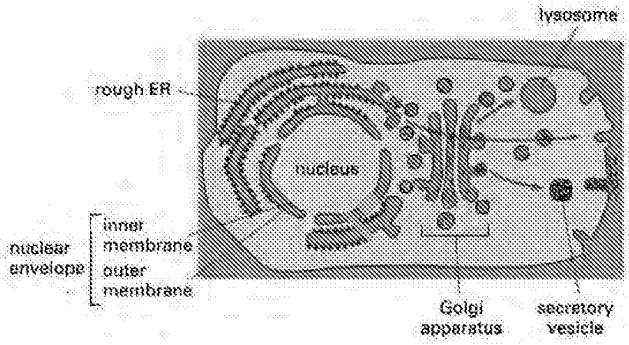


Figure 12–4 Topological relationships between compartments in a eucaryotic cell. Topologically equivalent spaces are shown in *red*. In principle, cycles of vesicle budding and fusion permit any lumen to communicate with any other and with the cell exterior. The *blue arrows* indicate the outward direction of vesicle traffic from the ER to Golgi apparatus to plasma membrane (or lysosomes), and the *black dots* represent protein molecules that are secreted by the cell. Some organelles, most notably mitochondria and (in plant cells) chloroplasts, however, do not take part in this vesicular communication and so are isolated from the traffic between organelles shown here.

adaptation to this increase in size: the eucaryotic cell has a much smaller ratio of surface to volume, and its area of plasma membrane is presumably too small to sustain the many vital functions for which membranes are required.

The evolution of internal membranes evidently has gone hand in hand with specialization of membrane function. Some present-day bacteria have specialized patches of plasma membrane in which a selected set of membrane proteins coalesce to carry out a group of related functions (Figure 12–3A). These specialized membrane patches, such as the "purple membrane" containing bacteriorhodopsin in *Halobacterium* (discussed in Chapter 10) and the chromatophores in photosynthetic bacteria (discussed in Chapter 14), represent primitive organelles. In some photosynthetic bacteria the patches have become elaborated into extensive invaginations of the plasma membrane (Figure 12–3B); in others the invaginations seem to have pinched off completely, forming sealed membrane-bounded vesicles specialized for photosynthesis (Figure 12–3C).

A eucaryotic organelle that originated by the type of pathway illustrated in Figure 12–3 might be expected to have an interior that is topologically equivalent to the exterior of the cell. We shall see that this is the case for the ER, Golgi apparatus, endosome, and lysosome—as well as for the many vesicular intermediates (*transport vesicles*) in the secretory and endocytic pathways. We can therefore think of all of these organelles as members of the same family, and, as we discuss in detail in the next chapter, their interiors communicate extensively with one another and with the outside of the cell via transport vesicles that bud off from one organelle and fuse with another (Figure 12–4). Ribosomes are found attached to the cytosolic side of the plasma membrane in bacteria, and so the evolutionary origin of the ER membrane from the plasma membrane may explain why ribosomes are attached to the ER membrane in eucaryotic cells.

This evolutionary scheme also offers a reasonable explanation for the architecture of the cell nucleus with its double membrane. In bacteria the single chromosome is attached at special sites to the inside of the plasma membrane. It is possible therefore that the double-layered nuclear envelope originated as a deep invagination of the plasma membrane, as shown in Figure 12–5A. This scheme would explain why the nuclear compartment is topologically equivalent to the cytosol. In fact, in higher eucaryotic cells the nuclear envelope breaks down during mitosis, allowing the nuclear contents to disperse in the cytosol, a situation that never occurs for the contents of any other membrane-bounded organelle. As the scheme in Figure 12–5A also predicts, the space between the two nuclear membranes is topologically equivalent to the exterior of the cell and is continuous with the lumen of the ER.

As discussed in Chapter 14, mitochondria and plastids (of which chloroplasts are one form) differ from the other membrane-bounded organelles in that they contain their own genomes. The nature of these genomes and the close resemblance of the proteins in these organelles to those in some present-day bacteria strongly suggest that mitochondria and plastids evolved from bacteria that were engulfed by other cells with which they initially lived in symbiosis (discussed in Chapters 1 and 14). According to the hypothetical scheme shown in Figure 12–3B, the inner membrane of mitochondria and plastids corresponds to the original

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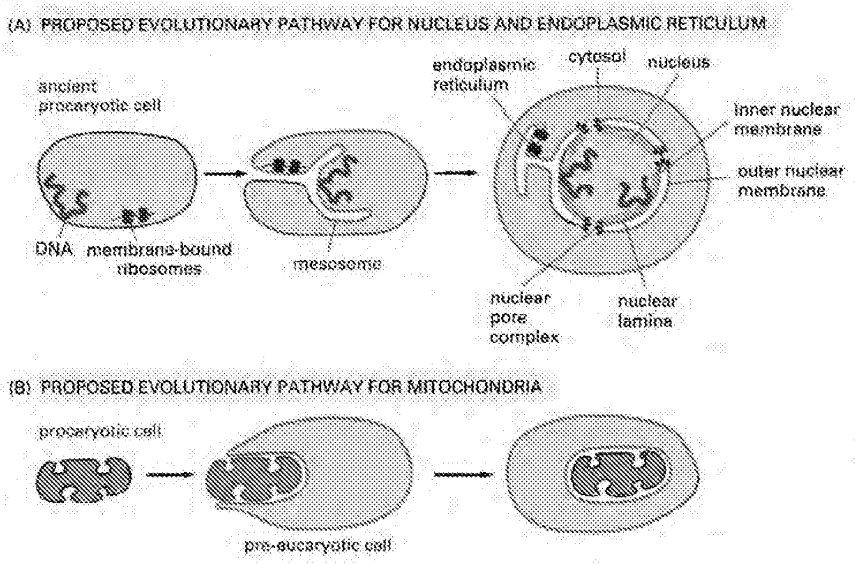


Figure 12–5 Hypotheses for the evolutionary origins of some membrane-bounded organelles. The origins of mitochondria, chloroplasts, ER, and the cell nucleus could explain the topological relationships of these intracellular compartments in eucaryotic cells. (A) A possible pathway for the evolution of the cell nucleus and the ER. In some bacteria the single DNA molecule is attached to an invagination of the plasma membrane, called a *mesosome*. Such an invagination in a very ancient procaryotic cell could have spread to form an envelope around the DNA while still allowing access of the DNA to the cell cytosol (as is required for DNA to direct protein synthesis). This envelope is presumed to have eventually pinched off completely from the plasma membrane, producing a nuclear compartment surrounded by a double membrane. As illustrated, the nuclear envelope is organized by a fibrous shell called the *nuclear lamina* and is penetrated by communicating channels called *nuclear pore complexes*. Because it is surrounded by two membranes that are in continuity where they are penetrated by these pores, the nuclear compartment is topologically equivalent to the cytosol. The lumen of the ER is continuous with the space between the inner and outer nuclear membranes and topologically equivalent to the extracellular space. (B) Mitochondria (and chloroplasts) are thought to have originated when a bacterium was engulfed by a larger pre-eucaryotic cell. They retain their autonomy. This may explain why the lumens of these organelles remain isolated from the vesicular traffic that interconnects the lumens of many other intracellular compartments.

plasma membrane of the bacterium, while the lumen of these organelles evolved from the bacterial cytosol. As might be expected from such origins, these two organelles remain isolated from the extensive vesicular traffic that connects the interiors of most of the other membrane-bounded organelles to one another and to the outside of the cell.

This evolutionary scheme groups the intracellular compartments in eucaryotic cells into five distinct families: (1) the nucleus and the cytosol, which communicate through the nuclear pores and are thus topologically continuous (although functionally distinct); (2) all organelles that function in the secretory and endocytic pathways—including the ER, Golgi apparatus, endosomes, lysosomes, and numerous classes of transport vesicles; (3) the mitochondria; (4) the plastids (in plants only); and (5) the peroxisomes (whose evolutionary origins are discussed later).

Proteins Can Move Between Compartments in Different Ways [3]

All proteins begin being synthesized on ribosomes in the cytosol, except for the few that are synthesized on the ribosomes of mitochondria and plastids. Their subsequent fate depends on their amino acid sequence, which can contain **sorting signals** that direct their delivery to locations outside the cytosol. Most proteins do not have a sorting signal and consequently remain in the cytosol as permanent residents. Many others, however, have specific sorting signals that direct their transport from the cytosol into the nucleus, the ER, mitochondria, plastids (in plants), or peroxisomes; sorting signals can also direct the transport of proteins from the ER to other destinations in the cell.

To understand the general principles by which sorting signals operate, it is important to distinguish three fundamentally different ways by which proteins move from one compartment to another. (1) The protein traffic between the cytosol and nucleus occurs between topologically equivalent spaces, which are in continuity through the nuclear pore complexes. This process is called **gated transport** because the nuclear pore complexes function as selective gates that can actively transport specific macromolecules and macromolecular assemblies, although they also allow free diffusion of smaller molecules. (2) In **transmembrane transport** membrane-bound *protein translocators* directly transport specific proteins across a membrane from the cytosol into a space that is topologically distinct. The transported protein molecule usually must unfold in order to snake

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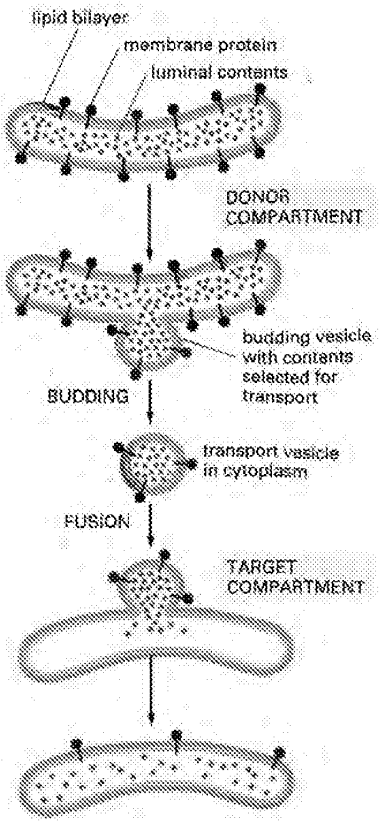


Figure 12–6 The "sidedness" of membranes is preserved during vesicular transport. Note that the original orientation of both proteins and lipids in the donor-compartment membrane is preserved in the target-compartment membrane and that soluble molecules are transferred from lumen to lumen.

through the membrane. The initial transport of selected proteins from the cytosol into the ER lumen or into mitochondria, for example, occurs in this way. (3) In **vesicular transport,** *transport vesicles* ferry proteins from one compartment to another. The vesicles become loaded with a cargo of molecules derived from the lumen of one compartment as they pinch off from its membrane; they discharge their cargo into a second compartment by fusing with its membrane. The transfer of soluble proteins from the ER to the Golgi apparatus, for example, occurs in this way. Because the transported proteins do not cross a membrane, they move only between compartments that are topologically equivalent (Figure 12–6). We discuss vesicular transport in more detail in Chapter 13. The three ways in which proteins are transported between different compartments are summarized in Figure 12–7.

Each of the three modes of protein transfer is usually selectively guided by sorting signals in the transported protein that are recognized by complementary receptor proteins in the target organelle. If a large protein is to be imported into the nucleus, for example, it must possess a sorting signal that is recognized by receptor proteins associated with the nuclear pore complex. If a protein is to be transferred directly across a membrane, it must possess a sorting signal that is recognized by the translocator in the membrane to be crossed. Likewise, if a protein is to be incorporated into certain types of transport vesicles or to be retained in certain organelles, its sorting signal must be recognized by a complementary receptor in the appropriate membrane.

Signal Peptides and Signal Patches Direct Proteins to the Correct Cellular Address [4]

There are at least two types of sorting signals on proteins. One type resides in a continuous stretch of amino acid sequence, typically 15 to 60 residues long. This **signal peptide** is often (but not always) removed from the finished protein by a specialized **signal peptidase** once the sorting process has been completed. The other type consists of a specific three-dimensional arrangement of atoms on the

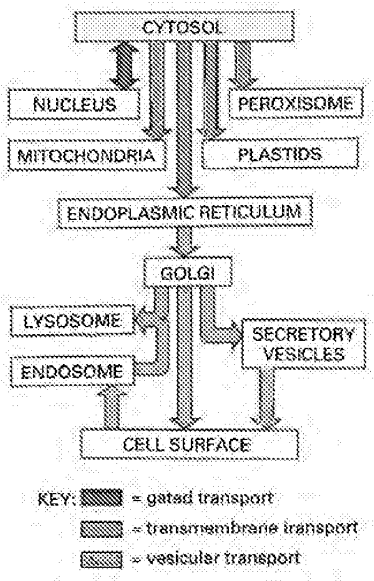


Figure 12–7 A simplified "road map" of protein traffic. Proteins can move from one compartment to another by gated transport (*red*), transmembrane transport (*blue*), or vesicular transport (*green*). The signals that direct a given protein's movement through the system, and thereby determine its eventual location in the cell, are contained in its amino acid sequence. The journey begins with the synthesis of a protein on a ribosome and terminates when the final destination is reached. At each intermediate station (*boxes*) a decision is made as to whether the protein is to be retained or transported further. In principle, a signal could be required either for retention in or for exit from each of the compartments shown, with the alternative fate being the *default pathway* (one that requires no signal). The vesicular transport of proteins from the ER through the Golgi apparatus to the cell surface, for example, appears not to require any specific sorting signals; specific sorting signals therefore are required to retain in the ER and the Golgi apparatus those specialized proteins that are resident there.

We shall use this figure repeatedly as a guide throughout this chapter and the next, highlighting the particular pathway being discussed.

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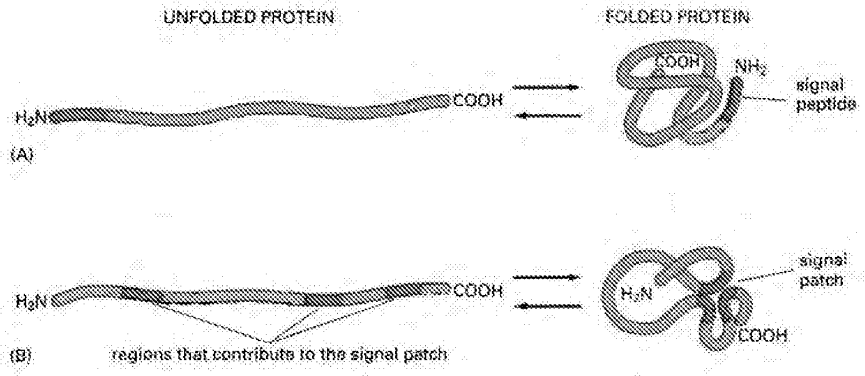


Figure 12–8 Two ways that a sorting signal can be built into a protein. (A) The signal resides in a single discrete stretch of amino acid sequence, called a *signal peptide*, that is exposed in the folded protein. Signal peptides often occur at the end of the polypeptide chain (as shown), but they can also be located elsewhere. (B) A *signal patch* can be formed by the juxtaposition of amino acids from regions that are physically separated before the protein folds (as shown); alternatively, separate patches on the surface of the folded protein that are spaced a fixed distance apart could form the signal. In either case the transport signal depends on the three-dimensional conformation of the protein, which makes it difficult to locate the signal precisely.

protein's surface that forms when the protein folds up. The amino acid residues that comprise this **signal patch** can be distant from one another in the linear amino acid sequence, and they generally remain in the finished protein (Figure 12–8). Signal peptides are used to direct proteins from the cytosol into the ER, mitochondria, chloroplasts, peroxisomes, and nucleus, and they are also used to retain soluble proteins in the ER. Signal patches identify certain enzymes that are to be marked with specific sugar residues that then direct them from the Golgi apparatus into lysosomes; signal patches are also used in other sorting steps that have been less well characterized.

Different types of signal peptides are used to specify different destinations in the cell. Proteins destined for initial transfer to the ER usually have a signal peptide at their amino terminus, which characteristically includes a sequence composed of about 5 to 10 hydrophobic amino acids. Most of these proteins will in turn pass from the ER to the Golgi apparatus, but those with a specific sequence of four amino acids at their carboxyl terminus are retained as permanent ER residents. Proteins destined for mitochondria have signal peptides of yet another type, in which positively charged amino acids alternate with hydrophobic ones. Proteins destined for peroxisomes usually have a specific signal sequence of three amino acids at their carboxyl terminus. Many proteins destined for the nucleus carry a signal peptide formed from a cluster of positively charged amino

Table 12–3 Some Typical Signal Peptides

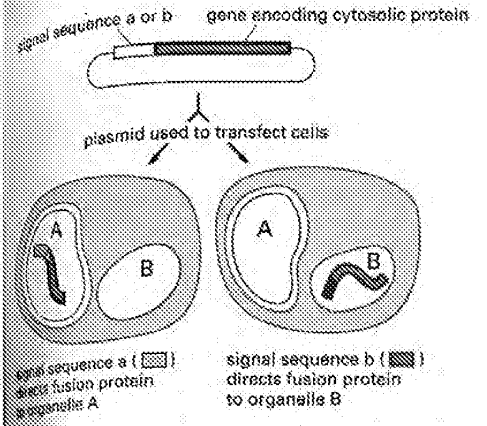
Function of Signal Peptide	Example of Signal Peptide
Import into ER	$^{+}H_3N$-Met-Met-Ser-Phe-Val-Ser-Leu-Leu-Leu-Val-Gly-Ile-Leu-Phe-Trp-Ala-Thr-Glu-Ala-Glu-Gln-Leu-Thr-Lys-Cys-Glu-Val-Phe-Gln-
Retain in lumen of ER	-Lys-Asp-Glu-Leu-COO^{-}
Import into mitochondria	$^{+}H_3N$-Met-Leu-Ser-Leu-Arg-Gln-Ser-Ile-Arg-Phe-Phe-Lys-Pro-Ala-Thr-Arg-Thr-Leu-Cys-Ser-Ser-Arg-Tyr-Leu-Leu-
Import into nucleus	-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val-
Import into peroxisomes	-Ser-Lys-Leu-
Attach to membranes via the covalent linkage of a myristic acid to the amino terminus	$^{+}H_3N$-Gly-Ser-Ser-Lys-Ser-Lys-Pro-Lys-

Positively charged amino acids are shown in *red* and negatively charged amino acids in *green*. An extended block of hydrophobic amino acids is enclosed in a *yellow* box. H_3N^{+} indicates the amino terminus of a protein; COO^{-} indicates the carboxyl terminus.

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A transfection approach for defining signal sequences

One way to show that a signal sequence is required and sufficient to target a protein to a specific intracellular compartment is to create a fusion protein in which the signal sequence is attached by genetic engineering techniques to a protein that is normally resident in the cytosol. After the cDNA encoding this protein is transfected into cells, the location of the fusion protein is determined by immunostaining or by cell fractionation.



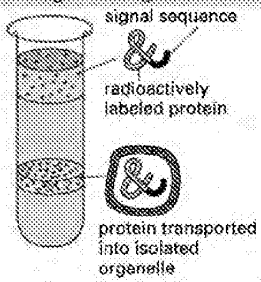
By altering the signal sequence using site-directed mutagenesis, one can determine which structural features are important for its function.

A biochemical approach for studying the mechanism of protein translocation

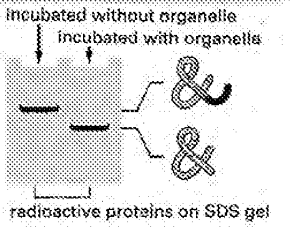
In this approach a labeled protein containing a specific signal sequence is transported into isolated organelles *in vitro*. The labeled protein is usually produced by cell-free translation of a purified mRNA encoding the protein; radioactive amino acids are used to label the newly synthesized protein so that it can be distinguished from the many other proteins that are present in the *in vitro* translation system.

Three methods are commonly used to test if the labeled protein has been translocated into the organelle:

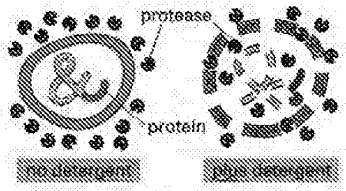
1. The labeled protein co-fractionates with the organelle during centrifugation.



2. The signal sequence is removed by a specific protease that is present inside the organelle.

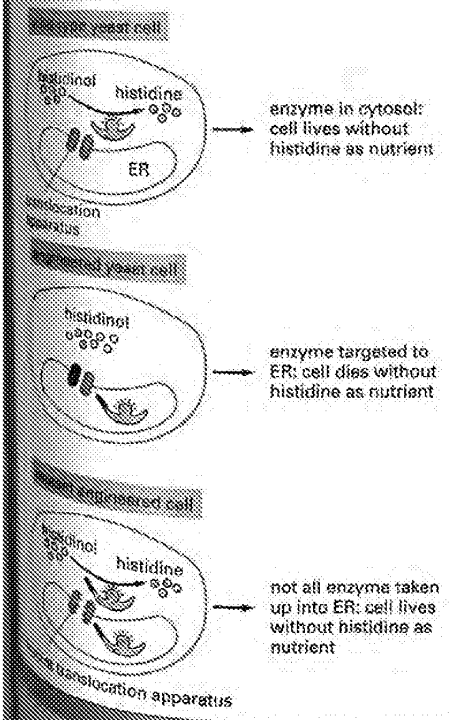


3. The protein is protected from digestion when proteases are added to the incubation medium but is susceptible if a detergent is first added to disrupt the organelle membrane.



By exploiting such *in vitro* assays, one can determine what components (proteins, ATP, GTP, etc.) are required for the translocation process.

Genetic approaches for studying the mechanism of protein translocation



Yeast cells with mutations in genes that encode components of the translocation machinery have been useful for studying protein translocation. Because mutant cells that cannot translocate proteins across their membranes will die, the trick is to design a strategy that allows weak mutations that cause only a partial defect in protein translocation to be isolated.

One way uses genetic engineering to design special yeast cells. The enzyme histidinol dehydrogenase, for example, normally resides in the cytosol, where it is required to produce the essential amino acid histidine from its precursor histidinol. A yeast strain is constructed in which the histidinol dehydrogenase gene is replaced by a re-engineered gene encoding a fusion protein with an added signal sequence that misdirects the enzyme into the endoplasmic reticulum (ER). When such cells are grown without histidine, they die because all of the histidinol dehydrogenase is sequestered in the ER, where it is of no use. Cells with a mutation that partially inactivates the mechanism for translocating proteins from the cytosol to the ER, however, will survive because enough of the dehydrogenase will be retained in the cytosol to produce histidine. Often one obtains a cell in which the mutant protein still functions partially at normal temperature but is completely inactive at higher temperature. A cell carrying such a temperature-sensitive mutation dies at higher temperature, whether or not histidine is present, as it cannot transport any protein into the ER. This allows the normal gene that was disabled by the mutation to be identified by transfecting the mutant cells with a yeast plasmid vector into which random yeast genomic DNA fragments have been cloned: the specific DNA fragment that rescues the mutant cells when they are grown at high temperature should encode the wild-type version of the mutant gene.

Panel 12-1 Approaches to studying signal sequences and protein translocation across membranes.

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acids, which is commonly found at internal sites of the polypeptide chain. Some typical signal peptides are listed in Table 12–3.

The importance of each of these signal peptides for protein targeting has been shown by experiments in which the peptide is transferred from one protein to another by genetic engineering techniques: placing the amino-terminal ER signal peptide at the beginning of a cytosolic protein, for example, redirects the protein to the ER. Even though their amino acid sequences can vary greatly, the signal peptides of all proteins having the same destination are functionally interchangeable: physical properties, such as hydrophobicity, often appear to be more important in the signal-recognition process than the exact amino acid sequence.

Signal patches are far more difficult to analyze than signal peptides, and so less is known about their structure. Because they result from a complex three-dimensional protein-folding pattern, they cannot be easily transferred experimentally from one protein to another.

The main ways of studying how proteins are directed from the cytosol to a specific compartment and how they are translocated across membranes are illustrated in Panel 12–1 (p. 559).

Cells Cannot Construct Their Membrane-bounded Organelles *de Novo*: They Require Information in the Organelle Itself [5]

When a cell reproduces by division, it has to duplicate its membrane-bounded organelles. In general, cells do this by enlarging the existing organelles by incorporating new molecules into them; the enlarged organelles then divide and are distributed to the two daughter cells. Thus each daughter cell inherits from its mother a complete set of specialized cell membranes. This inheritance is essential because a cell could not make such membranes *de novo*. If the ER were completely removed from a cell, for example, how could the cell reconstruct it? The membrane proteins that define the ER and carry out many of its functions are themselves products of the ER. A new ER could not be made without an existing ER or, at the very least, a membrane that contains the translocators required to import specific proteins into the ER (and lacks the translocators required to import the proteins that function in other organelles).

Thus it seems that the information required to construct a membrane-bounded organelle does not reside exclusively in the DNA that specifies the organelle's proteins. *Epigenetic* information in the form of at least one distinct protein that preexists in the organelle membrane is also required, and this information is passed from parent cell to progeny cell in the form of the organelle itself. Presumably, such information is essential for the propagation of the cell's compartmental organization, just as the information in DNA is essential for the propagation of its nucleotide and amino acid sequences.

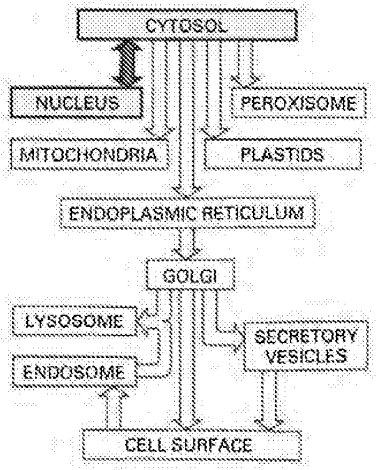
Summary

Eucaryotic cells contain intracellular membranes that enclose nearly half the cell's total volume in separate intracellular compartments called organelles. The main types of membrane-bounded organelles that are present in all eucaryotic cells are the endoplasmic reticulum, Golgi apparatus, nucleus, mitochondria, lysosomes, endosomes, and peroxisomes; plant cells also contain plastids, such as chloroplasts. Each organelle contains a distinct set of proteins that mediates its unique functions.

Each newly synthesized organelle protein finds its way from the ribosome where it is made to the organelle where it functions by following a specific pathway, guided by signals in its amino acid sequence that function as signal peptides or signal patches. The signal peptides and patches are recognized by complementary receptor proteins in the target organelle. Proteins that function in the cytosol do not contain signal peptides or signal patches and therefore remain in the cytosol after they are synthesized.

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The Transport of Molecules into and out of the Nucleus [6]



The **nuclear envelope** encloses the DNA and defines the nuclear compartment. It is formed from two concentric membranes that, as we have seen, are continuous with the endoplasmic reticulum. Although the inner and outer nuclear membranes are continuous, the two membranes maintain distinct protein compositions. The **inner nuclear membrane** contains specific proteins that act as binding sites for the feltlike *nuclear lamina* that supports it. The inner membrane is surrounded by the **outer nuclear membrane,** which closely resembles the membrane of the rough endoplasmic reticulum (Figure 12–9). Like the membrane of the rough ER, the outer nuclear membrane is studded with ribosomes engaged in protein synthesis. The proteins made on these ribosomes are transported into the space between the inner and outer nuclear membranes (the *perinuclear space*), which is continuous with the ER lumen (see Figure 12–9).

Bidirectional traffic occurs continuously between the cytosol and the nucleus. The many proteins that function in the nucleus—including histones, DNA and RNA polymerases, gene regulatory proteins, and RNA-processing proteins—are selectively imported into the *nuclear compartment* from the cytosol where they are made. At the same time, tRNAs and mRNAs are synthesized in the nuclear compartment and then exported to the cytosol. Like the import process, the export process is selective: mRNAs, for example, are exported only after they have been properly modified by RNA-processing reactions in the nucleus. In some cases the transport process is complex: ribosomal proteins, for instance, are made in the cytosol, imported into the nucleus—where they assemble with newly made ribosomal RNA into particles—and then exported again to the cytosol as part of a ribosomal subunit; each of these steps involves selective transport across the nuclear envelope.

Nuclear Pores Perforate the Nuclear Envelope [7]

The nuclear envelope in all eucaryotes, from yeasts to humans, is perforated by *nuclear pores.* Each pore is formed by a large, elaborate structure known as the **nuclear pore complex,** which has an estimated molecular mass of about 125 million and is thought to be composed of more than 100 different proteins, arranged with a striking octagonal symmetry (Figures 12–10 and 12–11).

Each pore complex contains one or more open aqueous channels through which water-soluble molecules that are smaller than a certain size can passively

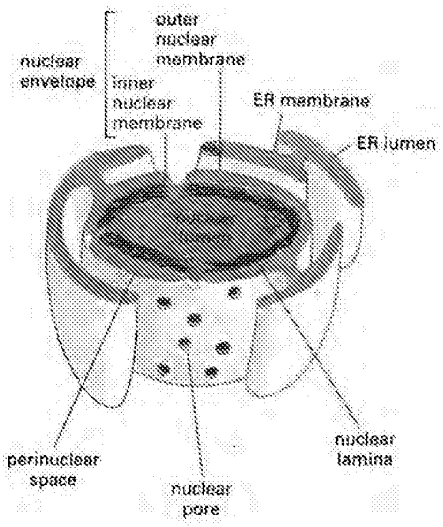
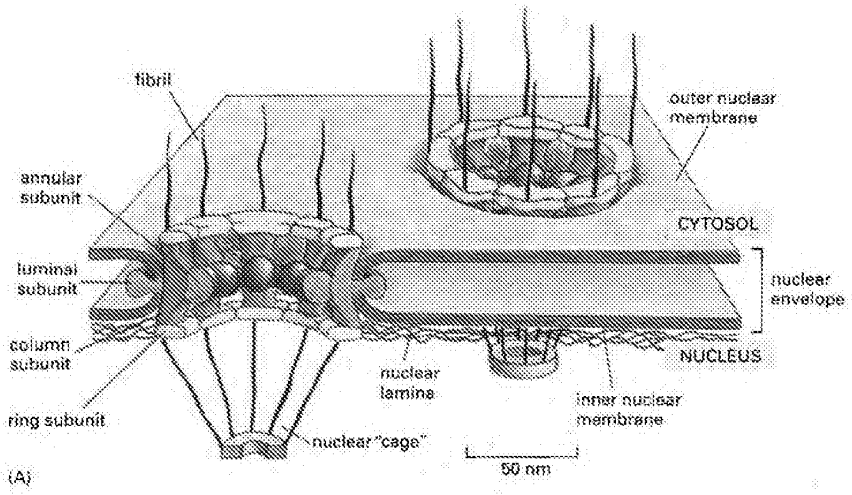


Figure 12–9 The nuclear envelope. The double-membrane envelope is penetrated by nuclear pores and is continuous with the endoplasmic reticulum. The ribosomes that are bound to the cytosolic surface of the ER membrane and outer nuclear membrane are not shown.

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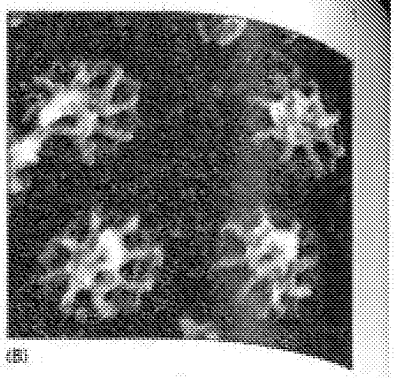


Figure 12–10 The arrangement of the nuclear pore complexes in the nuclear envelope. (A) A sketch showing a small region of the nuclear envelope. In cross-section the nuclear pore complex appears composed of three parts: (1) a column component which forms the bulk of the pore wall; (2) an annular component, which extends "spokes" toward the center of the pore; and (3) a luminal component, which is formed by a large transmembrane glycoprotein that is thought to help anchor the complex to the nuclear membrane. In addition, fibrils protrude from both the cytosolic and nucleus sides of the complex. On the nuclear side the fibrils converge to form cagelike structures, which are shown in a scanning electron micrograph of the nuclear side of the nuclear envelope of an oocyte in (B). (B, from M.W. Goldberg and T.D. Allen, *J. Cell Biol.* 119:1429–1440, 1992, by copyright permission of the Rockefeller University Press.)

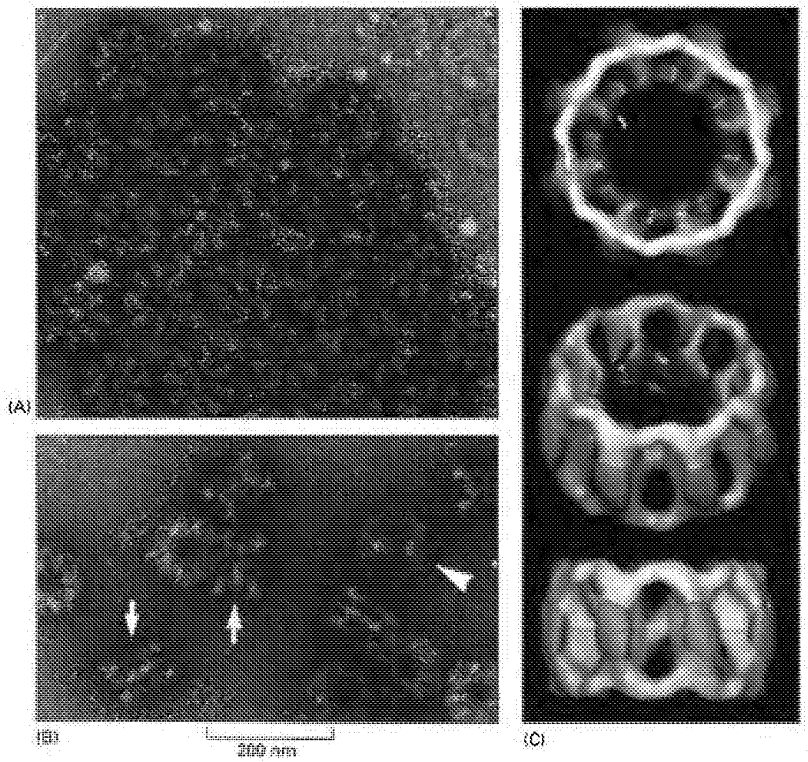


Figure 12–11 Electron micrograph and computer reconstruction of nuclear pore complexes. (A) and (B) Negatively stained views of nuclear pore complexes released from the envelope by detergent. In (B) some nuclear pore complexes can be seen on their side. (C) Three-dimensional computer reconstructions showing top, tilted, and side views of pore complexes. (From J.E. Hinshaw and B. Milligan, *Cell* 69:1133–1141, 1992. © Cell Press.)

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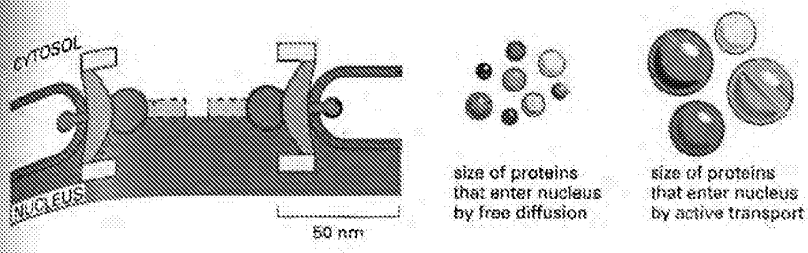


Figure 12–12 Possible paths for free diffusion through the nuclear pore complex. The drawing shows a hypothetical diaphragm inserted into the pore to restrict the size of the open channel to 9 nm, which is the pore size estimated from diffusion measurements. Nine nanometers is a much smaller diameter than that of the central opening apparent on the images of the nuclear pore complex derived from electron micrographs (or from that measured during active transport when the pore dilates to allow transport of particles of up to 26 nm in diameter). Thus it is likely that some pore components are lost during the preparation of specimens for electron microscopy and that these normally restrict free diffusion through the central opening. Such components may form a diaphragm (or plug) that opens and closes to allow passage of large objects during active transport, which is mediated by a sorting signal (discussed below). Although plugs can be seen in some preparations, it is not clear whether they are components of the pore complex or material that is being transported through it. Three-dimensional computer reconstructions suggest that the channels permitting free diffusion might not be located at the center of the pore complex but near its rim between the column components (see Figure 12–10A); this would mean that passive diffusion and active transport take place through different parts of the pore complex.

diffuse. The effective size of these channels has been determined by injecting labeled molecules (that are not nuclear components) into the cytosol and then measuring their rate of diffusion into the nucleus. Small molecules (5000 daltons or less) diffuse in so fast that the nuclear envelope can be considered to be freely permeable to them. A protein of 17,000 daltons takes 2 minutes to equilibrate between the cytosol and nucleus, while a protein of 44,000 daltons takes 30 minutes. A globular protein larger than about 60,000 daltons seems hardly able to enter the nucleus at all. A quantitative analysis of such data suggests that the nuclear pore complex contains a pathway for free diffusion equivalent to a water-filled cylindrical channel about 9 nm in diameter and 15 nm long; such a channel would occupy only a small fraction of the total pore volume (Figure 12–12).

Because many cellular proteins are too large to pass by diffusion through the nuclear pores, the nuclear envelope allows the nuclear compartment and the cytosol to maintain different complements of proteins. Mature cytosolic ribosomes, for example, are about 30 nm in diameter and thus cannot diffuse through the 9-nm channels; their exclusion from the nucleus ensures that all protein synthesis is confined to the cytosol. But how does the nucleus export newly made ribosomal subunits or import large molecules, such as DNA and RNA polymerases, which have subunit molecular weights of 100,000 to 200,000? As we discuss next, these and many other protein and RNA molecules bind to specific receptor proteins located in the pore complexes and are then actively transported across the nuclear envelope through the complexes.

Nuclear Localization Signals Direct Nuclear Proteins to the Nucleus [8]

In general, the more active the nucleus is in transcription, the greater the number of pore complexes its envelope contains. The nuclear envelope of a typical mammalian cell contains 3000 to 4000 pore complexes. If the cell is synthesizing DNA, it needs to import about 10^6 histone molecules from the cytosol every 3 minutes in order to package newly made DNA into chromatin, which means that, on average, each pore complex needs to transport about 100 histone molecules per minute. If the cell is growing rapidly, each pore complex also needs to transport about 6 newly assembled large and small ribosomal subunits per minute from the nucleus, where they are produced, to the cytosol, where they are used. And that is only a very small part of the total traffic that passes through the nuclear pores.

When proteins are experimentally extracted from the nucleus and microinjected back into the cytosol, even the very large ones efficiently reaccumulate in the nucleus. The selectivity of this nuclear protein import resides in **nuclear localization signals,** which are present only in nuclear proteins. The signals have been precisely defined in many nuclear proteins using recombinant DNA technology. They can be located almost anywhere in the amino acid sequence and generally consist of a short sequence (typically from four to eight amino acids) that varies for different nuclear proteins but is rich in the positively charged amino acids lysine and arginine and usually contains proline. In many nuclear proteins this sequence is split into two blocks of two to four amino acids each,

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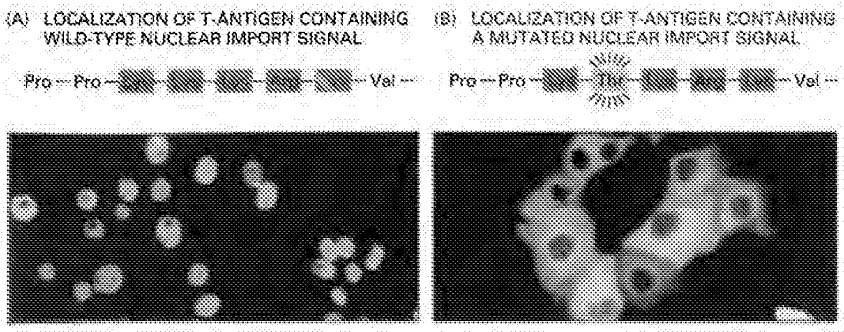


Figure 12–13 **The function of a nuclear localization signal.** Immunofluorescence micrographs showing the cellular location of SV40 virus T-antigen containing or lacking a short peptide that serves as a nuclear localization signal. The wild-type T-antigen protein contains the lysine-rich sequence indicated and is imported to its site of action in the nucleus, as indicated by immunofluorescence staining with antibody against the T-antigen (A). T-antigen with an altered nuclear localization signal (a threonine replacing a lysine) remains in the cytosol (B). (From D. Kalderon, B. Roberts, W. Richardson, and A. Smith, *Cell* 39:499–509, 1984. © Cell Press.)

with the blocks separated from each other by about ten amino acids. The signals are thought to form loops on the protein surface.

Nuclear localization signals were first identified in the large viral protein called *T-antigen*, which is encoded by the SV40 virus and is needed for viral DNA replication in the host cell nucleus. The T-antigen normally accumulates in the nucleus shortly after being synthesized in the cytosol. A mutation in a single amino acid, however, prevents nuclear import (Figure 12–13). On the assumption that this mutation is in a nuclear localization signal sequence, short lengths of the DNA encoding this region of the normal T-antigen were fused to a gene coding for a cytosolic protein. The shortest sequence that caused the resulting fusion protein to be imported into the nucleus encoded a stretch of eight contiguous amino acids, which is normally located in an internal region of the T-antigen polypeptide chain (see Figure 12–13). Further experiments showed that the signal sequence could function even when it was linked as a short peptide to selected lysine side chains on the surface of a cytosolic protein, suggesting that the precise location of a nuclear localization signal within the amino acid sequence of a nuclear protein is not important. In fact, many nuclear proteins contain more than one nuclear localization signal.

Macromolecules Are Actively Transported into and out of the Nucleus Through Nuclear Pores [9]

The active transport of nuclear proteins through nuclear pore complexes can be directly visualized by coating gold particles with a nuclear protein, injecting the particles into the cytosol, and then following their fate by electron microscopy (Figures 12–14 and 12–15).

The initial interaction of a nuclear protein with the nuclear pore complex requires one or more cytosolic proteins that bind to the nuclear localization signals and help direct the nuclear protein to the pore complex, where it appears to bind to the fibrils that project from the rim of the complex. The nuclear protein then moves to the center of the pore complex, where it is actively transported across the nuclear envelope by a process that requires ATP hydrolysis (Figure 12–16). Studies with various sizes of gold beads indicate that the opening can dilate up to about 26 nm in diameter during the transport process: a poorly defined structure in the center of the nuclear pore complex appears to function like a close-fitting diaphragm that opens just the right amount when activated by a signal on an appropriate large protein (see Figure 12–12). The molecular basis of this mechanism, and how it operates to pump macromolecules both into and out of the nucleus, is a mystery.

It seems likely that the export of new ribosomal subunits and messenger RNA molecules through the nuclear pores also depends on a selective transport system. If 20-nm-diameter gold spheres, similar to those used in the experiments shown in Figure 12–15, are coated with small RNA molecules (tRNA or 5S RNA) and then injected into the nucleus of a frog oocyte, they are rapidly transported through the nuclear pores into the cytosol. If they are injected into the cytosol

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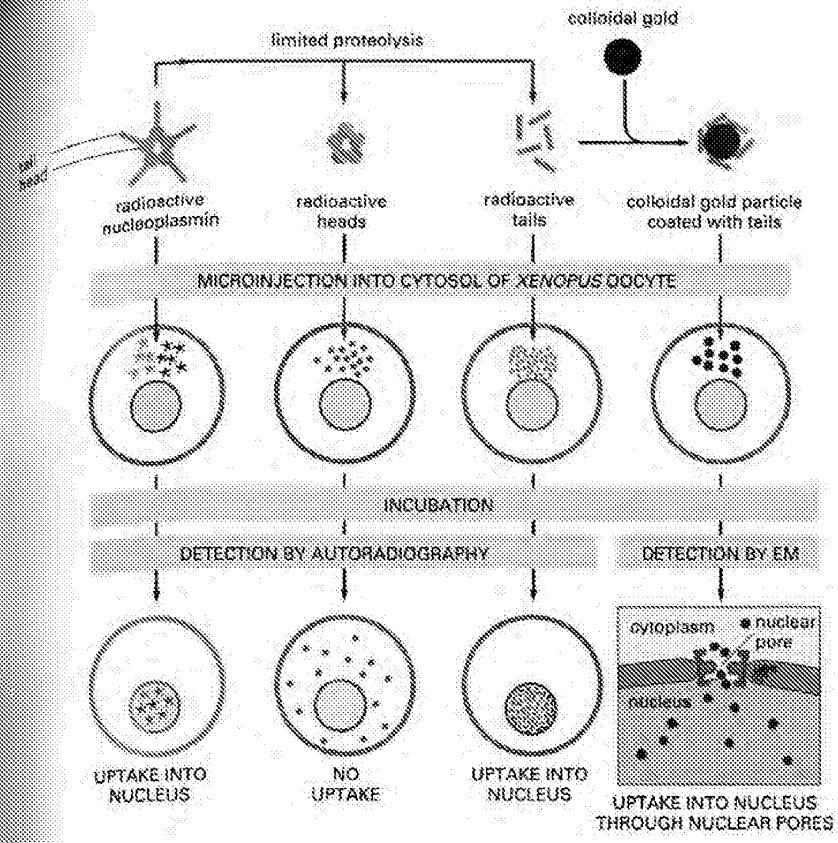


Figure 12–14 The uptake of nuclear proteins via nuclear pores. *Nucleoplasmin* is a large nuclear protein with distinct head and tail domains. The heads can be cleaved from the tails by limited proteolysis. When injected into the cytosol of a frog oocyte, intact nucleoplasmin molecules rapidly accumulate in the nucleus even though they are too large to diffuse passively through the pore complex. The signal for this nuclear import resides in the tail domains, since injected tails are taken up by the nucleus but heads are not. The role of nuclear pores in this signal-directed import is demonstrated by electron microscopy using nucleoplasmin tails coupled to spheres of colloidal gold, which are easily visualized because of their high electron density. The attached nucleoplasmin tails direct the entry of the gold particles into the nucleus via nuclear pores (see Figure 12–15).

of the oocyte, on the other hand, they remain there. Thus it seems that, in addition to receptors that recognize nuclear protein import signals, the pore contains one or more receptors that recognize RNA molecules (or the proteins bound to them) destined for the cytosol. Using differently sized gold particles, one set coated with RNA and injected into the nucleus and the other set coated with nuclear protein import signals, it can be shown that a single pore complex allows traffic in both directions.

The mechanism of macromolecular transport across nuclear pores is fundamentally different from the transport mechanisms involved in the transfer of proteins across the membranes of other organelles in that it occurs through a large, regulated aqueous pore rather than through a protein transporter that spans one or more lipid bilayers. It is thought that a nuclear protein is transported through the pores while it is in a fully folded conformation, just as a newly formed ribosomal subunit is transported as an assembled particle; by contrast, proteins have to be unfolded during their transport into other organelles, as we discuss later.

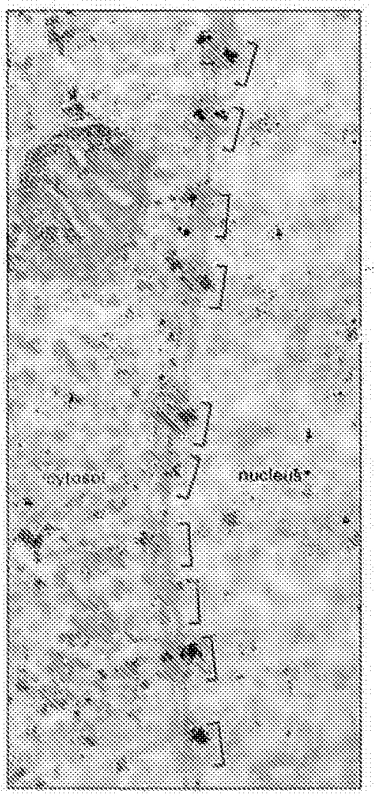


Figure 12–15 Visualizing the specific import of a protein through nuclear pores. This electron micrograph shows colloidal gold spheres coated with nucleoplasmin (see Figure 12–14) entering the nucleus by means of nuclear pores (indicated by *red brackets*). The same result is obtained when the gold spheres are coated with the tail regions of nucleoplasmin molecules. These gold particles are much larger in diameter than the diffusion channel in the pore complex, implying that a pore has been induced to widen to permit their passage. Because the gold particles line up in the cytosol before they contact and enter the pore complex, it has been suggested that the fibrils that extend into the cytosol from the pore complex (see Figure 12–10A) guide the particles to their destination. (From C. Feldherr, E. Kallenbach, and N. Schultz, *J. Cell Biol.* 99:2216–2222, 1984, by copyright permission of the Rockefeller University Press.)

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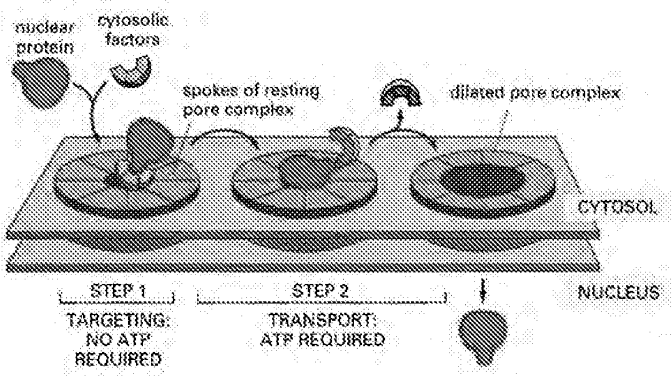


Figure 12–16 **Highly schematic view of the mechanism of active transport through nuclear pores.** The proteins and structures involved in the active transport process are not known. A diverse set of related cytosolic proteins, however, is required for the initial binding of nuclear proteins to the complex. These proteins, called *nucleoporins*, contain a simple sugar (*N*-acetylglucosamine) that aided their identification through the use of lectins and specific antibodies. The fibrils that project from the pore complex and are thought to help guide nuclear proteins to the center of the pore are not shown.

The Nuclear Envelope Is Disassembled During Mitosis [10]

The **nuclear lamina** is a meshwork of interconnected protein subunits called **nuclear lamins.** These are a special class of intermediate filament proteins (discussed in Chapter 16) that polymerize into a two-dimensional lattice (Figure 12–17). The nuclear lamina is thought to give shape and stability to the nuclear envelope, to which it is anchored by attachment to both the nuclear pore complexes and the inner nuclear membrane. As the chromatin is also thought to interact directly with the nuclear lamina, the lamina provides a structural link between the DNA and the nuclear envelope.

When a nucleus disassembles during mitosis, the nuclear lamina depolymerizes, at least partly as a consequence of the phosphorylation of the nuclear lamins at the onset of mitosis. At the same time the nuclear pore complexes disassemble into their various components. Depolymerization of the nuclear lamina is probably a prerequisite for the nuclear envelope to break up into membrane vesicles, which, together with the nuclear contents, disperse throughout the cytosol. Reassembly of the lamina occurs when the nuclear lamins are dephosphorylated and, as a result, repolymerize on the surface of the chromosomes; the re-assembled lamina then binds the vesicles of nuclear envelope membrane, which fuse with one another to re-form an envelope around each chromosome or group of chromosomes. During this process the nuclear pore complexes also re-assemble. The enveloped chromosomes then come together, and their membranes fuse to form a single nuclear envelope, which actively reimports all those proteins that contain nuclear localization signals (Figure 12–18). Because the new nuclear envelope is so closely applied to the surface of the chromosomes, it excludes all of the proteins in the cell except those bound to the mitotic chromosomes. Thus large proteins are kept out of the interphase nucleus unless they contain nuclear localization signals.

Nuclear localization signals are not cleaved off after transport into the nucleus. This is presumably because nuclear proteins need to be imported repeatedly, once after every cell division. In contrast, once a protein molecule has been imported into any of the other membrane-bounded organelles, it is passed on from generation to generation within that compartment and need never be translocated again; the signal peptide on these molecules is often removed following protein translocation.

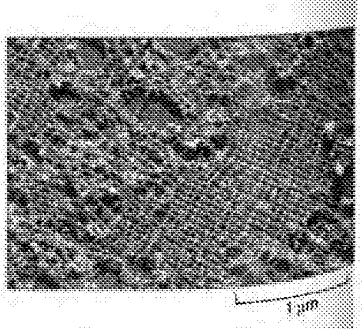


Figure 12–17 **The nuclear lamina.** Electron micrograph of a portion of the nuclear lamina in a *Xenopus* oocyte prepared by freeze drying and metal shadowing. The lamina is formed by a regular lattice of specialized intermediate filaments. (Courtesy of Ueli Aebi.)

Transport Between Nucleus and Cytosol Can Be Regulated by Preventing Access to the Transport Machinery [11]

As discussed in Chapter 9, the activity of some gene regulatory proteins is controlled by keeping them out of the nuclear compartment until they are needed there. The nuclear localization signal of some of these proteins can be inactivated by phosphorylation. Others are bound to inhibitory cytosolic proteins that either

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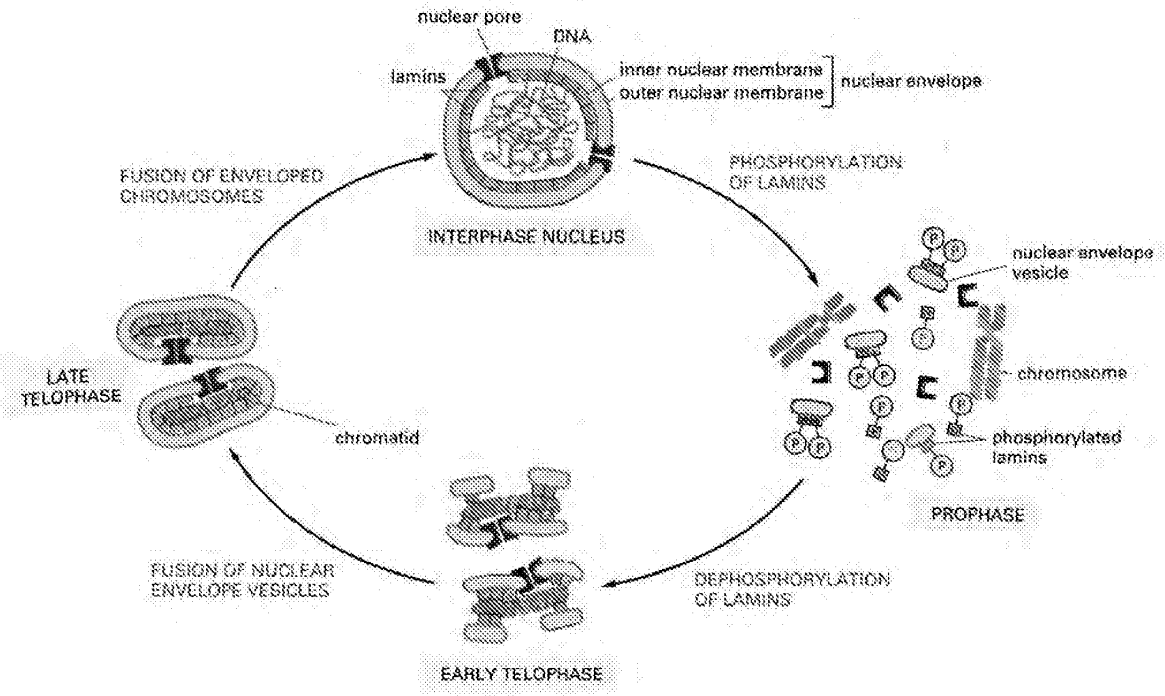


Figure 12–18 **Breakdown and re-formation of the nuclear envelope during mitosis.** The phosphorylation of the lamins is thought to help trigger the disassembly of the nuclear lamina, which in turn causes the nuclear envelope to break up into vesicles. Dephosphorylation of the lamins is thought to help reverse the process.

anchor them in the cytosol—presumably through interactions with the cytoskeleton or specific organelles—or mask their nuclear localization signals. When the cell receives an appropriate stimulus, the protein is released from its cytosolic anchor or mask and is transported into the nucleus (Figure 12–19).

Export of RNA from the nucleus may be controlled in a similar way. Like active import into the nucleus, export also requires a signal: in the case of most messenger RNA molecules, this is provided by a unique modification, the cap structure, at the 5′ end of the RNA (discussed in Chapter 8). Incompletely processed pre-messenger RNAs include this cap but are anchored to the nuclear transcription and splicing machinery, which releases an RNA molecule only after its processing is completed; genetic studies in yeast have shown that mutations that prevent the pre-messenger RNA from properly engaging with the splicing machinery lead to the export of the unspliced RNA. Other RNAs, like transfer RNA or ribosomal RNA, which lack a 5′ cap, must first be assembled with proteins and are then exported as part of these complexes. Presumably, nuclear export signals are contained in the protein subunits of these complexes, and these signals become activated after proper assembly with the RNA components, but the nature of these signals is not known.

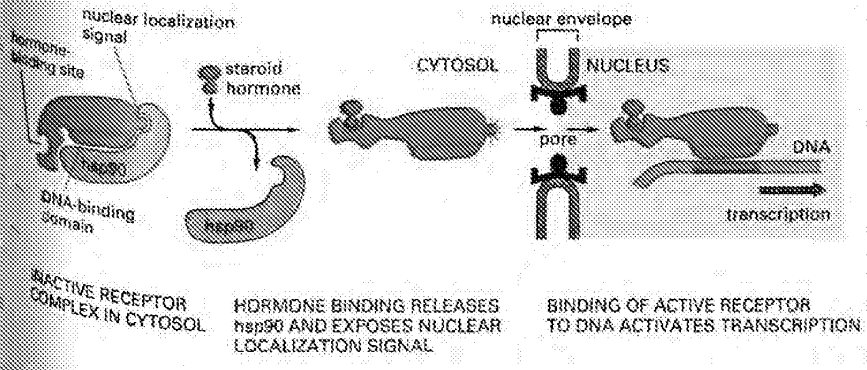


Figure 12–19 **The nuclear import of the glucocorticoid receptor.** The glucocorticoid receptor is a gene regulatory protein that, in the non-hormone-treated cell, is bound in the cytosol to the chaperone protein hsp90. When activated by the binding of the appropriate steroid hormone, it is released from hsp90 and is directed into the nucleus by a nuclear localization signal; once in the nucleus, it binds to specific DNA sequences and regulates the transcription of a discrete set of genes (discussed in Chapters 9 and 15).

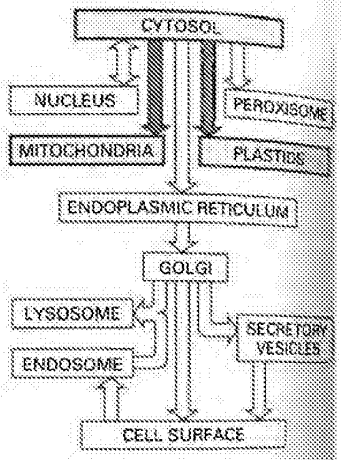
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Summary

The nuclear envelope consists of an inner and an outer nuclear membrane. The outer membrane is continuous with the ER membrane, and the space between it and the inner membrane is continuous with the ER lumen. RNA molecules, which are made in the nucleus, and ribosomal subunits, which are assembled there, are exported to the cytosol, while all of the proteins that function in the nucleus are synthesized in the cytosol and are then imported. The extensive traffic of materials between nucleus and cytosol occurs through nuclear pores that provide a direct passageway across the nuclear envelope.

Proteins containing nuclear localization signals are actively transported inward through the pores, while RNA molecules and newly made ribosomal subunits are actively transported outward through the pores. Because the nuclear localization signals are not removed, nuclear proteins can be imported repeatedly, as is required each time the nucleus reassembles following mitosis. The transport of nuclear proteins and RNA molecules through the pores can be regulated by denying these molecules access to the transport machinery in the nuclear pore complexes.

The Transport of Proteins into Mitochondria and Chloroplasts [12]



As discussed in Chapter 14, mitochondria and chloroplasts are double-membrane-bounded organelles that specialize in the synthesis of ATP, using energy derived from electron transport and oxidative phosphorylation in mitochondria and from photosynthetic phosphorylation in chloroplasts. Although both organelles contain their own DNA, ribosomes, and other machinery for protein synthesis, most of their proteins are encoded in the cell nucleus and imported from the cytosol. Moreover, each imported protein must reach the particular organelle subcompartment in which it functions. For mitochondria there are two subcompartments: the internal **matrix space** and the **intermembrane space.** These compartments are formed by the two distinct mitochondrial membranes: the **inner membrane,** which encloses the matrix space, and the **outer membrane,** which is in contact with the cytosol (Figure 12–20A). Chloroplasts have the same two subcompartments plus an additional subcompartment, the *thylakoid space,* which is surrounded by the *thylakoid membrane* (Figure 12–20B). Each of the subcompartments contains a distinct set of proteins. The growth of mitochondria and chloroplasts by the import of proteins from the cytosol is therefore a major feat, requiring that proteins be translocated across a number of membranes in succession and end up in the appropriate place.

The relatively few proteins encoded by the genomes of these organelles are located mostly in the inner membrane in mitochondria and in the thylakoid membrane in chloroplasts. These organelle-encoded polypeptides generally form subunits of protein complexes whose other subunits are encoded by nuclear genes and are imported from the cytosol. The formation of such hybrid protein complexes requires a balanced synthesis of the two types of subunits; how protein synthesis is coordinated on different types of ribosomes located two membranes apart is still largely a mystery.

Translocation into the Mitochondrial Matrix Depends on a Matrix Targeting Signal [13]

Proteins imported into the mitochondrial matrix are usually taken up from the cytosol within a minute or two of their release from polyribosomes. These **mitochondrial precursor proteins** almost always have a signal peptide (20–80 residues long) at their amino terminus that is rapidly removed after import by a protease (the *signal peptidase*) in the mitochondrial matrix. The signal peptide

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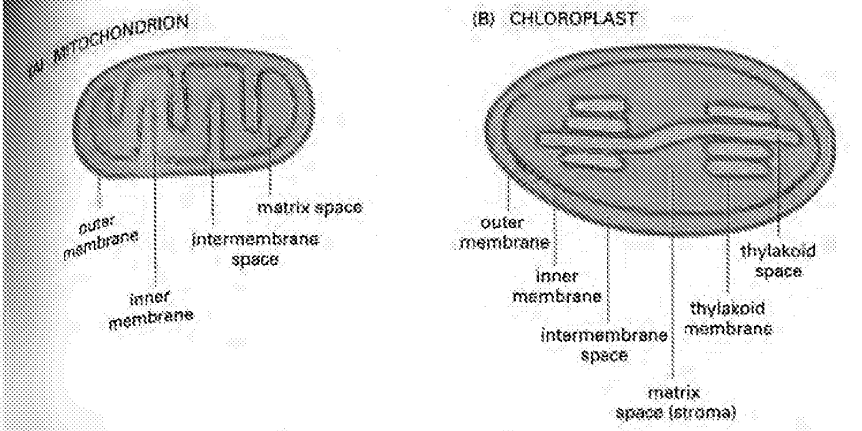


Figure 12–20 **The subcompartments of mitochondria and chloroplasts.** The topology of the chloroplast can be derived from that of the mitochondrion in a simple way: pinching off the invaginations of the inner mitochondrial membrane to create vesicles would generate a compartment that is topologically equivalent to the thylakoid vesicle in chloroplasts. Thylakoid vesicles may have evolved in this way.

can be remarkably simple. Molecular genetic experiments in which the signal peptide is progressively reduced in length have shown that, for one mitochondrial protein, only 12 amino acids at the amino terminus are needed to signal mitochondrial import. These 12 residues can be attached to any cytosolic protein and will direct the protein into the mitochondrial matrix. Physical studies of full-length signal peptides suggest that they can form amphipathic α-helical structures in which positively charged residues are clustered on one side of the helix while uncharged hydrophobic residues are clustered on the opposite side (Figure 12–21). This configuration is thought to be recognized by specific receptor proteins on the mitochondrial surface.

Translocation into the Mitochondrial Matrix Requires Both the Electrochemical Gradient Across the Inner Membrane and ATP Hydrolysis [14]

Almost everything we know about the molecular mechanism of protein import into mitochondria has been learned from analysis of cell-free, reconstituted transport systems. Mitochondria are first purified by differential centrifugation of homogenized cells and are then incubated with radiolabeled mitochondrial precursor proteins. The precursor proteins are generally taken up rapidly and efficiently into such mitochondria during a brief *in vitro* incubation. By changing the conditions in these experiments *in vitro*, it is possible to establish the biochemical requirements for protein transport into the mitochondria.

Vectorial movement and transport require energy. In most biological systems the energy is supplied by ATP hydrolysis. In the case of mitochondrial import, however, an electrochemical gradient across the inner mitochondrial membrane is required in addition to ATP hydrolysis. This gradient is maintained by the pumping of H^+ from the matrix to the intermembrane space, driven by electron transport processes in the inner membrane. The mitochondrial outer membrane,

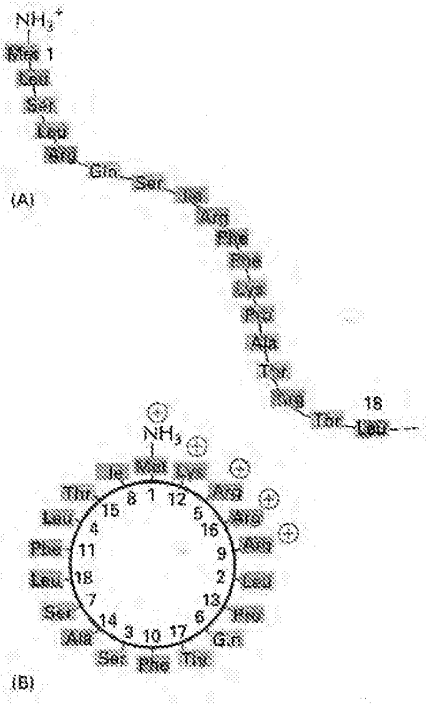


Figure 12–21 **A signal peptide for mitochondrial protein import.** Cytochrome oxidase is a large multiprotein complex located in the mitochondrial inner membrane, where it functions as the terminal enzyme in the electron-transport chain (discussed in Chapter 14). (A) The first 12 amino acids of the precursor to subunit IV of this enzyme serve as a signal peptide for the import of the subunit into the mitochondrion. (B) When the full-length signal peptide is folded as an α helix with 3.6 residues per turn and viewed from the top, the positively charged residues (*red*) are seen to be clustered on one face of the helix while the nonpolar residues (*green*) are clustered on the opposite face. Mitochondrial signal peptide sequences always have the potential to form such an amphipathic helix, which is thought to be recognized by specific receptor proteins on the mitochondrial surface.

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like that of gram-negative bacteria (see Figure 11–14), contains large amounts of a pore-forming protein called *porin* and is thus freely permeable to inorganic ions and metabolites (but not to most proteins), so that no gradient can be maintained across it. The energy in the electrochemical gradient across the inner membrane is tapped to help drive most of the cell's ATP synthesis, but it is also used to drive the translocation of proteins bearing a mitochondrial import signal peptide: when ionophores that collapse the mitochondrial membrane potential are added, import is blocked. It is still uncertain how the electrochemical gradient contributes to protein translocation. The role of ATP hydrolysis is much better understood, as we see below.

Mitochondrial Proteins Are Imported into the Matrix in a Two-Stage Process at Contact Sites That Join the Inner and Outer Membranes [15]

As a first step in mitochondrial import, the mitochondrial precursor proteins have to bind to receptor proteins that reside in the mitochondrial outer membrane and recognize the mitochondrial signal peptides. The next step is the translocation process itself.

A protein could reach the mitochondrial matrix by crossing the two membranes one at a time, or it could pass through both at once. To distinguish these possibilities, a cell-free import system is cooled to a low temperature, arresting the proteins at an intermediate step in the translocation process. The proteins that accumulate at this step have already had their amino-terminal signal peptide removed by the matrix signal peptidase, indicating that their amino terminus must be in the matrix space; yet the bulk of the protein can still be attacked from outside the mitochondria by externally added proteolytic enzymes (Figure 12–22). This result demonstrates that the precursor proteins can pass through both mitochondrial membranes at once to enter the matrix. It is thought that there are two protein translocators, one in the outer membrane and one in the inner membrane, whose functions are usually coupled to allow translocation across both membranes at the same time. Electron microscopists have noted numerous **contact sites** at which the inner and outer mitochondrial membranes appear to be joined, and it seems likely that translocation occurs at or near these sites.

Although precursor proteins are transported through both membranes at once, it is clear from the experiments described in Figure 12–22 that the import process occurs in two distinct stages, only the second of which is arrested at a low temperature. Of these, it is only the initial penetration, which is not affected by low temperatures, that requires the membrane potential; when cooled mitochondria containing partly translocated intermediates are warmed up, import is rapidly completed (see Figure 12–22) even if the potential across the inner membrane is collapsed. This second stage of the transport process, however, requires

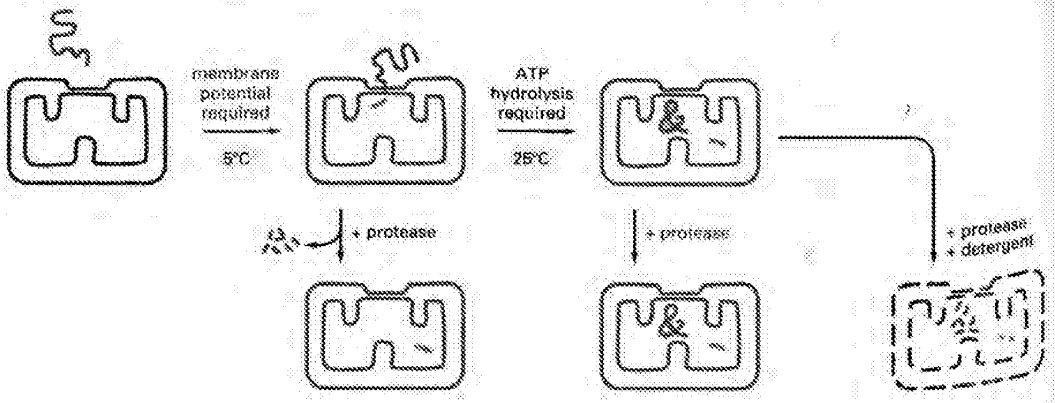


Figure 12–22 **Proteins transient span both the inner and outer mitochondrial membranes duri their translocation into the mat** When isolated mitochondria are incubated with a precursor prote 5°C, the precursor is only partiall translocated. The amino-termina signal peptide (*red*) is cleaved off the matrix; most of the polypepti chain remains outside the mitochondria, where it is accessi to proteolytic enzymes. Upon warming to 25°C, the translocati completed. Once inside the mitochondrion, the polypeptide is protected from externally adde proteolytic enzymes unless deter are added to disrupt the mitochondrial membranes, whic allows the imported proteins to b digested.

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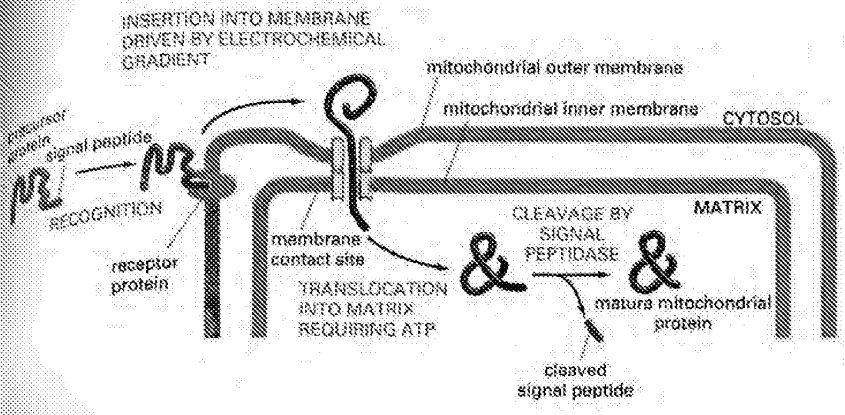


Figure 12–23 Protein import by mitochondria. The amino-terminal signal peptide of the precursor protein is recognized by receptors that reside in the outer membrane. The protein is thought to be translocated across both mitochondrial membranes at or near special contact sites, driven first by the electrochemical gradient across the inner membrane and then by ATP hydrolysis. The signal peptide is cleaved off by a signal peptidase in the matrix to form the mature protein; the free signal peptide is rapidly degraded.

ATP. Thus the first stage, which involves the insertion of the signal peptide and adjoining sequences into both mitochondrial membranes, is driven by the electrochemical gradient, and the second stage, in which the remainder of the polypeptide chain moves into the matrix, requires both ATP hydrolysis and a physiological temperature (Figure 12–23).

Proteins Are Imported into the Mitochondrial Matrix in an Unfolded State [16]

Transport of mitochondrial precursor proteins across the two mitochondrial membranes at a contact site is guided by members of the chaperone family of proteins, which are discussed in Chapter 5. It is difficult to envisage how a folded, water-soluble protein could straddle two (or even one) lipid bilayer while retaining its native three-dimensional conformation. It is now known that cytosolic chaperone proteins (called *chaperonins*) belonging to the hsp70 family, as well as helping to ensure the correct folding of cytosolic proteins, play an essential part in protein import into both mitochondria and the ER by binding the precursor in its unfolded state during translocation. As discussed in Chapter 5, the release of newly synthesized polypeptides from the hsp70 family of chaperone proteins requires ATP hydrolysis, and this partly accounts for the ATP dependence of the later stages of mitochondrial import.

The essential role of the chaperone proteins in translocation across internal cellular membranes was first indicated by genetic studies in yeasts. When the genes encoding certain members of the hsp70 family of chaperone proteins are inactivated, mitochondrial precursor proteins fail to be imported into mitochondria and accumulate in the cytosol instead. It is thought that newly synthesized precursor proteins, as they are released from polyribosomes in the cytosol, bind to hsp70 proteins, which prevent the precursor proteins from aggregating or folding up spontaneously before they bind to the protein translocator in the target membrane. The energy liberated by the hydrolysis of ATP is used to release the bound hsp70 proteins as the translocated protein is passed across the membrane. Experimentally, the requirement for hsp70 and ATP in the cytosol can be bypassed if the precursor proteins are artificially unfolded, for example, by a denaturation step in a concentrated solution of urea.

Sequential Binding of the Imported Protein to Mitochondrial hsp70 and hsp60 Drives Its Translocation and Assists Protein Folding [17]

Imported proteins are not only delivered to the mitochondrion by chaperone proteins: once they are extruded into the interior, they are received by closely related hsp70 proteins in the matrix space. *Mitochondrial hsp70* is crucial to the

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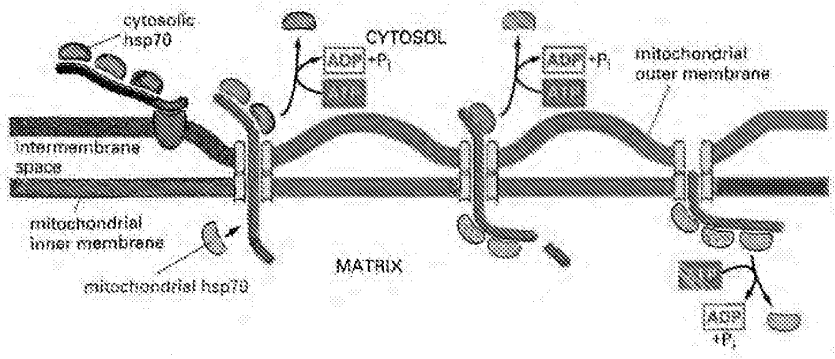


Figure 12–24 **Protein import into the mitochondrial matrix requires hsp70 proteins on both sides of the mitochondrial double membrane.** After the initial insertion of the signal peptide and of adjacent portions of the polypeptide chain, the unfolded chain slides in a channel that spans both membranes. Bound *cytosolic hsp70* is released from the protein in a step that depends on ATP hydrolysis; concomitantly, *mitochondrial hsp70* binds to regions of the polypeptide chain as they become exposed in the matrix, thereby pulling the protein into the interior of the mitochondrion.

import process, as mitochondria containing mutant forms of the protein fail to import precursor proteins. Like its cytosolic cousin, mitochondrial hsp70 has a high affinity for unfolded polypeptide chains, and it binds tightly to an imported protein as soon as it emerges from the translocator. The hsp70 then releases the protein in an ATP-dependent step. This energy-driven cycle of binding and subsequent release could provide the driving force for protein import after the protein has initially inserted into the translocator (Figure 12–24): the sequential binding of multiple mitochondrial hsp70 proteins may pull the unfolded protein through a transmembrane channel into the matrix.

After the initial interaction with mitochondrial hsp70, many imported proteins are passed on to another chaperone protein, *mitochondrial hsp60*. As discussed in Chapter 5, hsp60 attaches to the unfolded polypeptide chain and facilitates its folding in an ATP-consuming reaction. Much of our current understanding of the function of hsp60 in facilitating protein folding is derived from studies on import of proteins into mitochondria.

Protein Transport into the Mitochondrial Inner Membrane and the Intermembrane Space Requires Two Signals [18]

Many of the functions of mitochondria require proteins that either are integrated into the mitochondrial inner membrane or operate in the intermembrane space. These proteins are transported from the cytosol by the same mechanism that transports proteins into the matrix, but they are then prevented by various means from ending their journey in the matrix. In many cases the precursor proteins are first transferred all the way into the matrix, as was illustrated in Figure 12–23. A very hydrophobic amino acid sequence, however, is strategically placed after the amino-terminal signal peptide that initiates import. Once the amino-terminal signal is cleaved by the matrix signal peptidase, the hydrophobic sequence can function as a new amino-terminal signal peptide to translocate the protein back again from the matrix into or across the inner membrane. Presumably, the final step in this pathway involves a mechanism that is also used to direct proteins encoded in the mitochondrion to the inner membrane (Figure 12–25A), and we see later that a similar mechanism is used for translocating proteins into or across the ER membrane and the procaryotic plasma membrane, where it has been more extensively investigated.

An alternative route to the inner membrane may avoid the excursion into the matrix space altogether. The translocator in the inner membrane binds to the hydrophobic sequence that follows the amino-terminal signal peptide that initiates import and prevents further translocation across the inner membrane. The two translocators in the outer and inner membranes become uncoupled, which causes the remainder of the protein to be pulled into the intermembrane space (Figure 12–25B). Different proteins may use one or the other of these two pathways to the inner membrane or intermembrane space. It is not clear which one is more commonly used.

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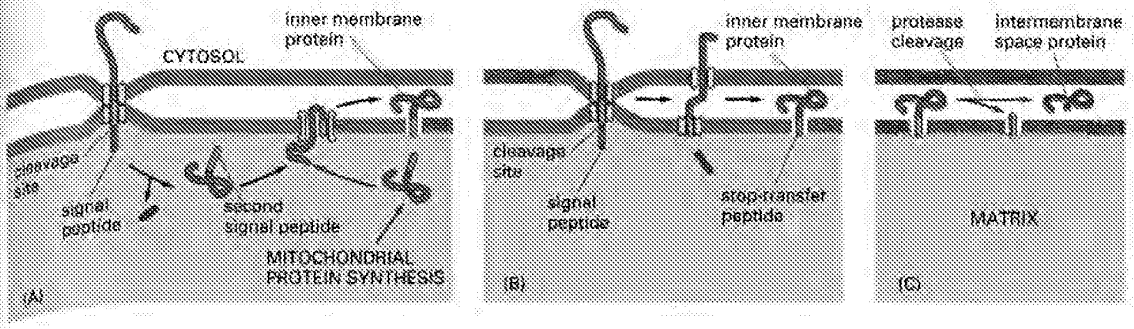


Figure 12–25 **Import of proteins from the cytosol to the mitochondrial intermembrane space or inner membrane.** (A) A pathway that requires two signal peptides and two translocation events is thought to be used to move some proteins from the cytosol to the inner membrane. The protein is first imported into the matrix space as in Figure 12–23. Cleavage of the signal peptide (*red*) used for the initial translocation, however, unmasks an adjacent hydrophobic signal peptide (*orange*) at the new amino terminus. This signal causes the protein to be integrated into the inner membrane by the same pathway that is used to insert proteins encoded by the mitochondrial genome into this membrane. (B) In an alternative mechanism, the hydrophobic sequence that follows the matrix targeting signal binds to the translocator and stops the translocation across the inner membrane. The remainder of the protein is then pulled into the intermembrane space and the hydrophobic sequence is released into the inner membrane. This mechanism is called the stop-transfer pathway and is discussed in detail later. (C) Some soluble proteins of the intermembrane space may also use the pathways shown in (A) and (B) before they are released into the intermembrane space by a second signal peptidase (with its active site in the intermembrane space), which removes the hydrophobic signal peptide.

After proteins destined for the intermembrane space have been inserted via their hydrophobic signal peptides into the inner membrane, some are cleaved by a signal peptidase in the intermembrane space to release the mature polypeptide chain as a soluble protein (Figure 12–25C). Many of these proteins ultimately become attached as peripheral membrane proteins to the outer surface of the inner membrane, where they form subunits of protein complexes that also contain transmembrane proteins.

Two Signal Peptides Are Required to Direct Proteins to the Thylakoid Membrane in Chloroplasts [19]

Protein transport into chloroplasts resembles transport into mitochondria in many respects: both occur posttranslationally, both require energy, and both utilize amphipathic amino-terminal signal peptides that are removed after use. There is at least one important difference, however: mitochondria exploit the electrochemical gradient across their inner membrane to help drive the transport, whereas chloroplasts, which have an electrochemical gradient across their thylakoid but not their inner membrane, appear to employ only ATP hydrolysis to power import across their double-membrane outer envelope.

Although the signal peptides for import into chloroplasts resemble those for import into mitochondria, mitochondria and chloroplasts are both present in the same plant cells, and proteins must choose appropriately between them. In plants, for example, a bacterial enzyme is directed specifically to mitochondria if it is experimentally joined to an amino-terminal signal sequence of a mitochondrial protein; the same enzyme joined to an amino-terminal signal sequence of a chloroplast protein ends up in chloroplasts. The different signal sequences, therefore, can be distinguished, presumably by the import receptors on each organelle.

Chloroplasts have an extra membrane-bounded compartment, the **thylakoid**. Many chloroplast proteins, including protein subunits of the photosynthetic system and of the ATP synthase, are embedded in the membrane of the thylakoid compartment. Like the precursors of some mitochondrial proteins, these proteins are transported from the cytosol to their final destination in two steps. First, they pass across the double membrane at contact sites into the matrix space of the chloroplast (termed the **stroma**), and then they are translocated into the thylakoid membrane (or across this membrane into the thylakoid space). The precursors of these proteins have a hydrophobic thylakoid signal peptide following the amino-terminal chloroplast signal peptide. After the amino-terminal signal peptide has been used to import the protein into the stroma, it is removed by a stromal signal peptidase (analogous to the matrix signal peptidase in mitochondria). This cleavage unmasks the thylakoid signal peptide, which then initiates transport across the thylakoid membrane (Figure 12–26). As with mitochondria, the second step is the pathway used to insert chloroplast-encoded proteins into the thylakoid membrane; the protein translocator required presumably originated in the chloroplast's bacterial ancestor.

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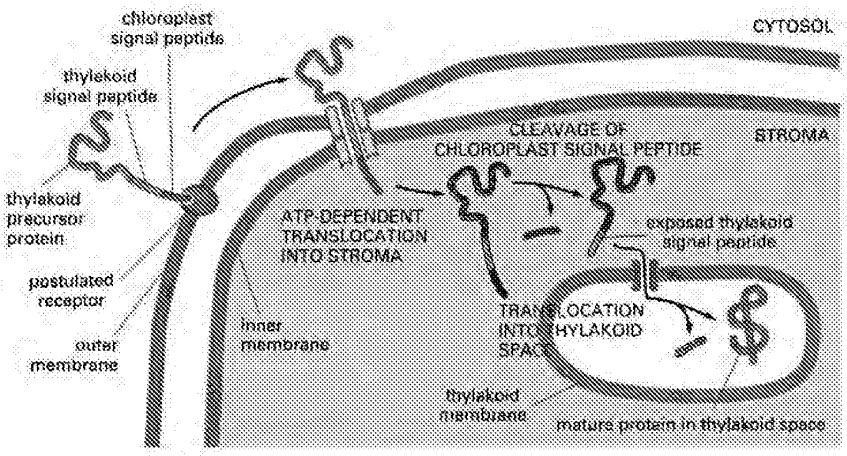


Figure 12–26 **Translocation into the thylakoid space of chloroplasts.** The precursor polypeptide contains an amino-terminal chloroplast signal peptide (*red*) followed immediately by a thylakoid signal peptide (*orange*). The chloroplast signal peptide initiates translocation into the stroma through a membrane contact site by a mechanism similar to that used for translocation into the mitochondrial matrix. The signal peptide is then cleaved off, unmasking the thylakoid signal peptide, which initiates translocation across the thylakoid membrane.

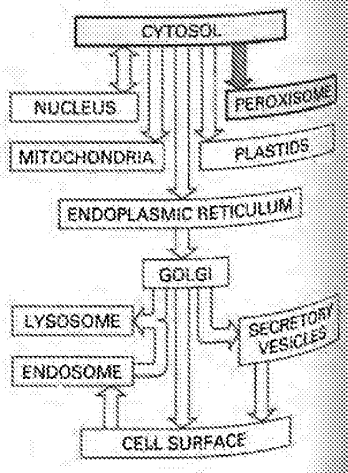
Summary

Although mitochondria and chloroplasts have their own genetic systems, they produce only a small proportion of their own proteins. Instead, the two organelles import most of their proteins from the cytosol using similar mechanisms. The transport processes involved have been most extensively studied in mitochondria, especially in yeasts. A protein is translocated into the mitochondrial matrix space by passing through sites of adhesion between the outer and inner membranes called contact sites. Translocation into mitochondria is driven by both ATP hydrolysis and the electrochemical gradient across the inner membrane, whereas translocation into chloroplasts is driven by ATP hydrolysis alone. The transported protein crosses the membranes of the mitochondrion or chloroplast in an unfolded state. Chaperone proteins of the cytosolic hsp70 family maintain the precursor proteins in an unfolded, translocation-competent state. Mitochondrial hsp70 in the matrix binds to the incoming polypeptide chain and is thought to pull the protein chain into the matrix. Once the protein is in the matrix, another stress protein, hsp60, helps the translocated protein fold up. Only proteins that contain a specific signal peptide are translocated into mitochondria or chloroplasts. The signal peptide is usually located at the amino terminus and is cleaved off after import. Transport across or into the inner membrane can occur as a second step if a hydrophobic signal peptide is also present in the imported protein; this second signal peptide is unmasked when the first signal peptide is removed. In the case of chloroplasts, import from the stroma into the thylakoid likewise requires a second signal peptide.

Peroxisomes [20]

Peroxisomes differ from mitochondria and chloroplasts in many ways. Most notably, they are surrounded by only a single membrane, and they do not contain DNA or ribosomes. In spite of these differences, peroxisomes are thought to acquire their proteins by a similar process of selective import from the cytosol. Because peroxisomes have no genome, however, *all* of their proteins must be imported. Peroxisomes thus resemble the ER in being self-replicating membrane-bounded organelles that exist without genomes of their own.

Because we do not discuss peroxisomes elsewhere, we shall digress to consider some of the functions of this diverse family of organelles before discussing their biosynthesis. Peroxisomes are found in all eucaryotic cells. They contain oxidative enzymes, such as *catalase* and *urate oxidase*, at such high concentrations that in some cells the peroxisomes stand out in electron micrographs because of the presence of a crystalloid core, largely composed of urate oxidase (Figure 12–27).



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Like the mitochondrion, the peroxisome is a major site of oxygen utilization. One hypothesis is that the peroxisome is a vestige of an ancient organelle that carried out all of the oxygen metabolism in the primitive ancestors of eucaryotic cells. When the oxygen produced by photosynthetic bacteria first began to accumulate in the atmosphere, it would have been highly toxic to most cells. Peroxisomes might have served to lower the intracellular concentration of oxygen while also exploiting its chemical reactivity to carry out useful oxidative reactions. According to this view, the later development of mitochondria rendered the peroxisome largely obsolete because many of the same reactions—which had formerly been carried out in peroxisomes without producing energy—were now coupled to ATP formation by means of oxidative phosphorylation. The oxidative reactions carried out by peroxisomes in present-day cells would therefore be those that have important functions not taken over by mitochondria.

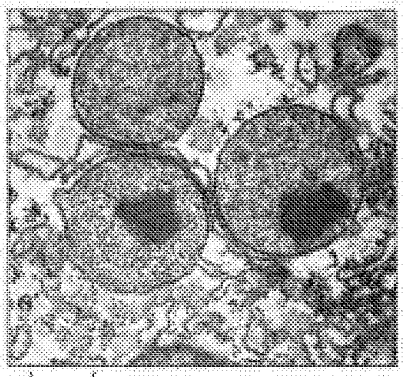


Figure 12–27 **Peroxisomes.** Electron micrograph of three peroxisomes in a rat liver cell. The paracrystalline electron-dense inclusions are the enzyme urate oxidase. (Courtesy of Daniel S. Friend.)

Peroxisomes Use Molecular Oxygen and Hydrogen Peroxide to Carry Out Oxidative Reactions [21]

Peroxisomes are so called because they usually contain one or more enzymes that use molecular oxygen to remove hydrogen atoms from specific organic substrates (designated here as R) in an oxidative reaction that produces *hydrogen peroxide* (H_2O_2):

$$RH_2 + O_2 \rightarrow R + H_2O_2$$

Catalase utilizes the H_2O_2 generated by other enzymes in the organelle to oxidize a variety of other substrates—including phenols, formic acid, formaldehyde, and alcohol—by the "peroxidative" reaction: $H_2O_2 + R'H_2 \rightarrow R' + 2H_2O$. This type of oxidative reaction is particularly important in liver and kidney cells, whose peroxisomes detoxify various toxic molecules that enter the bloodstream. About a quarter of the ethanol we drink is oxidized to acetaldehyde in this way. In addition, when excess H_2O_2 accumulates in the cell, catalase converts it to H_2O ($2H_2O_2 \rightarrow 2H_2O + O_2$).

A major function of the oxidative reactions carried out in peroxisomes is the breakdown of fatty acid molecules. In a process called *β oxidation*, the alkyl chains of fatty acids are shortened sequentially by blocks of two carbon atoms at a time that are converted to acetyl CoA and exported from the peroxisomes to the cytosol for reuse in biosynthetic reactions. β oxidation in mammalian cells occurs both in mitochondria and peroxisomes; in yeast and plant cells, however, this essential reaction is exclusively found in peroxisomes.

Peroxisomes are unusually diverse organelles and even in the different cells of a single organism may contain very different sets of enzymes. They also can adapt remarkably to changing conditions. Yeast cells grown on sugar, for example, have small peroxisomes. But when some yeasts are grown on methanol, they develop large peroxisomes that oxidize methanol; and when grown on fatty acids, they develop large peroxisomes that break down fatty acids to acetyl CoA by β oxidation.

Peroxisomes also have very important roles in plants. Two very different types have been studied extensively. One type is present in leaves, where it catalyzes the oxidation of a side product of the crucial reaction that fixes CO_2 in carbohydrate (Figure 12–28A). This process is called *photorespiration* because it uses up O_2 and liberates CO_2. The other type of peroxisome is present in germinating seeds, where it plays an essential role in converting the fatty acids stored in seed lipids into the sugars needed for the growth of the young plant. Because this conversion of fats to sugars is accomplished by a series of reactions known as the *glyoxylate cycle*, these peroxisomes are also called *glyoxysomes* (Figure 12–28B). In the glyoxylate cycle two molecules of acetyl CoA produced by fatty acid breakdown in the peroxisome are used to make succinic acid, which leaves the peroxisome and is converted into glucose. The glyoxylate cycle does not occur in animal cells, and animals are thus unable to convert the fatty acids in fats into carbohydrates.

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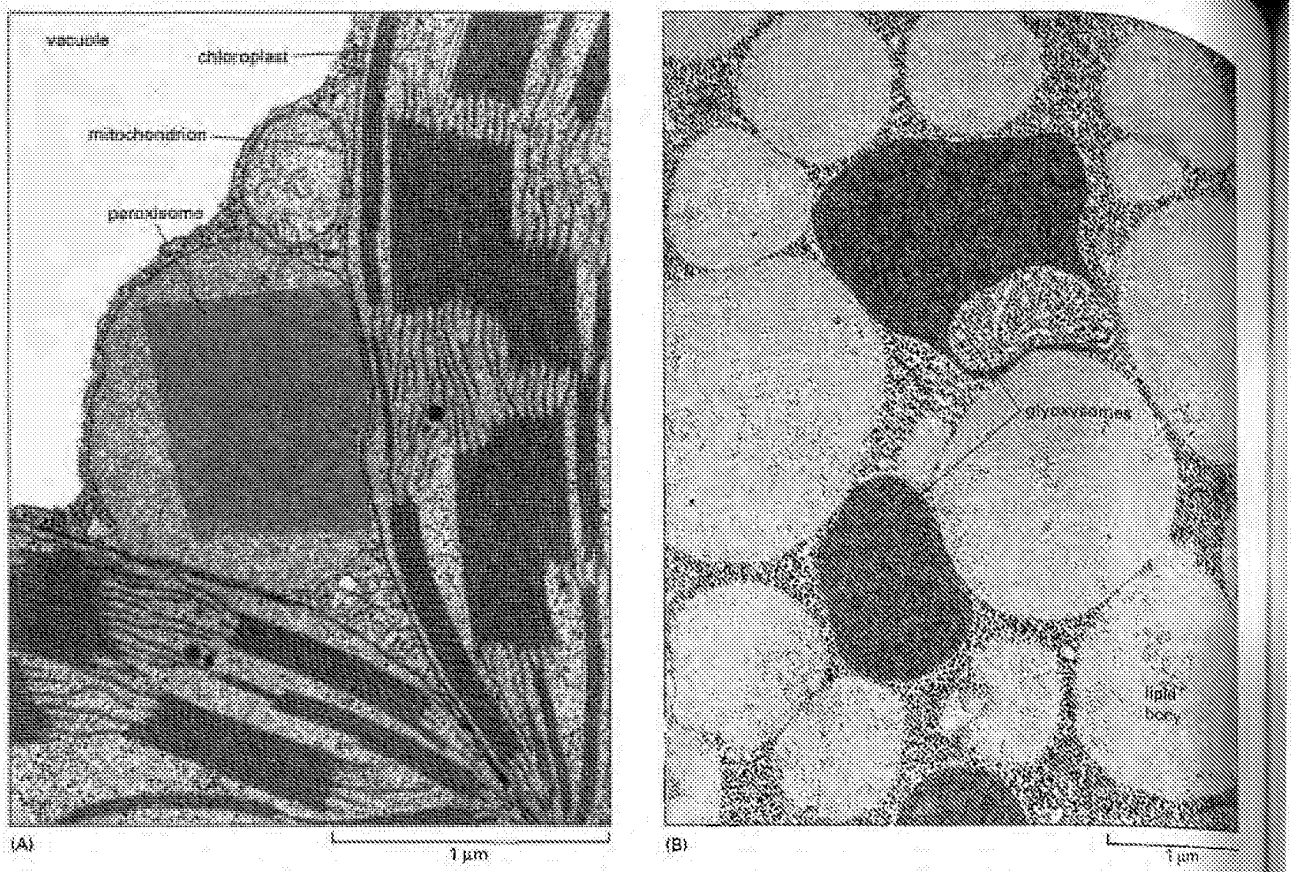


Figure 12–28 Electron micrographs of two types of peroxisomes found in plant cells. (A) A leaf peroxisome with a paracrystalline core in a tobacco leaf mesophyll cell. Its close association with chloroplasts is thought to facilitate the exchange of materials between these organelles during photorespiration. (B) Peroxisomes in a fat-storing cotyledon cell of a tomato seed 4 days after germination. Here the peroxisomes (*glyoxysomes*) are associated with the lipid bodies where fat is stored, reflecting their central role in fat mobilization and gluconeogenesis during seed germination. (A, courtesy of P.J. Gruber and E.H. Newcomb; B, courtesy of S.E. Frederick and E.H. Newcomb.)

A Short Signal Sequence Directs the Import of Proteins into Peroxisomes [22]

A specific sequence of three amino acids located near the carboxyl terminus of many peroxisomal proteins functions as an import signal (see Table 12–3); if this sequence is experimentally attached to a cytosolic protein, the protein is imported into peroxisomes. The importance of this import process and of peroxisomes is dramatically demonstrated by the inherited human disease *Zellweger syndrome*, in which a defect in importing proteins into peroxisomes leads to a severe peroxisomal deficiency. These individuals, whose cells contain "empty" peroxisomes, have severe abnormalities in their brain, liver, and kidneys, and they die soon after birth. One form of this disease has been shown to be due to a mutation in the gene encoding a peroxisomal integral membrane protein called peroxisome assembly factor-1.

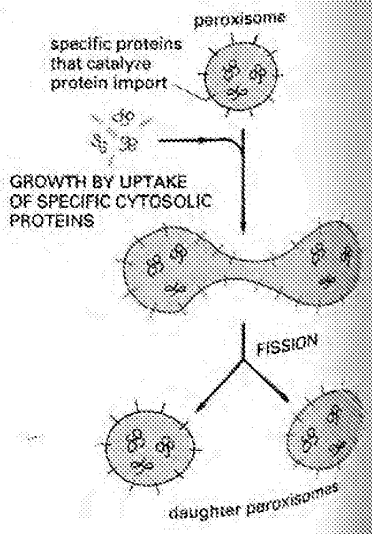


Figure 12–29 A model for how peroxisomes are assembled. The peroxisome membrane contains specific import receptor proteins. All peroxisomal proteins, including new copies of the import receptor, are synthesized by cytosolic ribosomes and then imported into the organelle. Thus peroxisomes form only from preexisting peroxisomes by a process of growth and fission. Presumably, the lipids required to make new peroxisome membrane are also imported. We discuss later how lipids made in the ER can be transported through the cytosol to other organelles.

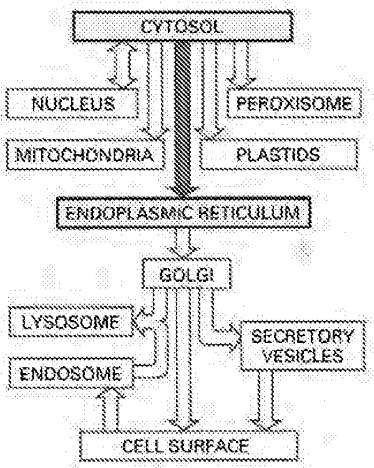
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Peroxisomes presumably have at least one unique protein exposed on their cytosolic surface to act as a receptor that recognizes the signal on the proteins to be imported. At one time it was thought that the membrane of the peroxisome forms by budding from the ER, while the content is imported from the cytosol. There is now evidence, however, suggesting that new peroxisomes arise only from preexisting ones, by organelle growth and fission, as described elsewhere for mitochondria and plastids and for the ER itself (Figure 12–29).

Summary

Peroxisomes are specialized for carrying out oxidative reactions using molecular oxygen. They generate hydrogen peroxide, which they also use for oxidative purposes—destroying the excess by means of the catalase they contain. Like mitochondria and plastids, peroxisomes are self-replicating organelles. Because they contain no DNA or ribosomes, they have to import all of their proteins from the cytosol. A specific three amino acid sequence near the carboxyl terminus of many of these proteins functions as a peroxisomal import signal.

The Endoplasmic Reticulum [23]



All eucaryotic cells have an **endoplasmic reticulum (ER).** Its membrane typically constitutes more than half of the total membrane of an average animal cell (see Table 12–2). It is organized into a netlike labyrinth of branching tubules and flattened sacs extending throughout the cytosol (Figure 12–30). The tubules and sacs are all thought to interconnect, so that the ER membrane forms a continuous sheet enclosing a single internal space. This highly convoluted space is called the **ER lumen** or the *ER cisternal space,* and it often occupies more than 10% of the total cell volume (see Table 12–1). The ER membrane separates the ER lumen from the cytosol, and it mediates the selective transfer of molecules between these two compartments.

The ER plays a central part in lipid and protein biosynthesis. Its membrane is the site of production of all the transmembrane proteins and lipids for most of the cell's organelles, including the ER itself, the Golgi apparatus, lysosomes, endosomes, secretory vesicles, and the plasma membrane. The ER membrane also makes a major contribution to mitochondrial and peroxisomal membranes by producing most of their lipids. In addition, almost all of the proteins that will be secreted to the cell exterior—as well as those destined for the lumen of the ER, Golgi apparatus, or lysosomes—are initially delivered to the ER lumen.

Membrane-bound Ribosomes Define the Rough ER [24]

The ER captures selected proteins from the cytosol as they are being synthesized. These proteins are of two types: (1) transmembrane proteins, which are only partly translocated across the ER membrane and become embedded in it, and (2) water-soluble proteins, which are fully translocated across the ER membrane and are released into the ER lumen. Some of the transmembrane proteins will remain in the ER, but many are destined to reside in the plasma membrane or the membrane of another organelle; the water-soluble proteins are destined either for the lumen of an organelle or for secretion. All of these proteins, regardless of their subsequent fate, are directed to the ER membrane by the same kind of signal peptide and are translocated across it by the same mechanism.

In mammalian cells the import of proteins into the ER begins before the polypeptide chain is completely synthesized—that is, it occurs **co-translationally.** This distinguishes the process from the import of proteins into mitochondria, chloroplasts, nuclei, and peroxisomes, which is **posttranslational** and requires different signal peptides. Since one end of the protein is usually translocated into the ER as the rest of the polypeptide chain is being made, the protein is never released into the cytosol and therefore is never in danger of folding up before

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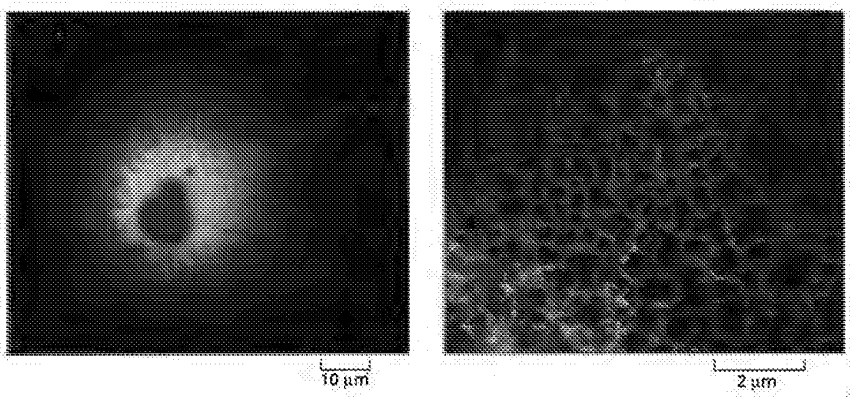


Figure 12–30 **The endoplasmic reticulum.** Fluorescence micrograph of a cultured mammalian cell stained with an antibody that binds to a protein retained in the ER. The ER extends as a network throughout the entire cytosol, so that all regions of the cytosol are close to some portion of the ER membrane. (Courtesy of Hugh Pelham.)

reaching the translocator in the membrane. In contrast to the posttranslational import of proteins into the mitochondria and chloroplasts, cytosolic chaperonins are therefore not required to keep the protein unfolded. The ribosome that is synthesizing the protein is directly attached to the ER membrane. These membrane-bound ribosomes coat the surface of the ER, creating regions termed **rough endoplasmic reticulum** (Figure 12–31).

There are, therefore, two spatially separate populations of ribosomes in the cytosol. **Membrane-bound ribosomes,** attached to the cytosolic side of the ER membrane, are engaged in the synthesis of proteins that are being concurrently translocated into the ER. **Free ribosomes,** unattached to any membrane, make all other proteins encoded by the nuclear genome. Membrane-bound and free ribosomes are structurally and functionally identical. They differ only in the proteins they are making at any given time. When a ribosome happens to be making a protein with an **ER signal peptide,** the signal directs the ribosome to the ER membrane. Since many ribosomes can bind to a single mRNA molecule, a **polyribosome** is usually formed, which becomes attached to the ER membrane via the signal peptides on multiple growing polypeptide chains (Figure 12–32). The individual ribosomes associated with such an mRNA molecule can return to the cytosol when they finish translation near the 3′ end of the mRNA molecule. The mRNA itself, however, tends to remain attached to the ER membrane by a changing population of ribosomes that are also held at the membrane by a ribosome receptor that helps to bind it there. In contrast, if an mRNA molecule encodes a protein that lacks an ER signal peptide, the polyribosome that forms remains free in the cytosol and its protein product is discharged there. Therefore, only those mRNA molecules that encode proteins with an ER signal peptide bind to rough ER membranes; those mRNA molecules that encode all other proteins remain free in the cytosol. The individual ribosomal subunits are thought to move

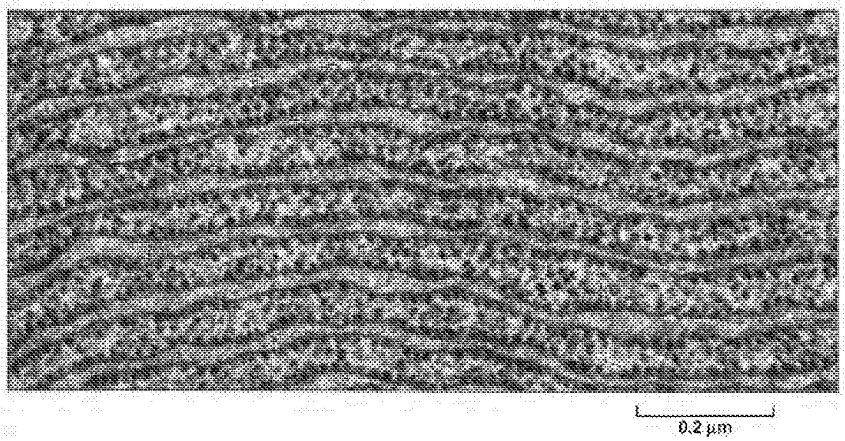


Figure 12–31 **The rough ER.** Electron micrograph of the rough ER, which receives its name from the many ribosomes on its cytosolic surface. (Courtesy of L. Orci.)

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randomly between these two segregated populations of mRNA molecules (Figure 12–33).

Smooth ER Is Abundant in Some Specialized Cells [25]

Regions of ER that lack bound ribosomes are called **smooth endoplasmic reticulum**, or **smooth ER**. In the great majority of cells such regions are scanty, and there is only a small region of the ER that is partly smooth and partly rough. This region is said to consist of **transitional elements** because it is from here that transport vesicles carrying newly synthesized proteins and lipids bud off for transport to the Golgi apparatus. In certain specialized cells, however, the smooth ER is abundant and has additional functions. In particular, it is usually prominent in cells that specialize in lipid metabolism: cells that synthesize steroid hormones from cholesterol, for example, have an expanded smooth ER compartment to accommodate the enzymes needed to make cholesterol and to modify it to form the hormones (Figure 12–34).

The main cell type in the liver, the *hepatocyte*, provides another example. It is the principal site of the production of lipoprotein particles; these particles carry lipids via the bloodstream to other sites in the body. The enzymes that synthesize the lipid components of lipoproteins are located in the membrane of the smooth ER, which also contains enzymes that catalyze a series of reactions to detoxify both lipid-soluble drugs and various harmful compounds produced by metabolism. The most extensively studied of the *detoxification reactions* are catalyzed by the *cytochrome P450* family of enzymes, which catalyze a series of reactions whereby water-insoluble drugs or metabolites that would otherwise accumulate to toxic levels in cell membranes are rendered sufficiently water-soluble to leave the cell and be excreted in the urine. Because the rough ER alone cannot house enough of these and other necessary enzymes, a major portion of the membrane in a hepatocyte normally consists of smooth ER (Figure 12–35 and see Table 12–2).

When large quantities of certain compounds, such as the drug phenobarbital, enter the circulation, detoxification enzymes are synthesized in the liver in unusually large amounts, and the smooth ER doubles in surface area within a few days. Once the drug disappears, the excess smooth ER membrane is specifically and rapidly removed by a lysosome-dependent process called *autophagocytosis* (discussed in Chapter 13). How these dramatic changes are regulated is not known.

Another function of the ER in most eucaryotic cells is to sequester Ca^{2+} from the cytosol. The release of Ca^{2+} into the cytosol from the ER, and its subsequent reuptake, mediate many rapid responses to extracellular signals, as discussed in Chapter 15. The storage of Ca^{2+} in the ER lumen is facilitated by the high concen-



Figure 12–32 Polyribosomes. Thin-section electron micrograph of polyribosomes attached to the ER membrane. The plane of section in some places cuts through the ER roughly parallel to the membrane, giving a face-on view of the rosettelike pattern of the polyribosomes. (Courtesy of George Palade.)

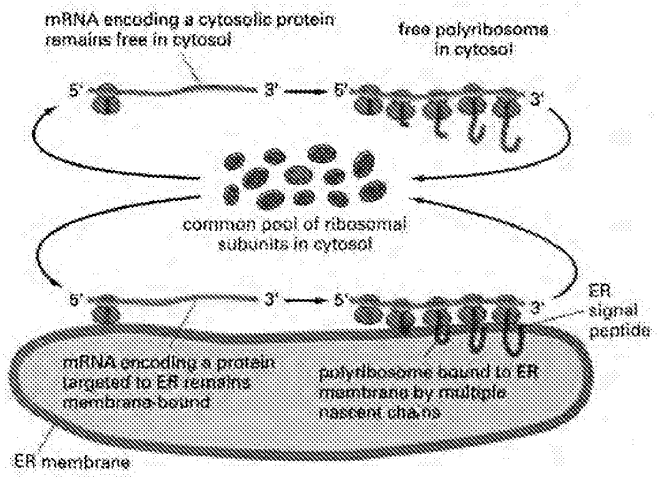


Figure 12–33 Free and membrane-bound ribosomes. A common pool of ribosomes is used to synthesize both the proteins that stay in the cytosol and those that are transported into the ER. It is the ER signal peptide on a newly formed polypeptide chain that directs the engaged ribosome to the ER membrane. The mRNA molecule may remain permanently bound to the ER as part of a polyribosome, while the ribosomes that move along it are recycled; at the end of each round of protein synthesis, the ribosomal subunits are released and rejoin the common pool in the cytosol.

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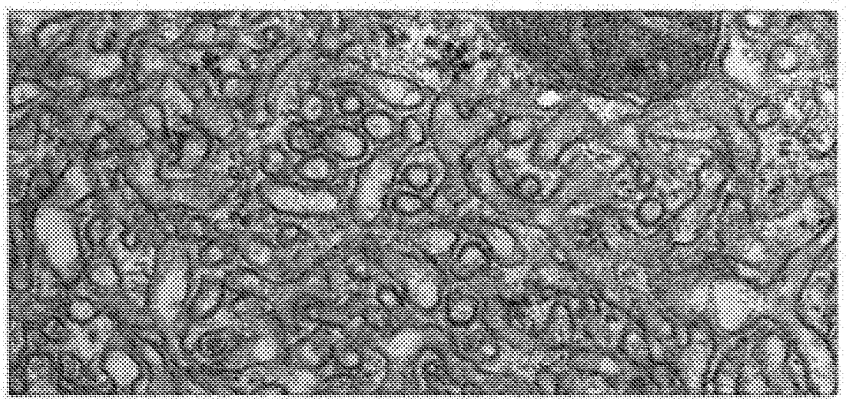


Figure 12–34 **Abundant smooth ER in a steroid-hormone-secreting cell.** This electron micrograph is of a testosterone-secreting Leydig cell in the human testis.

trations of Ca^{2+}-binding proteins there. In some cell types, and perhaps in most, specific regions of the ER are specialized for Ca^{2+} storage. Muscle cells, for example, have an abundant specialized smooth ER, called the *sarcoplasmic reticulum*, which sequesters Ca^{2+} from the cytosol by means of a Ca^{2+}-ATPase that pumps in Ca^{2+}; the release and reuptake of Ca^{2+} by the sarcoplasmic reticulum mediates the contraction and relaxation of the myofibrils during each round of muscle contraction (discussed in Chapter 16).

We shall now return to the two major roles of the ER: the synthesis and modification of proteins and the synthesis of lipids.

Rough and Smooth Regions of ER Can Be Separated by Centrifugation [26]

In order to study the functions and biochemistry of the ER, it is necessary to isolate the ER membrane. At first sight this seems a hopeless task since the ER is intricately interleaved with other components of the cytosol. Fortunately, when tissues or cells are disrupted by homogenization, the ER is fragmented and reseals into many small (~100 nm in diameter) closed vesicles called **microsomes**, which are relatively easy to purify. Microsomes derived from rough ER are studded with ribosomes and are called *rough microsomes*. The ribosomes are always found on the *outside* surface, so that the interior of the microsome is biochemically equivalent to the luminal space of the ER (Figure 12–36). Because they can be readily purified in functional form, rough microsomes are especially useful for studying the many processes carried out by the rough ER. To the biochemist they represent small authentic versions of the rough ER, still capable of protein synthesis, protein glycosylation, and lipid synthesis.

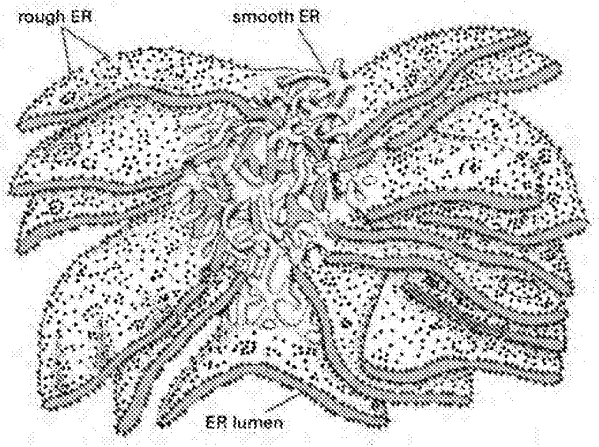


Figure 12–35 **Three-dimensional reconstruction of a region of the smooth and rough ER in a liver cell.** The rough ER forms oriented stacks of flattened cisternae, each having a luminal space 20 to 30 nm wide. The smooth ER membrane is connected to these cisternae and forms a fine network of tubules 30 to 60 nm in diameter. (After R.V. Krstić, Ultrastructure of the Mammalian Cell. New York: Springer-Verlag, 1979.)

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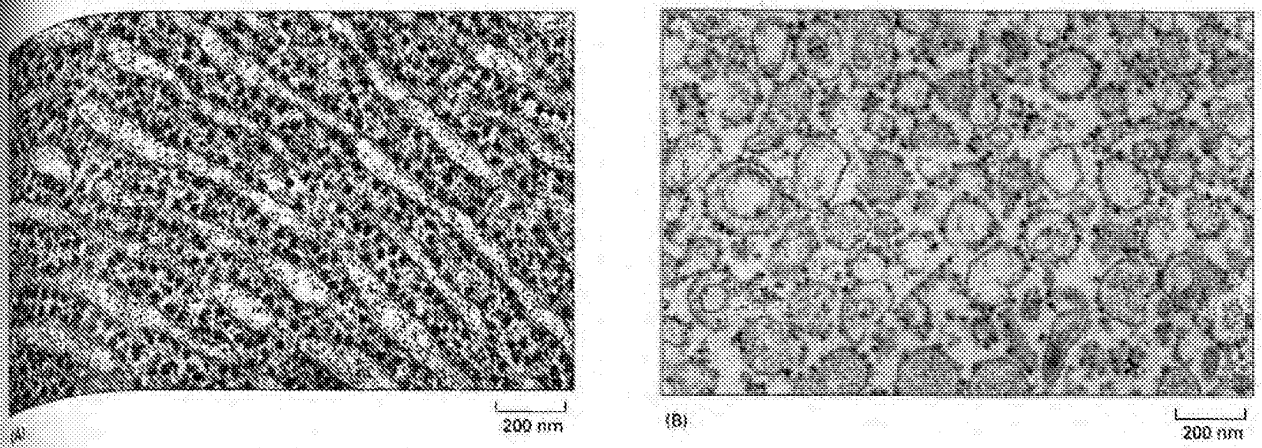


Figure 12–36 **Electron micrographs of microsomes.** When cells are disrupted by homogenization, the cisternae of rough ER (A) break up into small closed vesicles called *rough microsomes* (B). Similarly, the smooth ER breaks up into small vesicles that lack ribosomes and are called *smooth microsomes*. (A, courtesy of Daniel S. Friend; B, courtesy of George Palade.)

Many vesicles of a size similar to that of rough microsomes but lacking attached ribosomes are also found in these homogenates. Such *smooth microsomes* are derived in part from smooth portions of the ER and in part from vesiculated fragments of plasma membrane, Golgi apparatus, endosomes, and mitochondria (the ratio depending on the tissue). Thus, whereas rough microsomes can be equated with rough portions of ER, the origins of smooth microsomes cannot be so easily assigned. The microsomes of the liver are an exception. Because of the unusually large quantities of smooth ER in the hepatocyte, most of the smooth microsomes in liver homogenates are derived from smooth ER.

The ribosomes attached to them make rough microsomes more dense than smooth microsomes. As a result, the rough and smooth microsomes can be separated from each other by equilibrium centrifugation (Figure 12–37). When the separated rough and smooth microsomes of liver are compared with respect to such properties as enzyme activity or polypeptide composition, they are very similar, although not identical: apparently most of the components of the ER membrane can diffuse freely between the rough and smooth regions, as would be expected for a continuous fluid membrane. The rough microsomes, however, contain more than 20 proteins that are not present in smooth microsomes, showing that some restraining mechanism must exist for a subset of ER membrane proteins. Some of the proteins in this subset help bind ribosomes to the rough ER, while others presumably produce the flattened shape of this part of the ER (see Figure 12–35). It is not clear whether these membrane proteins are retained by forming large two-dimensional aggregates in the lipid bilayer or whether they are instead held in place by interactions with a network of structural proteins on one or the other face of the ER membrane.

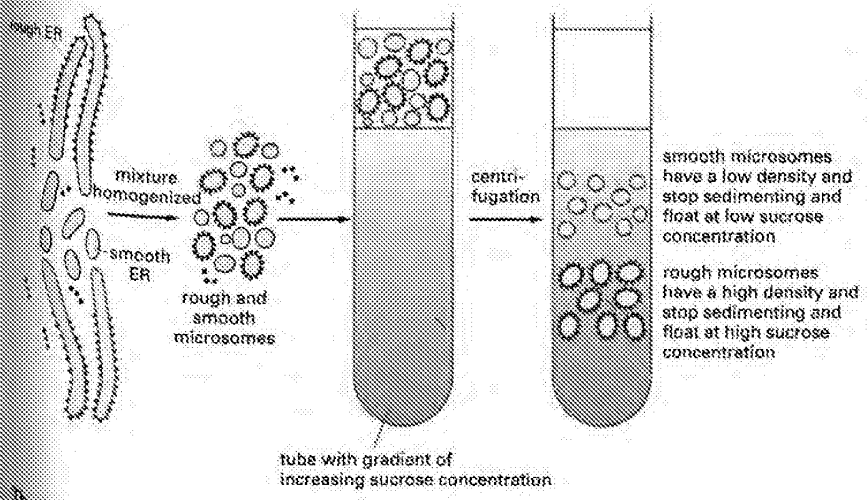


Figure 12–37 **The isolation procedure used to purify rough and smooth microsomes from the ER.** When sedimented to equilibrium through a gradient of sucrose, the two types of microsomes separate from each other on the basis of their different densities.

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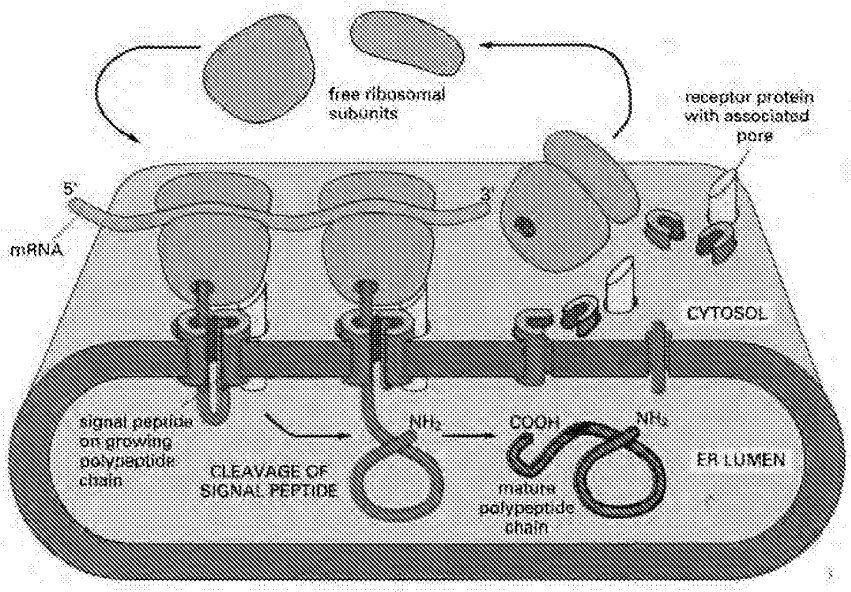


Figure 12–38 **The original signal hypothesis.** A simplified view of protein translocation across the ER membrane, as originally proposed. When the signal peptide emerges from the ribosome, it directs the ribosome to a receptor protein on the ER membrane. As it is synthesized, the polypeptide is postulated to be translocated across the ER membrane through a protein pore associated with the receptor. The signal peptide is clipped off during translation by a signal peptidase, and the mature protein is released into the lumen of the ER immediately after being synthesized. We now know that the hypothesis is correct in outline but that additional components besides those shown in this figure are required. The signal peptidase, for example, is a complex of five different membrane-bound polypeptide chains, with one complex apparently associated with every translocation pore.

Signal Peptides Were First Discovered in Proteins Imported into the Rough ER [27]

Signal peptides (and the signal peptide strategy of protein import) were first discovered in the early 1970s in secreted proteins that are translocated across the ER membrane as a first step toward their eventual discharge from the cell. In the key experiment the mRNA encoding a secreted protein was translated by ribosomes *in vitro*. When microsomes were omitted from this cell-free system, the protein synthesized was slightly larger than the normal secreted protein, the extra length being due to the presence of an amino-terminal *leader peptide*. In the presence of microsomes derived from the rough endoplasmic reticulum, however, a protein of the correct size was produced. These results were explained by the **signal hypothesis,** which postulated that the leader serves as a *signal peptide* that directs the secreted protein to the ER membrane and is then cleaved off by a signal peptidase in the ER membrane before the polypeptide chain is completed (Figure 12–38).

According to the signal hypothesis, the secreted protein should be extruded into the lumen of the microsome during its *in vitro* synthesis. This can be demonstrated with protease treatment: a newly synthesized protein made in the absence of microsomes is degraded when protease is added to the medium, whereas the same protein made in the presence of microsomes remains intact because it is protected by the microsomal membrane. When proteins without ER signal peptides are similarly synthesized *in vitro*, they are not imported into microsomes and therefore are degraded by protease treatment.

The signal hypothesis has been thoroughly tested by genetic and biochemical experiments and is found to apply to both plant and animal cells, as well as to protein translocation across the bacterial plasma membrane and, as we have seen, the membranes of mitochondria, chloroplasts, and peroxisomes. Amino-terminal ER signal peptides guide not only secreted proteins but also the precursors of all proteins made in the ER, including soluble proteins and membrane proteins. The signaling function of these peptides has been demonstrated directly by using recombinant DNA techniques to attach signal sequences to proteins that do not normally have them; the resulting fusion proteins are directed to the ER.

Cell-free systems in which proteins are imported into microsomes have provided powerful assay procedures for identifying, purifying, and studying the various components of the molecular machinery responsible for the ER import process.

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A Signal-Recognition Particle (SRP) Directs ER Signal Peptides to a Specific Receptor in the Rough ER Membrane [28]

The ER signal peptide is guided to the ER membrane by at least two components: a **signal-recognition particle (SRP)**, which cycles between the ER membrane and the cytosol and binds to the signal peptide, and an *SRP receptor*, also known as a *docking protein*, in the ER membrane. The SRP was discovered when it was found that washing microsomes with salt eliminated their ability to import secreted proteins. Import could be restored by adding back the supernatant containing the salt extract. The "translocation factor" in the salt extract was then purified and found to be a complex particle consisting of six different polypeptide chains bound to a single small RNA molecule (Figure 12–39). SRP and SRP receptor are present in all eucaryotic cells and probably in procaryotic cells as well.

The SRP binds to the ER signal peptide as soon as the peptide emerges from the ribosome. This causes a pause in protein synthesis, which presumably gives the ribosome enough time to bind to the ER membrane before the synthesis of the polypeptide chain is completed, thereby ensuring that the protein is not released into the cytosol. This may provide a safety mechanism as many secreted proteins and lysosomal proteins are hydrolases that could wreak havoc in the cytosol. Cells that secrete large amounts of hydrolases take the added precaution of having high concentrations of hydrolase inhibitors in their cytosol.

Once formed, the SRP ribosome complex binds to the **SRP receptor,** which is an integral membrane protein exposed on the cytosolic surface of the rough ER membrane. The SRP is then released, and a poorly characterized translocation apparatus transfers the growing polypeptide chain across the membrane (Figure 12–40). Because one of the SRP proteins and both chains of the SRP receptor contain GTP-binding domains, it is thought that conformational changes that occur during cycles of GTP binding and hydrolysis (discussed in Chapter 5) ensure that SRP release occurs only after the ribosome has become properly engaged with the translocation apparatus in the ER membrane.

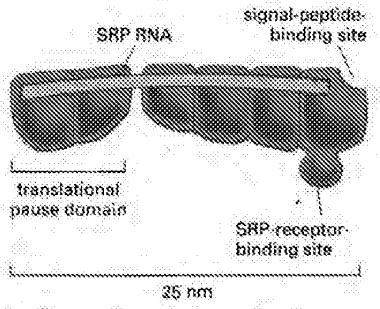


Figure 12–39 **A highly schematic drawing of a signal-recognition particle (SRP).** An SRP is an elongated complex containing six protein subunits and one RNA molecule (SRP RNA). One end of the SRP binds to an ER signal peptide on a growing polypeptide chain, while the other end binds to the ribosome itself and stops translation. The RNA in the particle may mediate an interaction with ribosomal RNA. (Adapted from V. Siegel and P. Walter, *Nature* 320:82–84, 1986.)

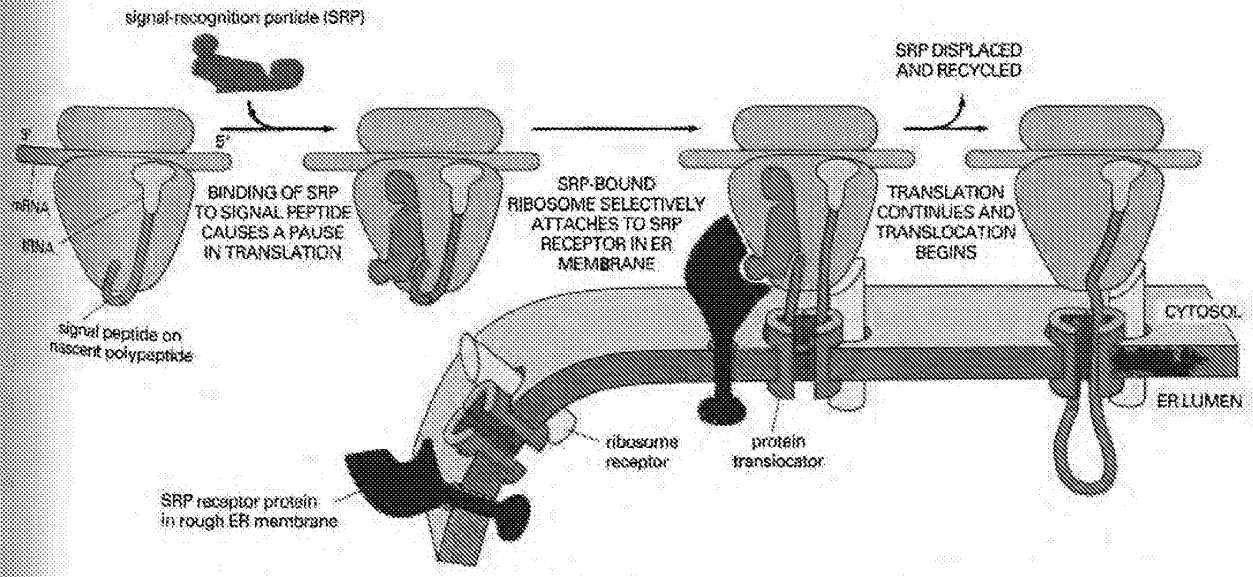


Figure 12–40 **How ER signal peptides and SRP direct ribosomes to the ER membrane.** The SRP and the SRP receptor are thought to act in concert. The SRP binds to the exposed ER signal peptide and to the ribosome, thereby inducing a pause in translation. The SRP receptor in the ER membrane, which is composed of two different polypeptide chains, binds the SRP ribosome complex. In a poorly understood reaction that involves multiple GTP-binding proteins, the SRP is released, leaving the ribosome on the ER membrane. A multisubunit protein translocation apparatus in the ER membrane then inserts the polypeptide chain into the membrane and transfers it across the lipid bilayer.

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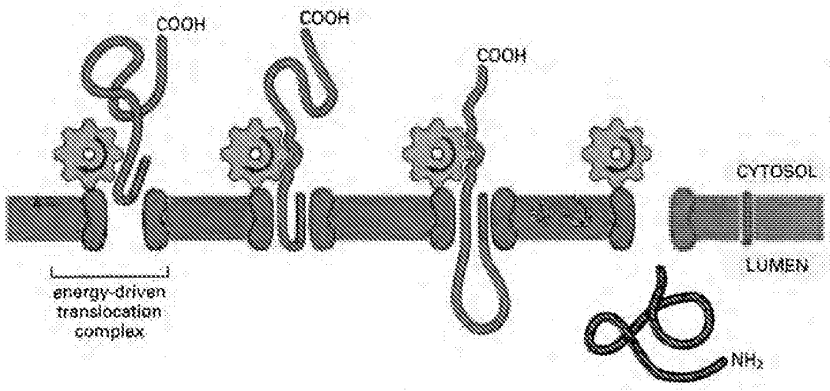


Figure 12–41 **Translocation of a protein across the bacterial plasma membrane.** In this schematic model, after a receptor in the translocation complex has bound the amino-terminal signal peptide, an energy-driven protein translocator threads the protein through the membrane, unfolding the polypeptide chain in the process. The energy is provided both by ATP hydrolysis and an electrochemical gradient across the membrane.

Translocation Across the ER Membrane Does Not Always Require Ongoing Polypeptide Chain Elongation [29]

As we have seen, translocation of proteins into mitochondria, chloroplasts, and peroxisomes occurs *posttranslationally*, after the protein is completed and released into the cytosol, whereas translocation across the ER membrane usually occurs during translation (*co-translationally*). This explains why ribosomes are bound to the ER but usually not to other organelles. A ribosome attached to the rough ER may utilize the energy of protein synthesis to force its growing polypeptide chain through a channel formed by a translocator in the ER membrane. Recent studies *in vitro*, however, have shown that a small minority of protein precursors can be imported into the ER after their synthesis has been completed, thus demonstrating that translocation does not always require ongoing translation. Posttranslational protein translocation may occur even more commonly across the ER membrane in yeast cells and across the bacterial plasma membrane (which is thought to be evolutionarily related to the ER; see Figure 12–5). In both cases the translocation requires ATP hydrolysis, and in bacteria an electrochemical gradient is needed as well. A translocation apparatus has been reconstituted from purified bacterial components, one of which is an ATPase that is thought to help thread the protein through the membrane (Figure 12–41). Since the proteins that use a posttranslational translocation pathway are first released into the cytosol, they are prevented from folding up by binding to cytosolic chaperone proteins, just as we have seen for the posttranslational import into mitochondria and chloroplasts.

The Polypeptide Chain Passes Through an Aqueous Pore in the Translocation Apparatus [30]

It has long been debated whether polypeptide chains are transferred across the ER membrane in direct contact with the lipid bilayer or through a pore in a pro-

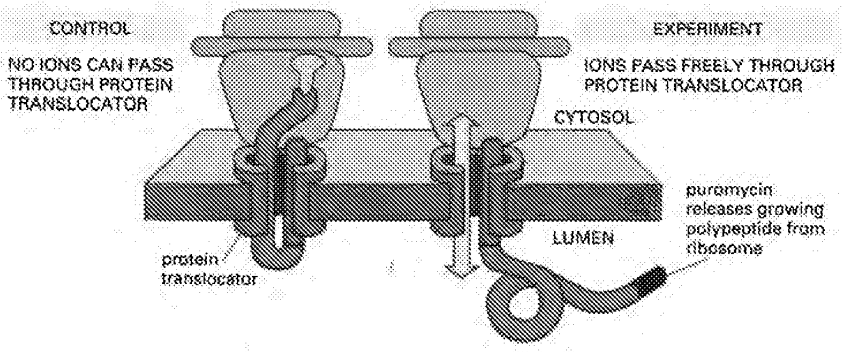


Figure 12–42 **The demonstration of protein-translocating aqueous pores in the ER membrane.** The experimental set-up is similar to that shown in Figure 10–5, with an artificial lipid bilayer separating two aqueous compartments. When rough microsomes are added to one of the compartments, they occasionally fuse with the lipid bilayer, incorporating a portion of ER membrane (with its bound ribosomes) into the bilayer. When the drug puromycin (*dark blue*) is added to the same compartment, it couples covalently to the carboxyl terminus of the growing polypeptide chain and releases it from the ribosome; pores of uniform size can now be detected as discrete increases in the electrical conductance across the membrane (the ion flow responsible for the increased electrical conductance is indicated by the *yellow arrow*). If the ribosomes are removed from the membrane with a high-salt wash, pores are no longer detected, indicating that ribosome binding is required to open (or assemble) the pore (not shown).

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tein translocator. There is now strong evidence for a protein translocator. Normally, the pore in the translocator is plugged with the growing polypeptide chain that is in transit across the membrane; when the nascent chains are experimentally released from the ribosomes with the drug puromycin, however, the pores can be detected by the ion currents that flow through them. Although the pore is large enough to allow the passage of an unfolded polypeptide chain, it closes when the ribosome is removed from the membrane (Figure 12–42). Thus the pore seems to be a dynamic structure, opening when a ribosome with a growing polypeptide chain attaches to the membrane and closing when the ribosome detaches after the synthesis of the protein is completed.

The ER Signal Peptide Is Removed from Most Soluble Proteins After Translocation [31]

We have seen that in chloroplasts and mitochondria the signal peptides are cleaved from the precursor proteins once they have crossed the membrane. Similarly, amino-terminal ER signal peptides are removed by a signal peptidase on the luminal side of the ER membrane. The peptide by itself, however, is not sufficient to signal cleavage by the peptidase; this requires an adjacent cleavage site that is specifically recognized by the peptidase. We shall see below that ER signal peptides that are contained within the polypeptide chain rather than at the amino terminus do not have these recognition sites and are never cleaved; instead, they can serve to retain transmembrane proteins in the lipid bilayer after the translocation process has been completed.

The amino-terminal ER signal peptide of a soluble protein itself has two signaling functions: in addition to directing the protein to the ER membrane, it is thought to serve as a **start-transfer signal,** which remains bound to the translocation apparatus while the rest of the protein is threaded continuously through the membrane as a large loop. Once the carboxyl terminus of the protein has passed through the membrane, the signal peptide is released from the translocator pore, cleaved off by the signal peptidase, and rapidly degraded to amino acids by other proteases in the ER while the protein is released into the ER lumen (Figure 12–43).

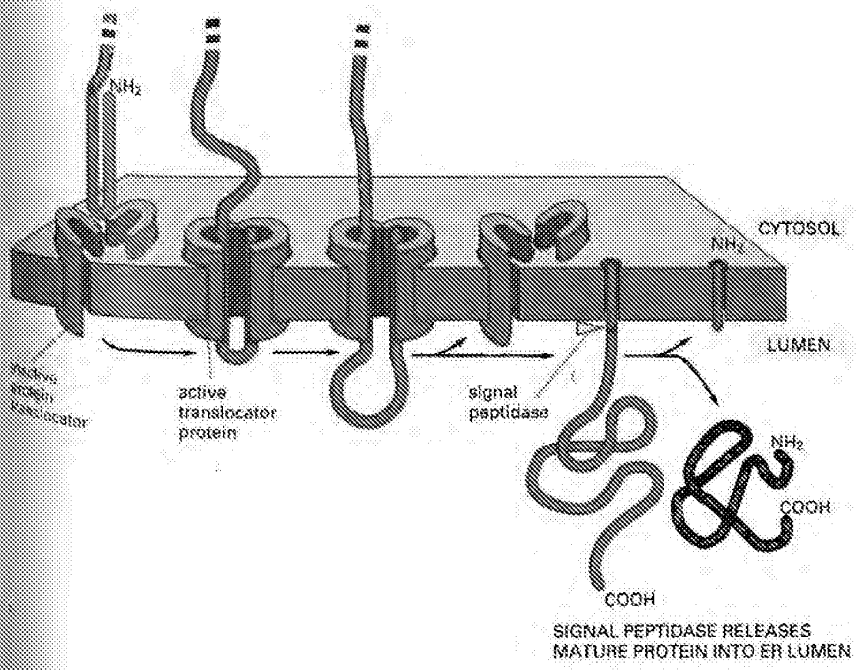


Figure 12–43 **The translocation of a soluble protein across the ER membrane.** In this hypothetical model the protein translocator in the membrane is postulated to exist in two alternative states—active or inactive. On binding an ER signal peptide (which acts as a start-transfer signal), the translocator adopts an active state and begins to transfer the polypeptide chain across the lipid bilayer as a loop. In this state it forms an aqueous pore across the membrane that can be detected electrophysiologically if the polypeptide chain is released (see Figure 12–42). After the protein has been completely translocated, the translocator reverts to an inactive conformation, which can no longer conduct ions across the membrane but is open to the lipid bilayer, allowing the hydrophobic signal peptide to diffuse out into the bilayer, where it is rapidly degraded. In this and the following two figures the ribosomes have been omitted for clarity.

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In Single-Pass Transmembrane Proteins a Single Internal ER Signal Peptide Remains in the Lipid Bilayer as a Membrane-spanning α Helix [32]

The translocation process for proteins destined to remain in the membrane is more complex than it is for soluble proteins, as some parts of the polypeptide chain are translocated across the lipid bilayer whereas others are not. Nevertheless, all modes of insertion of membrane proteins can be considered as variants of the sequence of events just described for transferring a soluble protein into the lumen of the ER. We begin by describing the three ways in which **single-pass transmembrane proteins** (see Figure 10–13) become inserted into the ER.

In the simplest case an amino-terminal signal peptide initiates translocation, just as for a soluble protein, but an additional hydrophobic segment in the polypeptide chain stops the transfer process before the entire polypeptide chain is translocated. This **stop-transfer peptide** anchors the protein in the membrane after the ER signal (start-transfer) peptide is released from the translocator and is cleaved off (Figure 12–44). The stop-transfer peptide forms a single α-helical membrane-spanning segment, with the amino terminus of the protein on the luminal side of the membrane and the carboxyl terminus on the cytosolic side.

In the other two cases the signal peptide is internal, rather than at the amino-terminal end of the protein. Like the amino-terminal ER signal peptides, the internal signal peptide is recognized by SRP, which brings the ribosome making the protein to the ER membrane and serves as a start-transfer signal that initiates the translocation of the protein. After release from the translocator, the internal start-transfer peptide remains in the lipid bilayer as a single membrane-spanning α helix. Internal start-transfer peptides, however, can bind to the translocation apparatus in either of two orientations, and the orientation of the inserted start-transfer peptide, in turn, determines which protein segment (the one preceding or the one following the start-transfer peptide) is moved across the membrane into the ER lumen. In one case the resulting membrane protein has its carboxyl terminus on the luminal side (Figure 12–45A), while in the other it has its amino terminus on the luminal side (Figure 12–45B). The orientation of the start-transfer peptide depends on the distribution of nearby charged amino acids, as described in the figure legend.

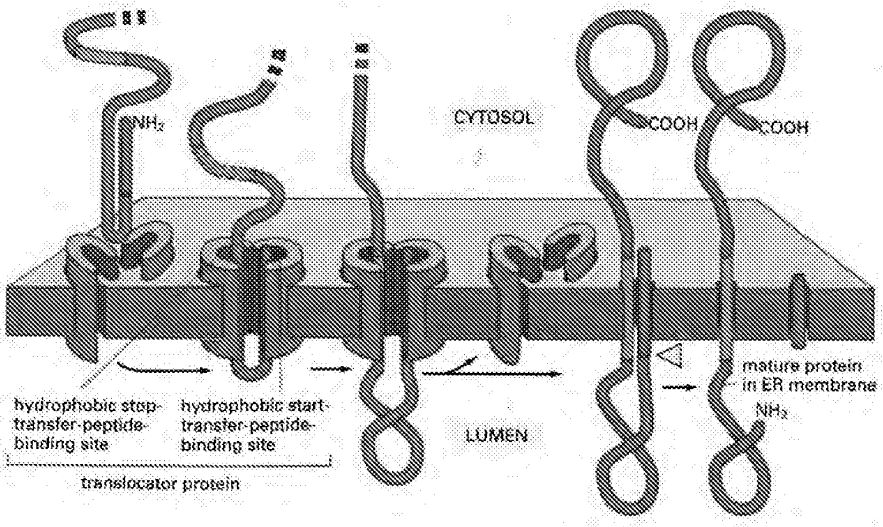


Figure 12–44 How a single-pass transmembrane protein with a cleaved ER signal peptide is integrated into the ER membrane. In this hypothetical model the co-translational translocation process is initiated by an amino-terminal ER signal peptide (*red*) that functions as a start-transfer signal as in Figure 12–43. In addition to the start-transfer peptide, however, the protein also contains a stop-transfer peptide (*orange*). When the stop-transfer peptide enters the translocator and interacts with a binding site, the translocator flips into its inactive state and discharges the protein laterally into the lipid bilayer.

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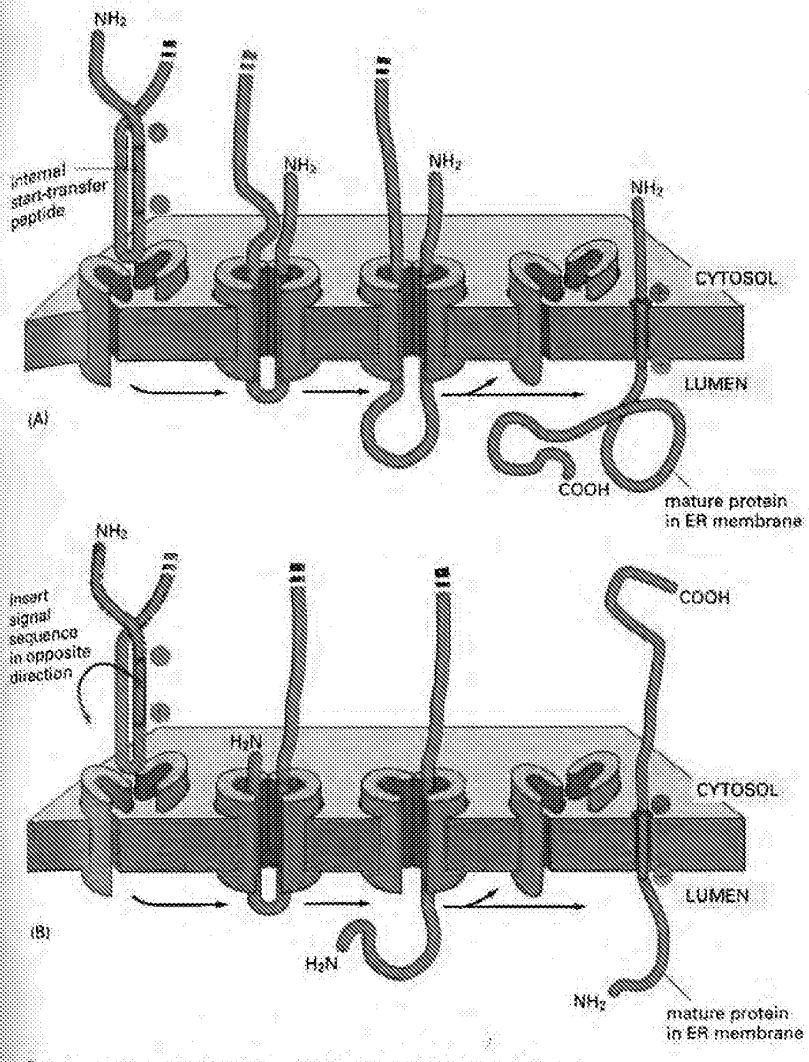


Figure 12–45 **How a single-pass membrane protein with an internal signal peptide is integrated into the ER membrane.** In this hypothetical model an internal ER signal peptide that functions as a start-transfer signal will bind to the translocator in such a way that its more positively charged end remains in the cytosol. If there are more positively charged amino acids immediately preceding the hydrophobic core of the start transfer peptide than there are following it on its carboxyl-terminal end, the start-transfer peptide will be inserted into the translocator in the orientation shown in (A), and the arm of the inserted loop carboxyl-terminal to the start-transfer sequence will be passed across the membrane. If, however, there are more positively charged amino acids immediately following the hydrophobic core of the start-transfer peptide than there are preceding it on its amino-terminal end, the start-transfer peptide will be inserted into the translocator in the orientation shown in (B), and the arm of the inserted loop amino-terminal to the start-transfer peptide will be passed across the membrane. Because translocation cannot start before a start-transfer sequence appears outside the ribosome, translocation of the amino-terminal portion of the protein shown in (B) can occur only after this portion has been fully synthesized. Note that there are two ways to insert a single-pass membrane-spanning protein whose amino terminus is located in the ER lumen: that shown in Figure 12–44 and that shown in (B) here.

Combinations of Start- and Stop-Transfer Signals Determine the Topology of Multipass Transmembrane Proteins [33]

In **multipass transmembrane proteins** the polypeptide chain passes back and forth repeatedly across the lipid bilayer (see Figure 10–13). It is thought that an internal signal peptide serves as a start-transfer signal in these proteins to initiate translocation, which continues until a stop-transfer peptide is reached. In double-pass transmembrane proteins, for example, the polypeptide is released into the bilayer at this point (Figure 12–46). In more complex multipass proteins, in which many hydrophobic α helices span the bilayer, a second start-transfer peptide terminates translocation further down the polypeptide chain until the next stop-transfer peptide causes polypeptide release, and so on for subsequent start-transfer and stop-transfer peptides (Figure 12–47).

Whether a given hydrophobic signal sequence will function as a start-transfer or stop-transfer peptide must depend on its location in a polypeptide chain, since its function can be switched by changing its location in the protein using recombinant DNA techniques. Thus the distinction between start-transfer and stop-transfer peptides results mostly from their relative order in the growing polypeptide chain. It seems that the SRP begins scanning an unfolded polypeptide chain for hydrophobic segments at its amino terminus and proceeds toward the car-

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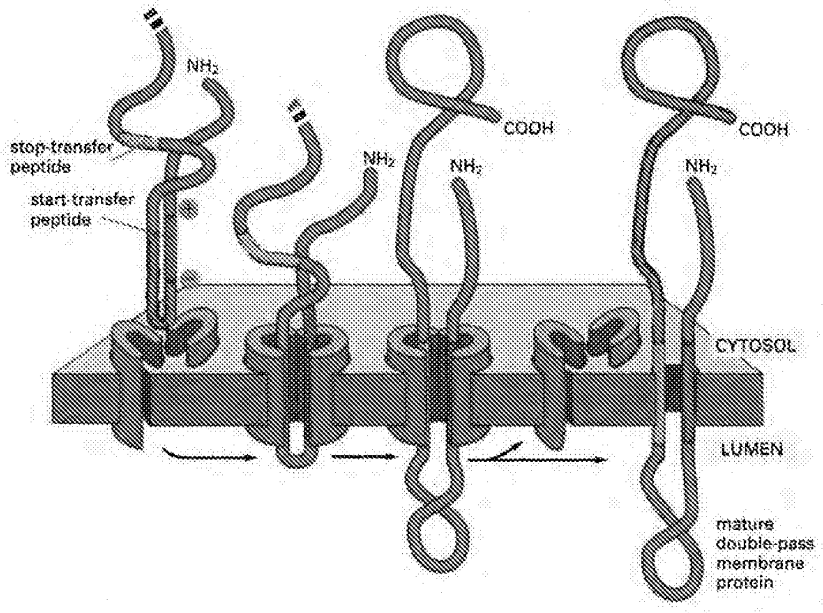


Figure 12–46 **How a double-pass membrane protein with an internal signal sequence is integrated into the ER membrane.** In this hypothetical model an internal ER signal peptide acts as a start-transfer signal (as in Figure 12–45) and initiates the transfer of the carboxyl terminal arm of the polypeptide chain. When a stop-transfer peptide enters the translocator, it discharges the protein laterally into the membrane.

boxyl terminus, in the direction that the protein is synthesized. By recognizing the first appropriate hydrophobic segment to emerge from the ribosome, the SRP sets the "reading frame"; if translocation is initiated, the next appropriate hydrophobic segment will be recognized as a stop-transfer peptide, causing the region of the polypeptide chain in between to be threaded across the membrane. A similar scanning process continues until all of the hydrophobic regions in the protein have been inserted into the membrane.

Because membrane proteins are always inserted from the cytosolic side of the ER in this programmed manner, all copies of the same polypeptide chain will have the same orientation in the lipid bilayer. This generates an asymmetrical ER membrane in which the protein domains exposed on one side are different from those domains exposed on the other. This asymmetry is maintained during the many membrane budding and fusion events that transport the proteins made in the ER to other cell membranes (discussed in Chapter 13). Thus the way in which a newly synthesized protein is inserted into the ER membrane determines the orientation of the protein in the other membranes as well.

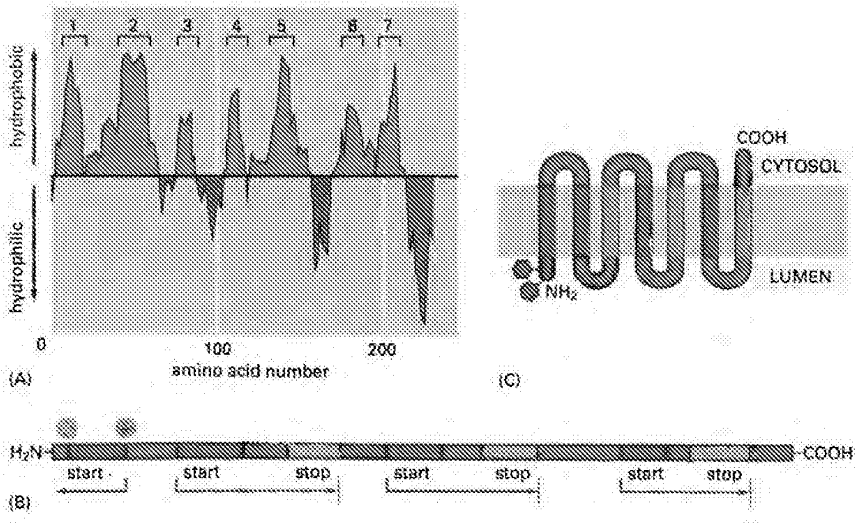


Figure 12–47 **The insertion of the multipass membrane protein rhodopsin into the ER membrane.** Rhodopsin is the light-sensitive protein in rod photoreceptor cells in the mammalian retina. (A) A hydrophobicity plot identifies seven short hydrophobic regions in rhodopsin. (B) The most amino-terminal region serves as a start-transfer peptide that causes the preceding amino-terminal portion of the protein to be passed across the ER membrane. Subsequent hydrophobic peptides will function in alternation as start-transfer and stop-transfer peptides. (C) The final integrated rhodopsin has its amino terminus located in the ER lumen and its carboxyl terminus located in the cytosol. The *blue hexagons* represent covalently attached oligosaccharides.

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When proteins are dissociated from a membrane and reconstituted in artificial lipid vesicles, a random mixture of right-side-out and inside-out protein orientations usually results. Thus the protein asymmetry observed in cell membranes seems not to be an inherent property of the protein but to result solely from the process by which proteins are inserted into the ER membrane from the cytosol.

Translocated Polypeptide Chains Fold and Assemble in the Lumen of the Rough ER [34]

Many of the proteins in the lumen of the ER are in transit, *en route* to other destinations; others, however, are normally resident there and are present at high concentrations. These **ER resident proteins** contain an **ER retention signal** of four amino acids at their carboxyl terminus that is responsible for retaining the protein in the ER (see Table 12–3). Some of these proteins function as catalysts that help the many proteins that are translocated into the ER to fold and assemble correctly. One such ER resident protein is *protein disulfide isomerase (PDI)*, which catalyzes the oxidation of free sulfhydryl (SH) groups to form disulfide (S—S) bonds. Almost all cysteine residues in protein domains exposed to either the extracellular space or the lumen of organelles in the secretory and endocytic pathways are disulfide bonded; disulfide bonds do not form, however, in domains exposed to the cytosol because of the reducing environment there.

Another ER resident protein is a chaperone protein known as **binding protein (BiP)**, which is structurally related to the hsp70 proteins and, like them, recognizes incorrectly folded proteins, as well as protein subunits that have not yet assembled into their final oligomeric complexes. BiP, like other chaperone proteins, is thought to bind to exposed amino acid sequences that would normally be buried in the interior of correctly folded or assembled polypeptide chains. The bound BiP both prevents the proteins from aggregating and helps to keep them in the ER (and thus out of the Golgi apparatus and later parts of the secretory pathway); it may also help them to fold normally. Like the hsp70 family of proteins, which bind unfolded proteins in the cytosol and facilitate their import into mitochondria and chloroplasts, BiP hydrolyzes ATP to provide the energy for its role in protein folding.

As we have seen earlier for mitochondrial hsp70 (see Figure 12–24), the binding of BiP to an unfolded protein chain emerging in the ER lumen may help pull the protein into the ER. This pulling process may be particularly important for proteins that enter the ER posttranslationally because in this case there is no ribosome attached to the membrane to help push the nascent protein through the translocator during the protein's synthesis.

Most Proteins Synthesized in the Rough ER Are Glycosylated by the Addition of a Common *N*-linked Oligosaccharide [35]

The covalent addition of sugars to proteins is one of the major biosynthetic functions of the ER. Most of the soluble and membrane-bound proteins that are made in the ER, including those destined for transport to the Golgi apparatus, lysosomes, plasma membrane, or extracellular space are **glycoproteins.** In contrast, very few proteins in the cytosol are glycosylated, and those that are carry a much simpler sugar modification in which a single *N*-acetylglucosamine group is added to a serine or threonine residue of the protein.

An important advance in understanding the process of **protein glycosylation** was the discovery that a preformed oligosaccharide (composed of *N*-acetylglucosamine, mannose, and glucose and containing a total of 14 sugar residues) is transferred *en bloc* to proteins in the ER. Because this oligosaccharide is transferred to the side-chain NH_2 group of an asparagine amino acid in the protein, it is said to be *N-linked* or *asparagine-linked* (Figure 12–48). The transfer is cata-

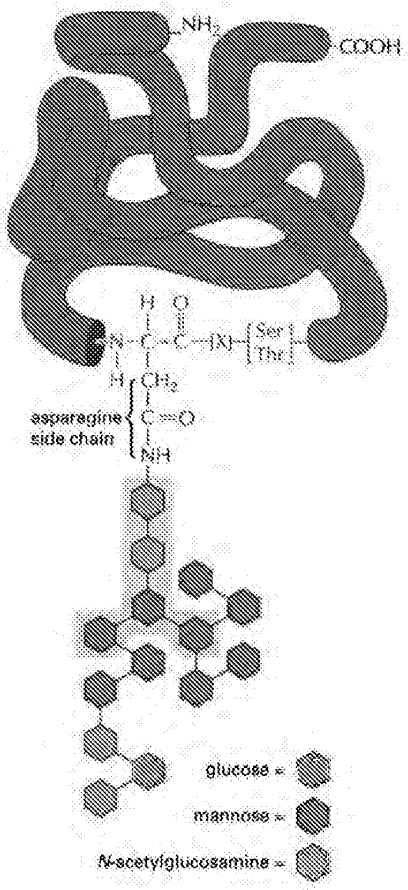


Figure 12–48 **The asparagine-linked (*N*-linked) oligosaccharide that is added to most proteins in the rough ER membrane.** The five sugar residues in the *gray box* form the "core region" of this oligosaccharide. For many glycoproteins only the core sugars survive the extensive oligosaccharide trimming process that takes place in the Golgi apparatus. Only asparagines in the sequences *Asn-X-Ser* or *Asn-X-Thr* (where X is any amino acid except proline) become glycosylated. These two sequences occur much less frequently in glycoproteins than in nonglycosylated cytosolic proteins; evidently there has been selective pressure against these sequences during protein evolution, presumably because glycosylation at too many sites would interfere with protein folding.

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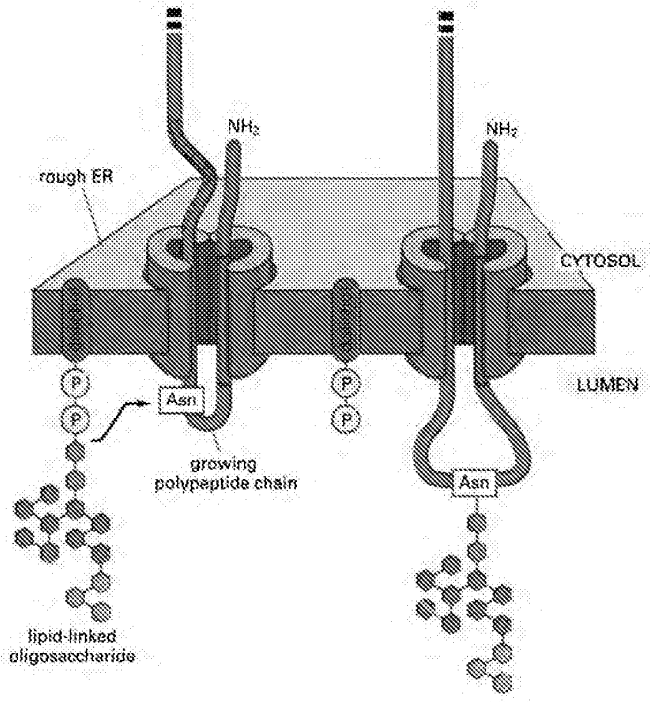


Figure 12–49 **Protein glycosylation in the rough ER.** Almost as soon as a polypeptide chain enters the ER lumen, it is glycosylated on target asparagine amino acids. The oligosaccharide shown in Figure 12–48 is transferred to the asparagine as an intact unit in a reaction catalyzed by a membrane-bound *oligosaccharyl transferase* enzyme. There is one copy of this enzyme associated with each protein translocator in the ER membrane.

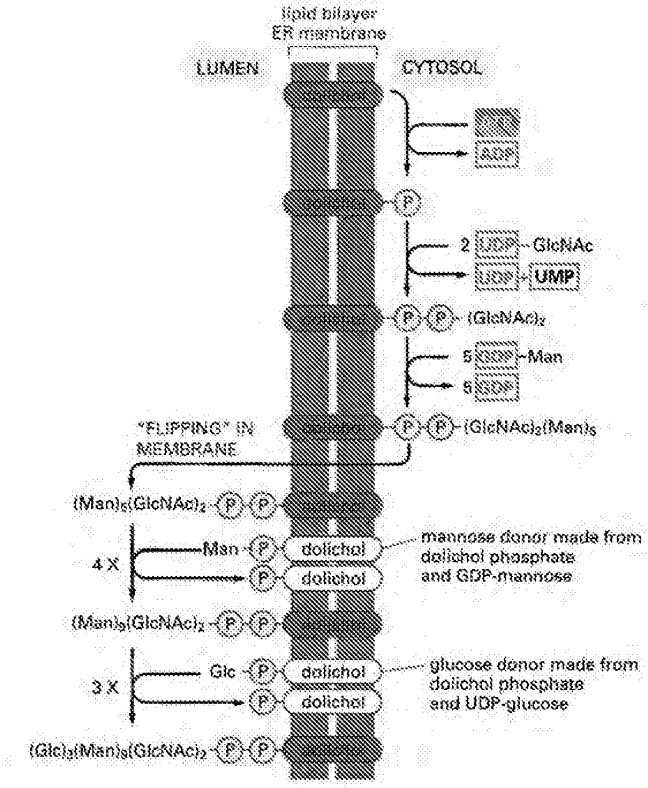


Figure 12–50 **Synthesis of the lipid-linked precursor oligosaccharide in the rough ER membrane.** The oligosaccharide is assembled sugar by sugar onto the carrier lipid dolichol (a polyisoprenoid—see Panel 2–4). Dolichol is long and very hydrophobic: its 22 five-carbon units can span the thickness of a lipid bilayer more than three times, so that the attached oligosaccharide is firmly anchored in the membrane. The first sugar group is linked to dolichol by a pyrophosphate bridge. This high-energy bond activates the oligosaccharide for its transfer from the lipid to an asparagine side chain of a nascent polypeptide on the luminal side of the rough ER. The synthesis of the oligosaccharide starts on the cytosolic side of the ER membrane and continues on the luminal face after the $(Man)_5(GlcNAc)_2$ lipid intermediate is flipped across the bilayer. All of the subsequent glycosyl transfer reactions on the luminal side of the ER involve transfers from dolichol-P-glucose and dolichol-P-mannose; these activated lipid-linked monosaccharides are synthesized from dolichol phosphate and UDP-glucose or GDP-mannose (as appropriate) on the cytosolic side of the ER and are then thought to be flipped across the ER membrane. GlcNAc = *N*-acetylglucosamine; Man = mannose; Glc = glucose.

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yzed by a membrane-bound enzyme, an *oligosaccharyl transferase*, which has its active site exposed on the luminal side of the ER membrane; this explains why cytosolic proteins are not glycosylated in this way. The precursor oligosaccharide is held in the ER membrane by a special lipid molecule called **dolichol**, and it is transferred to the target asparagine in a single enzymatic step immediately after that amino acid emerges in the ER lumen during protein translocation (Figure 12–49). Since most proteins are co-translationally imported into the ER, *N*-linked oligosaccharides are almost always added during protein synthesis.

The lipid-linked precursor oligosaccharide is linked to the dolichol by a high-energy pyrophosphate bond, which provides the activation energy that drives the glycosylation reaction illustrated in Figure 12–49. The entire oligosaccharide is built up sugar by sugar on this membrane-bound lipid molecule prior to its transfer to a protein. The sugars are first activated in the cytosol by the formation of *nucleotide-sugar intermediates*, which then donate their sugar (directly or indirectly) to the lipid in an orderly sequence. Partway through this process, the lipid-linked oligosaccharide is flipped from the cytosolic to the luminal side of the ER membrane (Figure 12–50).

All of the diversity of the *N*-linked oligosaccharide structures on mature glycoproteins results from later modification of the original precursor structure. While still in the ER, three glucose residues (see Figure 12–48) and one mannose residue are quickly removed from the oligosaccharides of most glycoproteins. This oligosaccharide "trimming" or "processing" continues in the Golgi apparatus and is discussed in Chapter 13.

The *N*-linked oligosaccharides are by far the most common ones found on glycoproteins. Less frequently, oligosaccharides are linked to the hydroxyl group on the side chain of a serine, threonine, or hydroxylysine amino acid. These *O-linked oligosaccharides* are formed in the Golgi apparatus by pathways that are not yet fully understood (discussed in Chapter 13).

Some Membrane Proteins Exchange a Carboxyl-Terminal Transmembrane Tail for a Covalently Attached Glycosylphosphatidylinositol (GPI) Anchor After Entry into the ER [36]

As discussed in Chapter 10, several cytosolic enzymes catalyze the covalent addition of a single fatty acid chain or prenyl group to selected proteins to help direct these proteins to cell membranes. A related process is catalyzed by enzymes in the rough ER: the carboxyl terminus of some membrane proteins destined for the plasma membrane is covalently attached to a sugar residue of a glycolipid. This linkage forms in the lumen of the ER by the mechanism illustrated in Figure 12–51, and it adds a **glycosylphosphatidylinositol (GPI) anchor,** which

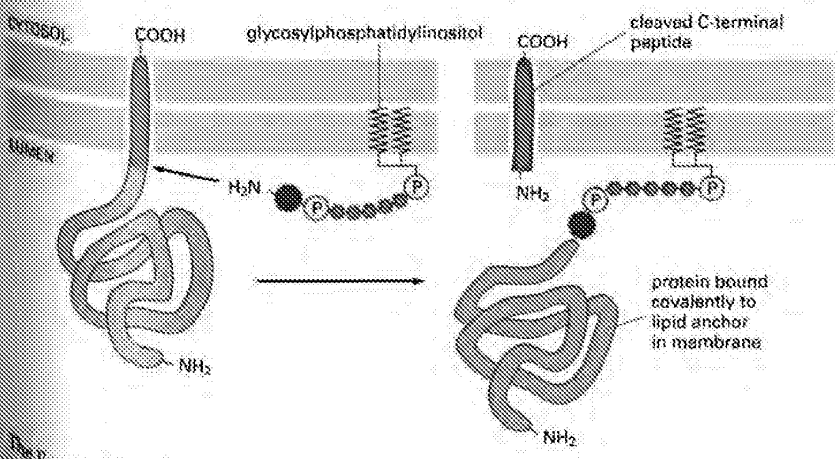


Figure 12–51 **The attachment of a glycosylphosphatidylinositol anchor.** Immediately after the completion of protein synthesis, the precursor protein remains anchored in the ER membrane by a hydrophobic carboxyl-terminal sequence of 15 to 20 amino acids, with the rest of the protein in the ER lumen. Within less than a minute, an enzyme in the ER cuts the protein free from its membrane-bound carboxyl terminus and simultaneously attaches the new carboxyl terminus to an amino group on a preassembled glycosylphosphatidylinositol intermediate. The signal that specifies this modification is contained within the hydrophobic carboxyl-terminal sequence and a few amino acids adjacent to it on the luminal side of the ER membrane; if this signal is added to other proteins, they too become modified in this way. Because of the covalently linked lipid anchor, the protein remains membrane-bound with all of its amino acids exposed initially on the luminal side of the ER and eventually on the cell exterior.

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contains two fatty acids, to the protein. At the same time the transmembrane segment of the protein is cleaved off. An increasing number of plasma membrane proteins have been shown to be modified in this way. Since these proteins are attached to the exterior of the plasma membrane only by their GPI anchors, in principle they can be released from cells in soluble form in response to signals that activate a specific phospholipase in the plasma membrane. Trypanosome parasites, for example, use this mechanism to shed their coat of GPI-anchored surface proteins if attacked by the immune system.

Most Membrane Lipid Bilayers Are Assembled in the ER [37]

The ER membrane produces nearly all of the lipids required for the elaboration of new cell membranes, including both phospholipids and cholesterol. The major phospholipid made is *phosphatidylcholine* (also called lecithin), which can be formed in three steps from choline, two fatty acids, and glycerol phosphate. Each step is catalyzed by enzymes in the ER membrane that have their active sites facing the cytosol, where all of the required metabolites are found. Thus phospholipid synthesis occurs exclusively in the cytosolic half of the ER bilayer. In the first step acyl transferases successively add two fatty acids to glycerol phosphate to produce phosphatidic acid, a compound sufficiently water-insoluble to remain in the lipid bilayer after it has been synthesized. It is this step that enlarges the lipid bilayer; the later steps determine the head group of a newly formed lipid molecule, and therefore the chemical nature of the bilayer, but do not result in net membrane growth (Figure 12–52). The two other major membrane phospholipids—phosphatidylethanolamine (PE) and phosphatidylserine (PS)—as well as the minor phospholipid phosphatidylinositol (PI), are all synthesized in this way.

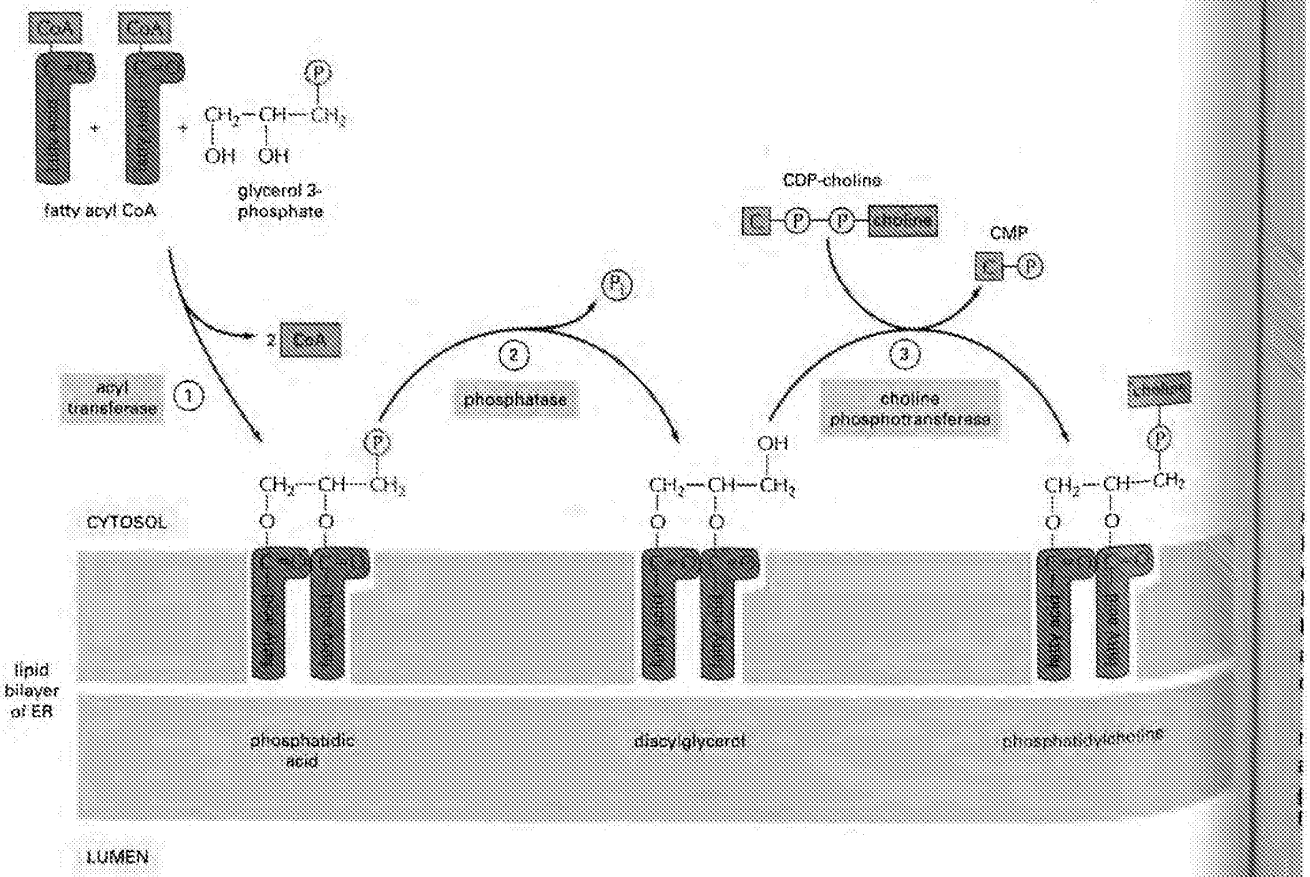


Figure 12–52 **The synthesis of phosphatidylcholine.** This phospholipid is synthesized from fatty acyl-coenzyme A (fatty acyl CoA), glycerol 3-phosphate, and cytidine bisphosphocholine (CDP-choline).

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As phospholipid synthesis takes place in the cytosolic half of the ER bilayer, there needs to be a mechanism that transfers some of the newly formed phospholipid molecules to the other half of the bilayer. In synthetic lipid bilayers, lipids do not "flip-flop" in this way. In the ER, however, phospholipids equilibrate across the membrane within minutes, which is almost 100,000 times faster than can be accounted for by spontaneous "flip-flop." This rapid transbilayer movement is thought to be mediated by **phospholipid translocators** that are head-group-specific. In particular, the ER membrane seems to contain a translocator (a "*flippase*") that transfers choline-containing phospholipids—but not ethanolamine-, serine-, or inositol-containing phospholipids—between cytosolic and luminal faces. This means that phosphatidylcholine reaches the luminal face much more readily than the other phospholipids. In this way the translocator is responsible for the asymmetric distribution of the lipids in the bilayer (Figure 12–53).

The ER also produces cholesterol and ceramide. *Ceramide* is made by condensing the amino acid serine with a fatty acid to form the amino alcohol *sphingosine;* a second fatty acid is then added to form ceramide. The ceramide is exported to the Golgi apparatus, where it serves as the precursor for the synthesis of two types of lipids: oligosaccharide chains are added to form *glycosphingolipids* (glycolipids), and phosphocholine head groups are transferred from phosphatidylcholine to other ceramide molecules to form *sphingomyelin.* Thus both glycolipids and sphingomyelin are produced relatively late in the process of membrane synthesis. Because they are produced by enzymes exposed to the Golgi lumen, they are found exclusively in the noncytosolic half of the lipid bilayers that contain them.

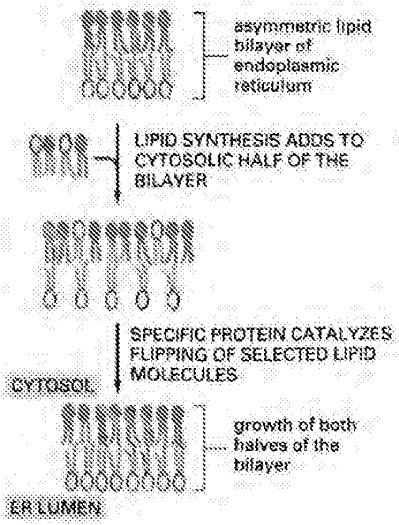


Figure 12–53 **The role of phospholipid translocators in lipid bilayer synthesis.** Since new lipid molecules are added only to the cytosolic half of the bilayer and lipid molecules do not flip spontaneously from one monolayer to the other, membrane-bound phospholipid translocator proteins ("flippases") are required to transfer selected lipid molecules from the cytosolic half to the luminal half so that the membrane grows as a bilayer. Because the flippase in the ER membrane preferentially recognizes and transfers choline-containing head groups, an asymmetric bilayer is generated, with the luminal monolayer (which produces the outer half of the plasma membrane bilayer) highly enriched for phosphatidylcholine.

Phospholipid Exchange Proteins Help Transport Phospholipids from the ER to Mitochondria and Peroxisomes [38]

As discussed in Chapter 13, the plasma membrane and the membranes of the Golgi apparatus, lysosomes, and endosomes all form part of a membrane system that communicates with the ER by means of transport vesicles that transfer both proteins and lipids. Mitochondria, plastids, and peroxisomes do not belong to this system, and they require different mechanisms for the import of proteins and lipids for growth. We have already seen that most (for mitochondria and plastids) or all (for peroxisomes) of the proteins in these organelles are imported from the cytosol. Although mitochondria modify some of the lipids they import, they do not synthesize lipids *de novo;* instead, their lipids have to be imported from the ER, either directly, or indirectly by way of other cellular membranes. In either case, special mechanisms are required for the transfer.

Water-soluble carrier proteins—called **phospholipid exchange proteins** (or *phospholipid transfer proteins*)—have been shown in *in vitro* experiments to have the ability to transfer individual phospholipid molecules between membranes. Each exchange protein recognizes only specific types of phospholipids. It functions by "extracting" a molecule of the appropriate phospholipid from a membrane and diffusing away with the lipid buried within its binding site. When it encounters another membrane, the exchange protein tends to discharge the bound phospholipid molecule into the new lipid bilayer (Figure 12–54). It has been proposed that phosphatidylserine is imported into mitochondria in this way and then decarboxylated to yield phosphatidylethanolamine, while phosphatidylcholine is imported intact.

Exchange proteins act to distribute phospholipids at random among all membranes present. In principle, such a random exchange process can result in a net transport of lipids from a lipid-rich to a lipid-poor membrane, allowing phosphatidylcholine and phosphatidylserine molecules, for example, to be transferred from the ER, where they are synthesized, to a mitochondrial or peroxisomal

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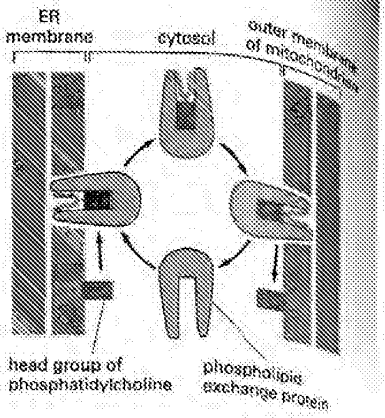


Figure 12–54 **Phospholipid exchange proteins.** Because phospholipids are insoluble in water, their passage between membranes requires a carrier protein. Phospholipid exchange proteins are water-soluble proteins that carry a single molecule of phospholipid at a time; they can pick up a lipid molecule from one membrane and release it at another and thereby redistribute phospholipids between membrane-bounded compartments. The transfer of phosphatidylcholine (PC) from ER to mitochondria can occur without the input of additional energy because the concentration of PC is high in the ER membrane (where it is made) and low in the mitochondrial outer membrane. One would predict that there must be a flippase in the outer mitochondrial membrane to equilibrate the lipids between the two leaflets of the bilayer, and there must be a mechanism to transfer lipids between the outer and inner mitochondrial membrane. These postulated pathways, however, remain to be discovered.

membrane. It might be that mitochondria and peroxisomes are the only "lipid-poor" organelles in the cytosol and that such an exchange process is sufficient, although other, more specific mechanisms probably also exist for transporting phospholipids to these organelles.

Summary

The extensive ER network serves as a factory for the production of almost all of the cell's lipids. In addition, a major portion of the cell's protein synthesis occurs on the cytosolic surface of the ER: all proteins destined for secretion and all proteins destined for the ER itself, the Golgi apparatus, the lysosomes, the endosomes, and the plasma membrane are first imported into the ER from the cytosol. In the ER lumen, the proteins fold and oligomerize, disulfide bonds are formed, and N-linked oligosaccharides are added.

Only proteins that carry a special hydrophobic signal peptide are imported into the ER. The ER signal peptide is recognized by a signal recognition particle (SRP), which binds both the growing polypeptide chain and the ribosome and directs them to a receptor protein on the cytosolic surface of the rough ER membrane. This binding to the membrane initiates the translocation process that threads a loop of polypeptide chain across the ER membrane through a hydrophilic pore in a protein translocator.

Soluble proteins destined for the ER lumen, for secretion, or for transfer to the lumen of other organelles pass completely into the ER lumen. Transmembrane proteins destined for the ER or for other cell membranes are translocated across the ER membrane but are not released into the lumen; instead, they remain anchored in the lipid bilayer by one or more membrane-spanning α-helical regions in their polypeptide chain. These hydrophobic portions of the protein can act either as start-transfer or stop-transfer signals during the translocation process. When a polypeptide contains multiple alternating start-transfer and stop-transfer signals, it will pass back and forth across the bilayer multiple times.

The asymmetry of lipid synthesis, protein insertion, and glycosylation in the ER establishes the polarity of the membranes of all of the other organelles that the ER supplies with lipids and membrane proteins.

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D20 Table 1 and D21 Table 1

Antibody	Type	Ab concentration mg/ml	Formulation
Satumomab pendetide	IgG1k (murine, conjugated to GYK-DTPA)	0.5	PBS
Indium-111 capromab pendetid	IgG1k (murine, conjugated to GYK-DTPA)	0.5	PBS
Muromomab-CD3	**IgG2a (murine)**	**1**	**Monobasic Na P, dibasic Na P, NaCl, PS80**
I-131 tositumab	**IgG2 gamma (murine)**	**1.1**	**Phosphate, povidone, maltose, NaCl, ascorbic acid**
Ibritumomab-Tiuxetan	IgG1k (murine, thiourea covalent linkage to Tiuxetan)	1.6	Na acetate, K P, NaCl
Cetuximab	IgG1k (chimeric human/mouse)	2	Dibasic Na P septahydrate, monobasic Na P monohydrate, NaCl
Abciximab	Fab, IgG1 (chimeric human/murine)	2	Na P, NaCl, PS80
Daclizimab	IgG1 (humanized)	5	Monobasic Na P monohydrate, dibasic Na P septahydrate, NaCl, PS80
Alemtuzumab	IgG1k (humanized)	10	Dibasic Na P, monobasic K P, NaCl, KCl, Na2EDTA, PS80
Ranibizumab	IgG1k (humanized)	10	His HCl, alpha-trehalose dihydrate, PS20
Rituximab	IgG1k (chimeric mouse/human)	10	Na citrate dihydrate, NaCl, PS80
Nofetumomab	Fab (murine)	10	PBS
Tositumomab	**IgG2 gamma (murine)**	**14**	**Phosphate, NaCl, maltose**
Natalizumab	**IgG4k (humanized)**	**20**	**Monobasic Na P, dibasic NaP septahydrate, NaCl, PS80**
Bevacizumab	IgG1 (humanized)	25	Monobasic Na P hydrate, dibasic Na P anhydrous, alpha-trehalose dihydrate, PS 20
Adalimumab	IgG1k (human)	50	Monobasic Na P dihydrate, dibasic Na P dihydrate, Na citrate, citric acid hydrate, NaCl, mannitol, PS80
Acrituomab	Murine Fab	powder	Stannous chloride, K Na tartrate tetrahydrate, Na acetate trihydrate, NaCl, glacial acetic acid, HCl, sucrose
Gemtuzumab ozogamicin	**IgG4k (conjugated with alicheamicin)**	**powder**	**Monobasic and dibasic Na P, dextran 40, sucrose, NaCl**
Basiliximab	IgG1k (chimeric)	powder	Monobasic K P, Na2HPO4, NaCl, sucrose, mannitol, glycine, PS80
Infliximab	IgG1 (chimeric human/murine)	powder	Monobasic Na P monohydrate, dibasic Na P dihydrate, sucrose, PS80
Trastuzumab	IgG1k (humanized)	powder	L-His HCl, L-His, alpha trehalose dihydrate, PS20 (benzyl alcohol as preservative)
Efalizumab*	IgG1 (humanized)	powder	L-His HCl monohydrate, L-His, sucrose, PS20
Palivizumab	IgG1k (humanized, CDR of murine Mab)	powder	His, Gly, mannitol
Omalizumab	IgG1k (humanized)	powder	L-His HCl, L-His, sucrose, PS20
Eculizumab	**IgG2/4**	**10 mg/ml solution**	**Na P, NaCl, PS80**

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28/11/2017

Medicine Name	Active Substance	Status	Authorisation date	Biosimilar	Product concentration mg/ml
Zevalin	ibritumomab tiuxetan	Authorised	16/01/2004	no	1.6
Dinutuximab beta EUSA (previously Dinutuximab beta Apeiron)	dinutuximab beta	Authorised	08/05/2017	no	4.5
Erbitux	cetuximab	Authorised	29/06/2004	no	5
Yervoy	ipilimumab	Authorised	13/07/2011	no	5
Stelara	ustekinumab	Authorised	16/01/2009	no	5
Lemtrada	alemtuzumab	Authorised	12/09/2013	no	10
Soliris	eculizumab	Authorised	20/06/2007	no	10
Opdivo	nivolumab	Authorised	19/06/2015	no	10
Lartruvo	olaratumab	Authorised	09/11/2016	no	10
Cyramza	ramucirumab	Authorised	19/12/2014	no	10
Lucentis	ranibizumab	Authorised	22/01/2007	no	10
Cinqaero	reslizumab	Authorised	16/08/2016	no	10
MabThera	rituximab	Authorised	02/06/1998	no	10
Portrazza	necitumumab	Authorised	15/02/2016	no	16
Bavencio	avelumab	Authorised	18/09/2017	no	20
Darzalex	daratumumab	Authorised	20/05/2016	no	20
Tysabri	natalizumab	Authorised	27/06/2006	no	20
Arzerra	ofatumumab	Authorised	19/04/2010	no	20
Vectibix	panitumumab	Authorised	03/12/2007	no	20
RoActemra	tocilizumab	Authorised	16/01/2009	no	20
Avastin	bevacizumab	Authorised	12/01/2005	no	25
Zinplava	bezlotoxumab	Authorised	18/01/2017	no	25

Gazyvaro	obinutuzumab	Authorised	23/07/2014	no	25
Perjeta	pertuzumab	Authorised	04/03/2013	no	30
Humira	adalimumab	Authorised	08/09/2003	no	50
Praxbind	idarucizumab	Authorised	20/11/2015	no	50
Tecentriq	atezolizumab	Authorised	21/09/2017	no	60
Prolia	denosumab	Authorised	26/05/2010	no	60
Xgeva	denosumab	Authorised	13/07/2011	no	70
Taltz	ixekizumab	Authorised	25/04/2016	no	80
Simponi	golimumab	Authorised	01/10/2009	no	100
Kyntheum	brodalumab	Authorised	17/07/2017	no	140
Repatha	evolocumab	Authorised	17/07/2015	no	140
Zinbryta	daclizumab	Authorised	01/07/2016	no	150
Dupixent	dupilumab	Authorised	27/09/2017	no	150
Cimzia	certolizumab pegol	Authorised	01/10/2009	no	200
Kevzara	sarilumab	Authorised	23/06/2017	no	131.6 or 175
Praluent	alirocumab	Authorised	23/09/2015	no	75 or 150
Simulect	basiliximab	Authorised	09/10/1998	no	powder
Benlysta	belimumab	Authorised	13/07/2011	no	powder
Scintimun	besilesomab	Authorised	11/01/2010	no	powder
Blincyto	blinatumomab	Authorised	23/11/2015	no	powder
Adcetris	brentuximab vedotin	Authorised	25/10/2012	no	powder
Ilaris	canakinumab	Authorised	23/10/2009	no	powder
Empliciti	elotuzumab	Authorised	11/05/2016	no	powder
Remicade	infliximab	Authorised	13/08/1999	no	powder
Besponsa	inotuzumab ozogamicin	Authorised	29/06/2017	no	powder
Nucala	mepolizumab	Authorised	02/12/2015	no	powder
Xolair	omalizumab	Authorised	25/10/2005	no	powder
Synagis	palivizumab	Authorised	13/08/1999	no	powder
Keytruda	pembrolizumab	Authorised	17/07/2015	no	powder
Cosentyx	secukinumab	Authorised	15/01/2015	no	powder
Sylvant	siltuximab	Authorised	22/05/2014	no	powder
LeukoScan	sulesomab	Authorised	14/02/1997	no	powder
Herceptin	trastuzumab	Authorised	28/08/2000	no	powder
Kadcyla	trastuzumab emtansine	Authorised	15/11/2013	no	powder
Entyvio	vedolizumab	Authorised	22/05/2014	no	powder

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET – Page 1 of 2

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. ED 481024650 US

112987 U.S.PTO 60/783070 031506

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Leonard	Bell	Woodbridge, Connecticut
Russell P.	Rother	Prospect, Connecticut

Additional inventors are being named on the _____ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max):

IMPROVEMENT IN QUALITY OF LIFE OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS TREATED WITH AN INHIBITOR OF COMPLEMENT

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☐ Application Data Sheet. See 37 CFR 1.76
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☑ Specification *Number of Pages* 32
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SIGNATURE Stephen A. Saxe Date March 15, 2006

TYPED or PRINTED NAME Stephen A. Saxe REGISTRATION NO. 38,609 *(if appropriate)*

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FEE TRANSMITTAL
For FY 2006

☑ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 100.00

Complete if Known	
Application Number	Not yet assigned
Filing Date	March 15, 2006
First Named Inventor	Leonard Bell
Examiner Name	Not yet assigned
Art Unit	N/A
Attorney Docket No.	114 CIP2 PRO

METHOD OF PAYMENT (check all that apply)

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1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
		Small Entity		Small Entity		Small Entity	
Application Type	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fees Paid ($)
Utility	300	150	500	250	200	100	
Design	200	100	100	50	130	65	
Plant	200	100	300	150	160	80	
Reissue	300	150	500	250	600	300	
Provisional	200	100	0	0	0	0	100.00

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims		Extra Claims		Fee ($)		Fee Paid ($)
_____	- 20 or HP =	_____	x	_____	=	0

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims		Extra Claims		Fee ($)		Fee Paid ($)
_____	- 3 or HP =	_____	x	_____	=	0

HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims	
Fee ($)	Fee Paid ($)
_____	0

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

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36	- 100 =	0	/ 50 =	0	(round up to a whole number) x	_____	=	0

4. OTHER FEE(S)

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Non-English Specification, $130 fee (no small entity discount)	0
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SUBMITTED BY

Signature	Stephen A. Saxe	Registration No. (Attorney/Agent) 38,609	Telephone 203-271-8289
Name (Print/Type)	Stephen A. Saxe		Date March 15, 2006

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Total Number of Pages in This Submission: 41

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First Named Inventor	Leonard Bell
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ENCLOSURES *(Check all that apply)*

- [✓] Fee Transmittal Form
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Provisional Application for Patent Cover Sheet (2 pages)
Provisional Application (36 pages)
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Remarks

Total pages (41) equals 32 pages of specification, 4 sheets of drawings, 2 pages of Provisional Application for Patent Cover Sheet, 1 page of Fee Transmittal, 1 page of Transmittal Form and 1 page of Certificate of Express Mailing. Additionally, a Return Receipt Postcard is enclosed.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	Stephen A. Saxe, Alexion Pharmaceuticals, Inc.		
Signature	Stephen a. Saxe		
Printed name	Stephen A. Saxe		
Date	March 15, 2006	Reg. No.	38,609

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Docket No.: 114 CIP2 PRO

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Leonard BELL and Russell P. ROTHER		
Serial No.:	Unassigned	Examiner:	Unassigned
Filed:	March 15, 2006	Art Unit:	Unassigned

For: **IMPROVEMENT IN QUALITY OF LIFE OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS TREATED WITH AN INHIBITOR OF COMPLEMENT**

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Stephen A. Saxe

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IMPROVEMENT IN QUALITY OF LIFE OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS TREATED WITH AN INHIBITOR OF COMPLEMENT

BACKGROUND

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematologic disease that results from clonal expansion of hematopoietic stem cells with somatic mutations in the X-linked gene called PIG-A.[1,2] Mutations in PIG-A lead to an early block in the synthesis of glycosylphosphatidylinositol (GPI)-anchors, which are required to tether many proteins to the cell surface. Consequently, PNH blood cells have a partial (type II) or complete (type III) deficiency of GPI-anchored proteins.

Intravascular hemolysis is a prominent feature of PNH and a direct result of the absence of the GPI-anchored complement regulatory protein CD59.[3,4] Under normal circumstances, CD59 blocks the formation of the terminal complement complex (also called the membrane attack complex) on the cell surface, thereby preventing erythrocyte lysis and platelet activation.[5-8] Excessive or persistent intravascular hemolysis in PNH patients not only results in anemia (normal ranges of hemoglobin are 14-18 g/dL for men and 12-16 g/dL for women, and persons with lower levels are considered to be anemic), but also hemoglobinuria and clinical sequelae related to the release of the erythrocyte contents into the circulation: fatigue, thrombosis, abdominal pain, dysphagia, erectile dysfunction, and pulmonary hypertension.[9,10,21,22] Indeed, impaired quality of life in PNH is disproportionate to the degree of anemia. Many PNH patients depend on blood transfusions to maintain adequate erythrocyte hemoglobin levels. There have been no therapies that effectively reduce intravascular hemolysis and improve the associated clinical morbidities in PNH.

Eculizumab is a humanized monoclonal antibody directed against the terminal complement protein C5.[11] In a preliminary, 12-week, open-label clinical study in 11 PNH patients, eculizumab was shown to reduce intravascular hemolysis and transfusion requirements.[12] However, this unblinded study involved a small number of patients with no control arm and without protocol-driven transfusion standards.

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SUMMARY

The present pivotal, phase III study, Transfusion Reduction Efficacy and Safety Clinical Investigation, Randomized, Multi-Center, Double-Blind, Placebo-Controlled, Using Eculizumab in Paroxysmal Nocturnal Hemoglobinuria (TRIUMPH), evaluated the effect of eculizumab on the stabilization of hemoglobin levels and transfusion requirements during 6 months of treatment in a cohort of 87 transfusion-dependent PNH patients. Measures of intravascular hemolysis and quality of life were also assessed. This is the first placebo controlled study of a PNH patient population to control hemolysis and to differentiate between the effects due to hemolysis and the effects due to anemia.

It has been surprisingly discovered that certain aspects of quality of life were unexpectedly improved by the treatment of PNH patients with eculizumab. Furthermore, these improvements in the quality of life were independent of transfusion. The improved aspects include, e.g., global health status, physical functioning, emotional functioning, cognitive functioning, role functioning, social functioning, fatigue, pain, dyspnea, appetite loss and insomnia. Improvement was also seen in nausea and vomiting, diarrhea, constipation, and financial difficulties but did not reach the level of statistical significance. Because the treated patients remained anemic throughout their treatment, it was unexpected that all of these improvements would have been seen because they were previously thought to be a result of the patient being anemic. Although not wishing to be bound by any theory, it appears that some of the symptoms are likely due, at least in part, to hemolysis and release of hemoglobin into the bloodstream and do not result solely from the patient being anemic. The treatment with eculizumab decreases the amount of lysis thereby limiting hemoglobin release into the bloodstream, thereby apparently resulting in the improvements seen in the treated patients' quality of life. The results presented herein indicate that any treatment that decreases hemolysis in a patient will result in an improvement in the quality of life of said patient.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-B show that eculizumab treatment decreases intravascular hemolysis and increases PNH type III erythrocytes. Figure 1A shows the degree of intravascular hemolysis in PNH patients, demonstrated by mean lactate dehydrogenase (LDH) levels.

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Figure 1B shows the mean proportion of PNH type III erythrocytes assessed for placebo- and eculizumab-treated patients.

Figure 2 shows the effect of eculizumab treatment on transfusion requirements in PNH patients. This is a Kaplan-Meier plot of time to first transfusion for eculizumab- and placebo-treated patients from baseline through week 26. The P value is from the log rank analysis.

Figure 3 shows the effect of eculizumab on fatigue assessed by the FACIT-Fatigue Instrument. Quality of Life scores were assessed using the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) instrument. Values for change from baseline to 26 weeks represent least-square means. A positive change indicates an improvement and a negative change indicates deterioration in the FACIT-Fatigue measures of quality of life.

DETAILED DESCRIPTION

The present disclosure relates to a method of treating paroxysmal nocturnal hemoglobinuria ("PNH"), more specifically to improving certain aspects of quality of life which are impaired in PNH patients, 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

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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 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.

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.

The complement system, useful complement inhibitors, and use of these inhibitors to treat PNH and other patients are more fully described in PCT Patent Application PCT/US2005/003225 filed February 3, 2005 and published as International Publication Number WO 2005/074607 A2 on August 18, 2005, the contents of which are incorporated herein by reference in their entirety.

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I. METHODS

Patient Selection

The TRIUMPH trial consisted of a 2-week screening period, an observation period of up to 3 months duration, and a 26-week treatment period.

During the screening period, patients were evaluated with respect to inclusion and exclusion criteria. Men and women, 18 years or older, diagnosed as having PNH with a type III erythrocyte population of ≥10%, and who had received at least 4 transfusions in the previous 12 months were eligible. Concomitant administration of erythropoietin, immunosuppressants, corticosteroids, coumadin, low molecular weight heparin, iron supplements, and folic acid were not reasons for exclusion, provided the doses were steady prior to the first visit and throughout the duration of the study. Because of the increased frequency of neisserial infections in individuals genetically deficient in terminal complement proteins, all patients were vaccinated against *Neisseria meningitides*. Patients were to avoid conception. The protocol was approved by an Investigational Review Board at each clinical site and written informed consent was obtained from all patients enrolled.

Patients transfused with a mean pre-transfusion hemoglobin level >10.5 g/dL over the previous 12 months, and those who showed evidence of having a suppressed immune response, complement deficiency, or active bacterial infection, including any history of meningococcal disease, were excluded from the study. Patients were also not eligible if they had previously received a bone marrow transplant or if they had participated in another trial or received another investigational drug within 30 days of the first visit. An individualized transfusion algorithm was calculated for each patient based on their prior 12-month transfusion history; the written algorithm documented the number of packed red blood cell (PRBC) units transfused for given hemoglobin values and served as a prospectively determined guide for transfusion during observation and treatment periods.

Each patient considered eligible entered an observation period of up to 13 weeks in order to confirm their PBRC transfusion dependence. At least one transfusion --termed the "qualifying" transfusion-- during the 13-week observation period at a hemoglobin value at or below 9 g/dL with symptoms, or at or below 7 g/dL with or without symptoms, in accordance with the transfusion algorithm indicated for each patient, was a

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requirement to proceed to randomization. The hemoglobin value at which each individual's qualifying transfusion was administered, was defined as the hemoglobin "set point" for that individual for the purpose of the primary efficacy variable. A platelet count ≥100,000/mL and a LDH level ≥1.5 times the upper limit of the normal range were also required either at screening or during the observation period for eligibility.

Study Design

Patients were randomly assigned on a one-on-one basis to receive either placebo or eculizumab (Soliris™, Alexion Pharmaceuticals, Inc.) within 10 days of the qualifying transfusion. Study medication was dosed in a blinded fashion as follows: 600 mg eculizumab for patients randomly assigned to active drug, or placebo for those patients randomly assigned to placebo, respectively via IV infusion every 7±1 days for 4 doses; followed by 900 mg eculizumab, or placebo, respectively, via IV infusion 7±1 day later; followed by a maintenance dose of 900 mg eculizumab, or placebo, respectively, via IV infusion every 14±2 days for a total of 26 weeks of treatment.

Measures of Clinical Efficacy

There were two co-primary endpoints in the study: (1) stabilization of hemoglobin levels, defined as a hemoglobin value maintained above the individual hemoglobin set point in the absence of transfusions for the entire 26-week treatment period, and (2) reduction in units of PRBCs transfused during the 26-week treatment phase of the study. The trigger for transfusion during the study period remained unchanged for each patient, as compared with their care before entry into the study: patients received blood transfusions when they had symptoms resulting from anemia and reached their individualized, pre-determined "set point". Pre-specified secondary endpoints included transfusion avoidance, hemolysis as measured by LDH area under the curve from baseline to 26 weeks, and QoL changes as measured from baseline to 26 weeks using the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) instrument.[13] Pre-specified exploratory analyses included assessment of the EORTC QLQ-C30 instrument,[14] the change in LDH from baseline through week 26, and thrombosis. Other pre-specified measurements included pharmacokinetics, pharmacodynamics, and immunogenicity of eculizumab. Time to first transfusion during

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the 26-week treatment phase and the proportion of PNH type III blood cells were also assessed.

Safety Assessments

Treatment-emergent adverse events, clinical laboratory tests (e.g., serum chemical analyses and complete blood counts), electrocardiogram data, and vital signs were assessed. Adverse events were defined using the MedDRA preferred terms and tabulated as incidence rates per treatment group.

Statistical Analysis

For co-primary endpoints, analyses were performed according to the intention to treat using the data from all patients who were randomized and received study drug; stabilization of hemoglobin levels was analyzed using the Fisher's exact test and total PRBC units transfused were analyzed with the Wilcoxon's rank sum test. For comparison of treatment effect on transfusion avoidance, the Fisher's exact test was used on the incidence and the log rank test was used for time to first transfusion. For LDH area under the curve the Wilcoxon's rank sum test was used.

Quality of life measure of fatigue was assessed using the scoring guidelines for the FACIT-Fatigue instrument.[15] Assessment of quality of life measures based on the EORTC QLQ-C30 instrument was conducted in accordance with the appropriate scoring guidelines.[16] The changes of FACIT-Fatigue and EORTC QLQ-C30 scores from baseline through 26 weeks were analyzed using a mixed model, with baseline as covariate, treatment and time as fixed effects, and patient as a random effect. Changes in LDH levels and PNH type III erythrocytes from baseline through 26 weeks were analyzed using the same mixed model. Two-sided tests were used for all analyses. The adverse events and long-term safety checklist were tabulated separately and compared between treatments using the Fisher's exact test. A p-value ≤ 0.05 was considered to be statistically significant.

II. RESULTS

Characteristics of Patients

A total of 115 PNH patients were screened. Six patients did not meet the inclusion/exclusion criteria during the screening period. Twenty-one other patients did

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not receive a qualifying transfusion and were not randomized into the treatment phase. One patient who did not meet the inclusion criteria was inadvertently randomized, but did not receive study medication. Thus 87 hemolytic PNH patients (35 men and 52 women) were enrolled and randomized to receive either eculizumab (N=43) or placebo (N=44), exceeding the original target of 75 randomized patients.

Patient characteristics were similar in the eculizumab- and placebo-treated cohorts: median age, 41 (range 20-85) and 35 (range 18-78) years; median duration of PNH, 4.2 (range 0.8 to 29.7) and 9.2 (range 0.4 to 38.3) years; patients with history of aplastic anemia, 4 and 11; history of myelodysplastic syndrome, 1 and 0; and history of thrombosis, 9 (16 events) and 8 (11 events). Stable usage of concomitant medications at baseline in the eculizumab- and placebo-treated groups included the following: erythropoietin, 3 patients and 0 patients; cyclosporine, 1 and 1; anticoagulants (coumarins or heparins) 21 and 11; and steroids (glucocorticoids or androgenic steroids), 12 and 12, respectively.

Of the 87 patients randomized, 85 completed the trial. Two patients who did not complete the trial had been randomized to the eculizumab arm: one patient discontinued due to the inconvenience of travel to the study site and the second patient became pregnant. Ten patients in the placebo-treatment group discontinued infusions, in all cases due to perceived lack of efficacy, but they remained in the study for monitoring purposes.

Pharmacokinetics/Pharmacodynamics

In 42 of the 43 eculizumab-treated patients the levels of drug during the maintenance period (900 mg every 2 weeks±2 days) were sufficient to completely block serum hemolytic activity (mean trough value at week 26 of 101.8 μg/mL). A single patient did not sustain therapeutic trough levels of eculizumab and demonstrated a breakthrough in complement blockade during the last few days of each dosing interval. These breakthroughs were clinically manageable and quickly resolved following the next dose.

Hemolytic Efficacy Variables

The 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 (Figure 1A). The median LDH

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area under the curve during the 26-week study period was reduced 85.8% in eculizumab- relative to placebo-treated patients (P<0.001). The mean LDH level decreased from 2199.7±157.7 IU/L at baseline to 327.3±67.6 IU/L by 26 weeks in eculizumab-treated patients while levels in placebo-treated patients remained consistently elevated with values of 2259.0±158.5 IU/L at baseline and 2418.9±140.3 IU/L at 26 weeks (P<0.001, for eculizumab versus placebo). A second biochemical measure of hemolysis, serum aspartate aminotransferase (AST), also showed a statistically significant improvement following eculizumab- versus placebo-treatment (data not shown). Haptoglobin levels were statistically significantly increased in eculizumab- as compared to placebo-treated patients but mean levels of haptoglobin were still below normal levels in eculizumab-treated patients (data not shown).

Figure 1A shows the degree of intravascular hemolysis in PNH patients, demonstrated by mean lactate dehydrogenase (LDH) levels (±standard error) from baseline (study week 0) to week 26 for both eculizumab- and placebo-treated patient populations. Screening occurred up to 3 months prior to study week 0. The upper limit of the normal range (103-223 IU/L) for LDH is indicated by a dashed line. LDH was reduced to a mean level just above the upper limit of normal at week 26 for eculizumab-treated patients; 15 of 41 patients who completed the study demonstrated LDH levels within the normal range. All placebo-treated patients remained at least 5 times above the upper limit of normal at week 26. The P value is based on a mixed model analysis from baseline through week 26. Figure 1B shows the mean proportion (±standard error) of PNH type III erythrocytes assessed for placebo- and eculizumab-treated patients. The screening visit occurred up to 3 months prior to study week 0. The P value is based on a mixed model analysis from baseline through week 26.

A corollary to the reduction in intravascular hemolysis during eculizumab treatment was an observed increase in the PNH type III erythrocyte population (Figure 1B). The mean proportions of type III erythrocytes increased from 28.1±2.0% at baseline to 56.9±3.6% by week 26 for eculizumab-treated patients while proportions in the placebo group remained constant with mean values of 35.7±2.8% before treatment to 35.5±2.8% at 26 weeks (P<0.001, for eculizumab versus placebo). By contrast, the proportions of PNH type III granulocytes and monocytes did not change significantly

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between the treatment groups during the treatment period and were greater than 90% at week 26.

III. CLINICAL EFFICACY

Co-primary endpoints

The co-primary efficacy endpoints in the TRIUMPH trial were stabilization of hemoglobin levels and reduction in PRBC units transfused. At the end of the treatment period, 48.8% of eculizumab-treated patients had maintained levels of hemoglobin above the pre-specified set-point (median set-point value of 7.7 g/dL for both treatment groups) in the absence of transfusions, whereas stabilization of hemoglobin did not occur in any of the patients in the placebo group ($P<0.001$; Table 1). By week 26, the median of PRBC units transfused per patient was 0 in the eculizumab group and 10.0 in the placebo cohort ($P<0.001$), while the mean of PRBC units transfused was 3.0 and 11.0 in the eculizumab and placebo cohorts, respectively. In the 6-month period prior to the study, the median of PRBC units transfused per patient was 9.0 in the eculizumab cohort and 8.5 in placebo patients while the mean of PRBC units transfused was 9.6±0.6 and 9.7±0.7, respectively. Mean hemoglobin levels were similar between the treatment groups at baseline (10.0±1.8 g/dL in eculizumab-treated patients and 9.7±1.8 g/dL in placebo-treated patients) and did not substantially change by week 26 (10.1±2.5 g/dL and 8.9±2.2 g/dL in eculizumab and placebo cohorts, respectively).

The median time to first transfusion was not reached during the study period in eculizumab-treated patients (it was greater than 26 weeks) while the placebo group reached the median time to first transfusion in only 4 weeks ($P<0.001$; Figure 2). Transfusion avoidance was achieved in 51.2% and 0% of the eculizumab and placebo cohorts, respectively ($P<0.001$). By the end of the 26-week treatment period, the total PRBC units transfused were 131 in eculizumab-treated patients versus 482 in the placebo group (Table 1). By contrast, in the 6-month period prior to the study, total PRBC units transfused in the eculizumab- and placebo-cohorts were 413 and 417, respectively.

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Table 1

Stabilization of Hemoglobin Levels and Reduction

in Transfusion Requirements during Eculizumab Treatment

	PRE-TREATMENT HISTORY*		TREATMENT PERIOD		
	Placebo	Eculizumab	Placebo	Eculizumab	P Value
†Stabilization of Hemoglobin Levels in the absence of transfusions (percent of patients)	NA	NA	0	48.8	<0.001‡
†Units Transfused per Patient					
Median	8.5	9.0	10	0	<0.001§
Mean±SE	9.7±0.7	9.6±0.6	11.0±0.83	3.0±0.67	
Total Units Transfused per Group	417	413	482	131	

*Twelve month historical transfusion data normalized to 6 months

†Co-primary endpoints

‡Base on 2-sided Fisher's exact test

§Based on Wilcoxon's rank sum test

NA, not applicable

Improvements in quality of life measures

Assessments of quality of life in PNH patients during eculizumab treatment were performed using two different instruments, FACIT-Fatigue and the EORTC QLQ-C30. Eculizumab-treated patients showed a mean increase (improvement) in the FACIT-Fatigue score of 6.4±1.2 points from baseline to 26-weeks while the mean score in placebo patients decreased by 4.0±1.7 points, a total difference between the treatment groups of 10.4 points (Figure 3). Mixed model analysis of covariance demonstrated a statistically significant difference between treatment groups (P<0.001).

For the EORTC instrument, improvements were observed with eculizumab-treatment in each subscale. Statistically significant improvements with eculizumab-

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compared with placebo-treated groups were observed in the following quality of life subscales (Table 2): global health status (P<0.001), physical functioning (P<0.001), emotional functioning (P=0.008), cognitive functioning (P=0.002), role functioning (P<0.001), social functioning (P=0.003), fatigue (P<0.001), pain (P=0.002), dyspnea (P<0.001), appetite loss (P<0.001), and insomnia (P=0.014). The improvements with eculizumab treatment in the other scales, including nausea and vomiting, diarrhea, constipation, and financial difficulties, did not reach statistical significance.

Table 2

Effect of Eculizumab Treatment on

Quality of Life Assessed by the EORTC QLQ-C30 Instrument

	Mean Change from Baseline to Week 26*			
	Placebo	**Eculizumab**	**Absolute Difference**	**P Value†**
Global Health Status	-8.5	10.9	19.4	<0.001
Functional				
Role	-6.9	17.9	24.8	<0.001
Social	2.0	16.7	14.6	=0.003
Cognitive	-6.1	7.9	14.0	=0.002
Physical	-3.5	9.4	13.0	<0.001
Emotional	-3.7	7.5	11.2	=0.008
Symptoms/Single Items				
Fatigue	10.0	-16.9	27.0	<0.001
Pain	5.3	-12.3	17.6	=0.002
Dyspnea	8.9	-7.9	16.9	<0.001
Appetite Loss	3.3	-10.3	13.6	<0.001
Insomnia	4.9	-7.9	12.8	=0.014

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Financial Difficulties	0.0	-10.3	10.3	=0.186
Constipation	0.0	-6.3	6.3	=0.199
Nausea/vomiting	2.8	-0.4	3.2	=0.056
Diarrhea	5.7	4.8	0.9	=0.147

*A positive change indicates an improvement in global health status and functional scales and a negative change indicates an improvement in symptom and single item scales.
†Based on a mixed analysis-of-covariance model with visit as a fixed effect, patient as a random effect and baseline as a covariate.

Relationship between FACIT-Fatigue quality of life and intravascular hemolysis

In order to determine if there was a treatment independent relationship between the FACIT-Fatigue quality of life instrument and intravascular hemolysis, an analysis was performed whereby the mean LDH level (through the 26 week study period) for each TRIUMPH patient was analyzed as a function of the patient's respective mean change in FACIT-Fatigue score from baseline (through the 26 week study period) (see Table 3). For this analysis, mean levels of LDH were divided into 4 groups that included: normal levels, 1-2 times the upper limit of normal (ULN), 2-10 times the upper limit of normal, and greater than 10 times the upper limit of normal. The analysis demonstrated that patients who maintained normal LDH levels throughout the study experienced significant improvements in fatigue when compared to patients who had incrementally higher levels of LDH throughout the study ($p=0.0048$). These data establish a clear link between increasing intravascular hemolysis as measured by LDH levels and decreased quality of life as measured by the FACIT-Fatigue quality of life instrument.

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Table 3

Relationship between FACIT-Fatigue and Intravascular Hemolysis

		Change of FACIT from Baseline		
Treatment Group	LDH Category	< 4	>= 4	P Value
Combined	Normal	4 (30.77%)	9 (69.23%)	.0048
	1 - 2 X ULN	16 (61.54%)	10 (38.46%)	
	2 - 10 X ULN	18 (72.00%)	7 (28.00%)	
	> 10 X ULN	16 (80.00%)	4 (20.00%)	

Safety

There were no deaths in the study. Serious adverse events (SAEs) were reported for 13 patients, of which 4 occurred in the eculizumab-treated cohort and 9 were in the placebo-treated cohort (see Table 4). All patients recovered without sequelae.

The most common AEs reported for eculizumab-treated patients were headache, nasopharyngitis, back pain, and upper respiratory tract infection. Headache and back pain occurred more commonly in the eculizumab-treatment group compared with the placebo group. However, the increase in headaches was limited to the first 2 weeks of therapy and was mild to moderate. There were no statistically significant differences in incidents rates between treatment groups for any AEs reported.

One episode of thrombosis (Budd-Chiari) occurred in a placebo-treated patient. There were no thromboses in eculizumab-treated patients.

Only one patient showed a detectable level of anti-eculizumab antibodies in the eculizumab-treated cohort; this response was weak (did not titrate), occurred at only one time point and did not result in a disruption of complement blockade.

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Table 4

Adverse Event Reporting

SERIOUS ADVERSE EVENTS*	**Placebo Patients n (percent)**	**Eculizumab Patients n (percent)**
Total	9 (20.5)	4 (9.3)
Eculizumab treatment emergent		
Exacerbation of PNH	3 (6.8)	1 (2.3)
Renal colic	0	1 (2.3)
Lumbar sacral disc prolapse	0	1 (2.3)
Alpha streptococcal bacteremia	0	1 (2.3)
Central line infection and UTI	1 (2.3)	0 (0)
Upper respiratory tract infection	1 (2.3)	0 (0)
Probable viral infection	1 (2.3)	0 (0)
Neutropenia	1 (2.3)	0 (0)
Cellulitis / folliculitis / neutropenia	1 (2.3)	0 (0)
Anemia and pyrexia	1 (2.3)	0 (0)
MOST FREQUENT ADVERSE EVENTS*†		
Headache§	12 (27.3)	19‡ (44.2)
Nasopharyngitis	8 (18.2)	10 (23.3)
Upper respiratory tract infection	10 (22.7)	6 (14)
Back pain	4 (9.1)	8 (18.6)
Nausea	5 (11.4)	7 (16.3)
Cough	4 (9.1)	5 (11.6)
Diarrhea	5 (11.4)	4 (9.3)
Arthralgia	5 (11.4)	3 (7.0)
Abdominal pain	5 (11.4)	2 (4.7)
Dizziness	5 (11.4)	2 (4.7)
Vomiting	5 (11.4)	2 (4.7)

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Fatigue	1 (2.3)	5 (11.6)
Viral infection	5 (11.4)	1 (2.3)

*By preferred terms

†Occurring in 10% or more of patients

‡Sixteen of 19 patients experienced headache within 48 hours of infusion

§Following the first 2 weeks of dosing, 20.9% of eculizumab- and 22.7% of placebo-treated patients experienced headache

IV. DISCUSSION

Chronic intravascular hemolysis with periods of acute exacerbation are the classical manifestations of PNH, frequently resulting in anemia, the need for transfusions to sustain hemoglobin levels, and deterioration in quality of life. In the phase III pivotal TRIUMPH study, we examined the effect of terminal complement inhibition with eculizumab on hemoglobin levels and transfusion requirements in patients with PNH. Forty-nine percent of patients treated with eculizumab over the 6-month period demonstrated stabilization of hemoglobin in the absence of transfusions compared to no patients in the placebo arm of the trial. Over 50% of eculizumab-treated patients were transfusion independent during the entire study compared to none in the placebo arm, and the overall mean transfusion rate was reduced by 73%. Moreover, even in patients who did not achieve transfusion independence, eculizumab treatment was associated with a 44% reduction in the rate of transfusion (data now shown).

Lactate dehydrogenase, a biochemical marker of hemolysis in PNH,[9] was immediately and consistently decreased in all eculizumab-treated patients, while patients in the placebo cohort continued to hemolyze with levels of LDH exceeding 5 times the upper limit of the normal range in all patients at the study end. Levels of LDH were reduced into the normal range in approximately one-third of eculizumab-treated patients, while the remainder stabilized at a level just above the upper limit of normal suggesting residual low level hemolysis in some patients. Levels of haptoglobin, a more sensitive marker of the presence of cell free hemoglobin in the circulation, were undetectable in most patients. Low level hemolysis in a subset of eculizumab-treated patients is possibly

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due to an inherent decrease in survival of these cells or C3b-mediated, extravascular clearance of PNH erythrocytes through the reticuloendothelial system.[17]

Before eculizumab treatment, hemoglobin levels in study patients were artificially maintained by frequent transfusion. Therefore, stabilization of hemoglobin levels with a concomitant cessation of or reduction in transfusions represents a net increase in endogenous hemoglobin levels. Our data suggest that resolution of hemolysis with eculizumab results in a new steady state hemoglobin level determined by a balance between the extent of the underlying bone marrow dysfunction, the number of PNH erythrocytes that are preserved by eculizumab therapy and the new level (if any) of transfusion requirement.

Patients with PNH generally experience markedly impaired quality of life characterized by fatigue, anemia, thrombosis, and pulmonary hypertension as well as smooth muscle dystonia including abdominal pain, dysphagia, and erectile dysfunction.[9,10,18] These symptoms have been attributed to both excessive intravascular hemolysis and downstream scavenging of nitric oxide by cell free hemoglobin in plasma. The reduction of intravascular hemolysis in eculizumab-treated patients in the current study was associated with significant improvements in the fatigue component of quality of life relative to placebo-treated patients as assessed via the FACIT-Fatigue instrument. Further, eculizumab therapy was associated with a median increase of 6.4 points over baseline values established before treatment. It has previously been demonstrated that an increase of 3 or more points from baseline represents a clinically important difference in this quality of life instrument.[19] Patients who received eculizumab also experienced a significant improvement in most domains of the EORTC QLQ-30 relative to the placebo-treated cohort including global health status, physical functioning, emotional functioning, cognitive functioning, role functioning, social functioning, fatigue, pain, dyspnea, appetite loss, and insomnia. Improvement in the fatigue component of the EORTC QLQ-30 provides support for the improvement demonstrated in the FACIT-Fatigue instrument during eculizumab therapy. Importantly, these improvements in quality of life in the eculizumab-treated patients occurred despite similar levels of erythrocyte hemoglobin in the two treatment groups, further supporting the contribution of hemolysis per se, as opposed to anemia, in mediating the poor quality of life in PNH patients. Clinical

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assessment of additional life quality-related symptoms of PNH, including abdominal pain, dysphagia, and erectile dysfunction, have also been reported to improve during eculizumab therapy.[20]

Eculizumab was safe and well-tolerated. There were no deaths in the study and only a single thrombotic event which occurred in a placebo patient in a site (the hepatic veins) which is typical of the thrombosis in PNH. The relative brief duration of this study was not sufficient to address the relevant issue of a possible protection from thrombosis by terminal complement inhibition with eculizumab.

Adverse events were generally mild with headache occurring at increased frequency in the eculizumab-treated patients; however, this increased frequency did not persist following the first two doses of therapy. There were 4 SAEs in the eculizumab treatment group and 9 SAEs in the placebo group. There was no evidence of increased infection risk in eculizumab-treated patients during the study period. One eculizumab-treated patient showed a low level of anti-eculizumab antibodies at one time point during the study which did not persist and did not result in a disruption of complement blockade. There were no AEs associated with eculizumab withdrawal in the 2 eculizumab-treated patients who did not complete the trial. Additional safety assessments, as well as efficacy measures, are being examined in an ongoing multi-center, open-label Phase III safety trial of eculizumab (SHEPHERD) in approximately 95 patients with PNH.

Results from the current randomized, double-blind, placebo-controlled, global study show that terminal complement inhibition with eculizumab appears to be a safe and effective therapy for patients with the rare disorder PNH. Treatment with eculizumab reduced intravascular hemolysis, and stabilized hemoglobin levels despite a reduction of transfusions, to the point where most PNH patients were rendered transfusion independent. Substantial and clinically meaningful improvements in fatigue and other key quality of life parameters were also demonstrated. All of the 85 patients who completed the study elected to receive eculizumab in an open-label extension study and all currently remain on drug. The results of the TRIUMPH study indicate that terminal complement inhibition with eculizumab safely and effectively addresses an important consequence of the underlying genetic defect in PNH hematopoietic stem cells by providing a therapeutic replacement for the terminal complement inhibitor deficiency.

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The present invention provides among other things treatment with an inhibitor of complement. Many variations of the invention will become apparent to those skilled in the art upon review of this specification. The full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

All publications and patents mentioned herein, including those items listed below, 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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WHAT IS CLAIMED IS:

1. A method to improve at least one aspect of the quality of life of a patient suffering from paroxysmal nocturnal hemoglobinuria, said method comprising administering to said patient a compound which inhibits complement or inhibits formation of C5b-9.

2. The method of claim 1 wherein said quality of life is measured by a FACIT-Fatigue score.

3. The method of claim 2 wherein the FACIT-Fatigue score increases by at least 3 points.

4. The method of claim 2 wherein the FACIT-Fatigue score increases by ≥ 4 points.

5. The method of claim 1 wherein said quality of life is measured by an EORTC QLQ-C30 score.

6. The method of claim 5 wherein said EORTC QLQ-C30 score improves by $\geq 10\%$ of the pretreatment score.

7. The method of claim 5 wherein said aspect of the quality of life as measured by an EORTC QLQ-C30 score is selected from the group consisting of a) global health status, b) physical functioning, c) emotional functioning, d) cognitive functioning, e) role functioning, f) social functioning, g) fatigue, h) pain, i) dyspnea, j) appetite loss, and k) insomnia.

8. The method of claim 7 wherein said aspect of quality of life is fatigue.

9. The method of claim 1 wherein said 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.

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10. The method of claim 1 wherein said compound is a steroid that suppresses complement.

11. The method of claim 1 wherein said compound is selected from the group consisting of antibodies, active antibody fragments, soluble complement inhibitory compounds, proteins, soluble complement inhibitors with a lipid tail, protein fragments, peptides, small organic compounds, 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.

12. The method of claim 11 wherein said compound is an antibody or an active antibody fragment.

13. The method of claim 12 wherein said antibody or active antibody fragment is selected from the group consisting of a) polyclonal antibodies, b) monoclonal antibodies, c) single chain antibodies, d) chimeric antibodies, e) humanized antibodies, f) Fabs, g) F(ab')s, h) $F(ab')_2$s, i) Fvs, j) diabodies, and k) human antibodies.

14. The method of claim 12 wherein said antibody or active antibody fragment blocks C5 cleavage.

15. The method of claim 12 wherein said antibody or active antibody fragment inhibits the formation of C5b-9.

16. The method of claim 12 wherein said antibody is eculizumab.

17. The method of claim 12 wherein said antibody or active antibody fragment is administered for at least 6 months.

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18. The method of claim 1 wherein said patient has aplastic anemia or myelodysplastic syndrome.

19. A method to improve at least one aspect of the quality of life of an anemic patient suffering from paroxysmal nocturnal hemoglobinuria, said method comprising administering to said patient a compound which inhibits complement or inhibits formation of C5b-9, wherein said patient remains anemic.

20. The method of claim 19 wherein said patient has a hemoglobin level less than i) 14 g/dL if a man or ii) 12 g/dL if a woman.

21. The method of claim 19 wherein said patient has a hemoglobin level less than i) 13 g/dL if a man or ii) 11 g/dL if a woman.

22. The method of claim 19 wherein said patient has a hemoglobin level less than i) 12 g/dL if a man or ii) 10 g/dL if a woman.

23. The method of claim 19 wherein said quality of life is measured by a FACIT-Fatigue score.

24. The method of claim 23 wherein the FACIT-Fatigue score increases by at least 3 points.

25. The method of claim 23 wherein the FACIT-Fatigue score increases by $\geq$ 4 points.

26. The method of claim 19 wherein said quality of life is measured by an EORTC QLQ-C30 score.

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27. The method of claim 26 wherein said EORTC QLQ-C30 score improves by ≥ 10% of the pretreatment score.

28. The method of claim 26 wherein said aspect of the quality of life as measured by an EORTC QLQ-C30 score is selected from the group consisting of a) global health status, b) physical functioning, c) emotional functioning, d) cognitive functioning, e) role functioning, f) social functioning, g) fatigue, h) pain, i) dyspnea, j) appetite loss, and k) insomnia.

29. The method of claim 28 wherein said aspect of quality of life is fatigue.

30. The method of claim 19 wherein said 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.

31. The method of claim 19 wherein said compound is a steroid that suppresses complement.

32. The method of claim 19 wherein said compound is selected from the group consisting of antibodies, active antibody fragments, soluble complement inhibitory compounds, proteins, soluble complement inhibitors with a lipid tail, protein fragments, peptides, small organic compounds, 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.

33. The method of claim 32 wherein said compound is an antibody or an active antibody fragment.

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34. The method of claim 33 wherein said antibody or active antibody fragment is selected from the group consisting of a) polyclonal antibodies, b) monoclonal antibodies, c) single chain antibodies, d) chimeric antibodies, e) humanized antibodies, f) Fabs, g) F(ab')s, h) $F(ab')_2s$, i) Fvs, j) diabodies, and k) human antibodies.

35. The method of claim 33 wherein said antibody or active antibody fragment blocks C5 cleavage.

36. The method of claim 33 wherein said antibody or active antibody fragment inhibits the formation of C5b-9.

37. The method of claim 33 wherein said antibody is eculizumab.

38. The method of claim 33 wherein said antibody or active antibody fragment is administered for at least 6 months.

39. The method of claim 19 wherein said patient has aplastic anemia or myelodysplastic syndrome.

40. A method to improve at least one aspect of the quality of life of a patient suffering from paroxysmal nocturnal hemoglobinuria, said method comprising administering to said patient a compound which inhibits intravascular hemolysis.

41. The method of claim 40 wherein said method results in a greater than 30% reduction in LDH in said patient.

42. The method of claim 40 wherein said quality of life is measured by a FACIT-Fatigue score.

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43. The method of claim 42 wherein the FACIT-Fatigue score increases by at least 3 points.

44. The method of claim 42 wherein the FACIT-Fatigue score increases by $\geq$ 4 points.

45. The method of claim 40 wherein said quality of life is measured by an EORTC QLQ-C30 score.

46. The method of claim 45 wherein said EORTC QLQ-C30 score improves by $\geq$ 10% of the pretreatment score.

47. The method of claim 45 wherein said aspect of the quality of life as measured by the EORTC QLQ-C30 score is selected from the group consisting of a) global health status, b) physical functioning, c) emotional functioning, d) cognitive functioning, e) role functioning, f) social functioning, g) fatigue, h) pain, i) dyspnea, j) appetite loss, and k) insomnia.

48. The method of claim 47 wherein said aspect of quality of life is fatigue.

49. The method of claim 40 wherein said 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 40 wherein said compound is a steroid that suppresses complement.

51. The method of claim 40 wherein said compound is selected from the group consisting of antibodies, active antibody fragments, soluble complement inhibitory compounds, proteins, soluble complement inhibitors with a lipid tail, protein fragments, peptides, small organic compounds, RNA aptamers, L-RNA

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aptamers, spiegelmers, antisense compounds, serine protease inhibitors, double stranded RNA, small interfering RNA, locked nucleic acid inhibitors, and peptide nucleic acid inhibitors.

52. The method of claim 51 wherein said compound is an antibody or an active antibody fragment.

53. The method of claim 52 wherein said antibody or active antibody fragment is selected from the group consisting of a) polyclonal antibodies, b) monoclonal antibodies, c) single chain antibodies, d) chimeric antibodies, e) humanized antibodies, f) Fabs, g) F(ab')s, h) $F(ab')_2$s, i) Fvs, j) diabodies, and k) human antibodies.

54. The method of claim 52 wherein said antibody or active antibody fragment blocks C5 cleavage.

55. The method of claim 52 wherein said antibody or active antibody fragment is administered for at least 6 months.

56. The method of claim 40 wherein said compound inhibits complement or inhibits the formation of C5b-9.

57. The method of claim 56 wherein said compound is an antibody or active antibody fragment.

58. The method of claim 57 wherein said antibody is eculizumab.

59. The method of claim 40 wherein said patient has aplastic anemia or myelodysplastic syndrome.

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60. A method to improve at least one aspect of the quality of life of an anemic patient whose anemia results at least in part from hemolysis, said method comprising administering to said patient a compound which inhibits intravascular hemolysis, wherein said patient remains anemic.

61. The method of claim 60 wherein said method results in a greater than 30% reduction in LDH in said patient.

62. The method of claim 60 wherein said patient has a hemoglobin level less than i) 14 g/dL if a man or ii) 12 g/dL if a woman.

63. The method of claim 60 wherein said patient has a hemoglobin level less than i) 13 g/dL if a man or ii) 11 g/dL if a woman.

64. The method of claim 60 wherein said patient has a hemoglobin level less than i) 12 g/dL if a man or ii) 10 g/dL if a woman.

65. The method of claim 60 wherein said patient suffers from paroxysmal nocturnal hemoglobinuria.

66. The method of claim 60 wherein said quality of life is measured by a FACIT-Fatigue score.

67. The method of claim 66 wherein the FACIT-Fatigue score increases by at least 3 points.

68. The method of claim 66 wherein the FACIT-Fatigue score increases by $\geq$ 4 points.

69. The method of claim 60 wherein said quality of life is measured by an EORTC QLQ-C30 score.

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70. The method of claim 69 wherein said EORTC QLQ-C30 score improves by ≥ 10% of the pretreatment score.

71. The method of claim 69 wherein said aspect of the quality of life as measured by an EORTC QLQ-C30 score is selected from the group consisting of a) global health status, b) physical functioning, c) emotional functioning, d) cognitive functioning, e) role functioning, f) social functioning, g) fatigue, h) pain, i) dyspnea, j) appetite loss, and k) insomnia.

72. The method of claim 71 wherein said aspect of quality of life is fatigue.

73. The method of claim 60 wherein said 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.

74. The method of claim 60 wherein said compound is a steroid that suppresses complement.

75. The method of claim 60 wherein said compound is selected from the group consisting of antibodies, active antibody fragments, soluble complement inhibitory compounds, proteins, soluble complement inhibitors with a lipid tail, protein fragments, peptides, small organic compounds, 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.

76. The method of claim 75 wherein said compound is an antibody or an active antibody fragment.

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77. The method of claim 76 wherein said antibody or active antibody fragment is selected from the group consisting of a) polyclonal antibodies, b) monoclonal antibodies, c) single chain antibodies, d) chimeric antibodies, e) humanized antibodies, f) Fabs, g) F(ab')s, h) $F(ab')_2$s, i) Fvs, j) diabodies, and k) human antibodies.

78. The method of claim 76 wherein said antibody or active antibody fragment blocks C5 cleavage.

79. The method of claim 76 wherein said antibody or active antibody fragment is administered for at least 6 months.

80. The method of claim 60 wherein said compound inhibits complement or inhibits the formation of C5b-9.

81. The method of claim 80 wherein said compound is an antibody or an active antibody fragment.

82. The method of claim 81 wherein said antibody is eculizumab.

83. The method of claim 60 wherein said patient has aplastic anemia or myelodysplastic syndrome.

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ABSTRACT

Eculizumab, a humanized monoclonal antibody against C5 that inhibits terminal complement activation, showed activity in a preliminary 12-week open-label trial in a small cohort of patients with paroxysmal nocturnal hemoglobinuria (PNH). The present study examined whether chronic eculizumab therapy could reduce intravascular hemolysis, stabilize hemoglobin levels, reduce transfusion requirements, and improve quality of life in a double-blind, randomized, placebo-controlled, multi-center global Phase III trial. It has been found that eculizumab stabilized hemoglobin levels, decreased the need for transfusions,, and improved quality of life in PNH patients via reduced intravascular hemolysis. Chronic eculizumab treatment appears to be a safe and effective therapy for PNH.

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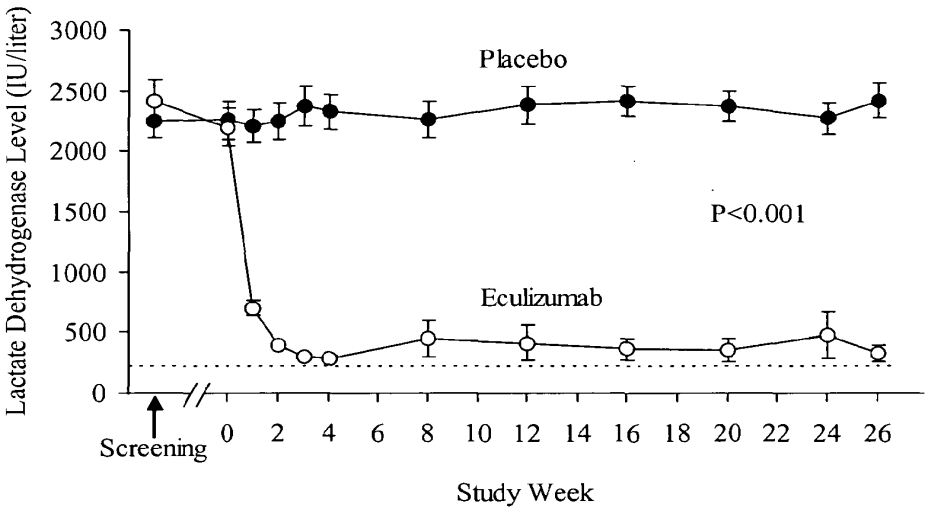


FIGURE 1A

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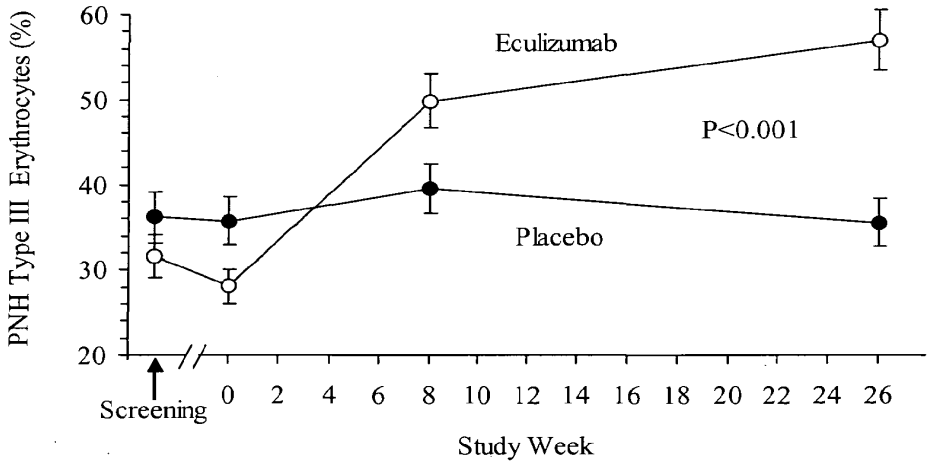


FIGURE 1B

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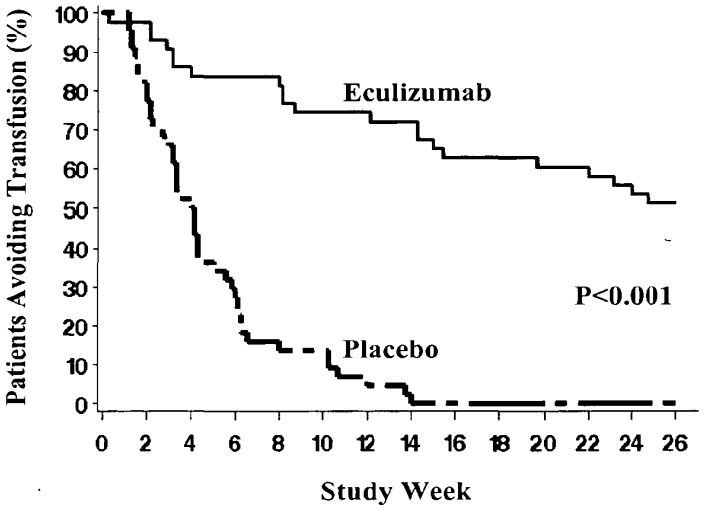


FIGURE 2

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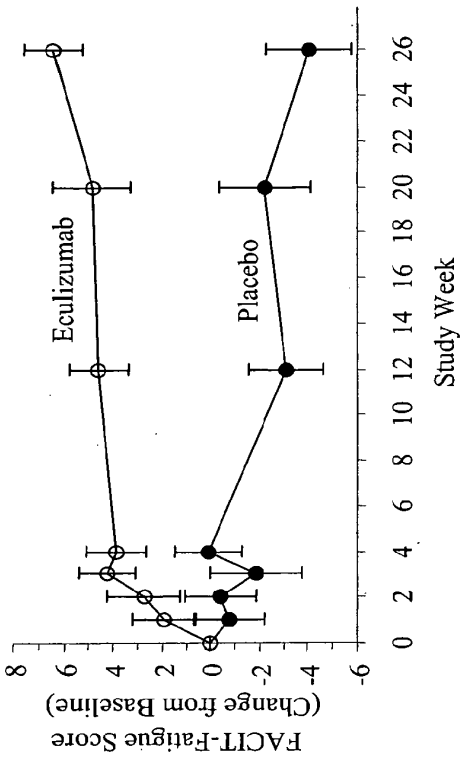


FIGURE 3

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Europäisches Patentamt
European Patent Office
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Request for grant of a European patent

For official use only		
1 Application number:	MKEY	**MKEY - EP16195355.9**
2 Date of receipt (Rule 35(2) EPC):	DREC	**DREC - 24 Oct 2016**
3 Date of receipt at EPO (Rule 35(4) EPC):	RENA	
4 Date of filing:		

5 Grant of European patent, and examination of the application under Article 94, are hereby requested. ☒

5.1 The applicant waives his right to be asked whether he wishes to proceed further with the application (Rule 70(2)) ☐

Procedural language: en

Description and/or claims filed in: en

6 Applicant's or representative's reference: P2913 EP/3

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14.1 The/Each applicant hereby declares that he is an entity or a natural person under Rule 6(4) EPC. ☐

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Inventor(s)

23 Inventor details filed separately ☒

24 **Title of invention**

Title of invention: Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement

25 **Declaration of priority (Rule 52)**

A declaration of priority is hereby made for the following applications

	State	Filing date	Kind	Application number:	Search results under Rule 141(1) are attached
Priority 01	US	15.03.2006	pr	60/783,070	☐

25.2 This application is a complete translation of the previous application ☐

25.3 It is not intended to file a (further) declaration of priority ☐

26 **Reference to a previously filed application**

27 **Divisional application** ☒

Application number of earlier application: EP11001632.6

Date of Filing (Art. 80/Rule 40 EPC): 15 March 2007

27.1 This divisional application is of the following generation: ☐ 1 ☒ 2 ☐ 3 ☐ 4 ☐ 5 or subsequent

28 **Article 61(1)(b) application** ☐

29 **Claims**

Number of claims: 3

29.1 ☒ as attached

29.2 ☐ as in the previously filed application (see Section 26.2)

29.3 ☐ The claims will be filed later

30 **Figures**

It is proposed that the abstract be published together with figure No.

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31 Designation of contracting states

All the contracting states party to the EPC and valid for the parent application at the time of filing of this divisional application are deemed to be designated (see Article 76(2)).

32 Different applicants for different contracting states

33 Extension/Validation

This application is deemed to be a request to extend the effects of the European patent application and the European patent granted in respect of it to all non-contracting states to the EPC with which extension or validation agreements are in force on the date on which the application is filed. However, the request is deemed withdrawn if the extension fee or the validation fee, whichever is applicable, is not paid within the prescribed time limit.

33.1 It is intended to pay the extension fee(s) for the following state(s): BA AL HR MK RS

33.2 It is intended to pay the validation fee(s) for the following state(s):

34 Biological material

38 Nucleotide and amino acid sequences

The European patent application contains a sequence listing as part of the description ☒

The sequence listing is attached in computer-readable format in accordance with WIPO Standard ST.25 ☒

The sequence listing is attached in PDF format ☐

Further indications

39 Additional copies of the documents cited in the European search report are requested

Number of additional sets of copies:

40 Refund of the search fee under to Article 9 of the Rules relating to Fees is requested ☒

Application or publication number of earlier search report: EP11001632.6

42 Payment

Mode of payment: Debit from deposit account

The European Patent Office is hereby authorised, to debit from the deposit account with the EPO any fees and costs indicated on the fees section below.

Currency: EUR

Deposit account number: 28000321

Account holder: Vossius & Partner

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Deferred execution date / total amount to be debited on: (or on the date of receipt if the submission date is after the deferred execution date you have indicated)	24.11.2016

Important information:
• This functionality allows you to indicate explicitly that your payment order is to be executed at a later date than the submission date. In this case, the payment date will be deemed to be the deferred execution date, provided that you have sufficient funds on your deposit account on that date.
• Please choose the deferred execution date option only if the fees are to be debited on a future date, as otherwise the default execution date is date of receipt.
• The deferred execution date can be a maximum of 40 days in the future.
• Please ensure that the deferred execution date is within the time limit set for paying the selected fees.
• Please note that any debit orders (standard or deferred execution) can only be revoked until 00.00 hrs CET on the date of submission (Arrangements for deposit accounts, 7)

43 Refunds

Fees	Factor applied	Fee schedule	Amount to be paid
001 Filing fee - EP direct - online	1	120.00	120.00
002 Fee for a European search - Applications filed on/after 01.07.2005	1	1 300.00	1 300.00
015 Claims fee - For the 16th to the 50th claim	0	235.00	0.00
015e Claims fee - For the 51st and each subsequent claim	0	585.00	0.00
501 Additional filing fee for the 36th and each subsequent page	26	15.00	390.00
552 Additional fee for divisional 2nd generation	1	210.00	210.00
Total:		EUR	2 020.00

44-A Forms		Details:	System file name:
A-1	Request		as ep-request.pdf
A-2	1. Designation of inventor	1. Inventor	as f1002-1.pdf

44-B Technical documents		Original file name:	System file name:
B-1	Specification	P2913 EP_3 Specification.pdf Description; 3 claims; 3 figure(s); abstract	SPECEPO-1.pdf
B-2	Sequence listings, ASCII	P2913 EP_3 Sequence listing.txt	SEQLTXT.txt

44-C Other documents		Original file name:	System file name:
C-1	Additional sheet	P2913 EP_3 Additional sheet.pdf	OTHER-1.pdf

45 General authorisation:

46 Signature(s)

Place:	**München**
Date:	**24 October 2016**
Signed by:	**/Dr. Joachim Wachenfeld/**
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European Patent Office

80298 M U N I C H

New EP divisional application
based on EP 11 00 1632.6
Alexion Pharmaceuticals, Inc.
Our Ref.: P2913 EP/3

München, October 24, 2016
WA/FLG

ADDITIONAL SHEET

This is to indicate support for the claims of the above-referenced new divisional application in the parent application as filed (i.e. EP 11 00 1632.6).

1. Claims 1 and 2 are directed to an antibody that binds C5. The antibody of claim 2 comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain CDRs of SEQ ID NOs 8, 9 and 10. The antibody of claim 2 comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4. The antibodies of claims 1 and 2 have a basis at page 5, last paragraph of the parent application as filed.

2. Claim 3 is directed to a pharmaceutical composition comprising the antibody as defined in claim 1 or 2. This pharmaceutical composition has a basis at page 3, lines 18-19 in conjunction with page 5, lines 30-33 of the parent application as filed.

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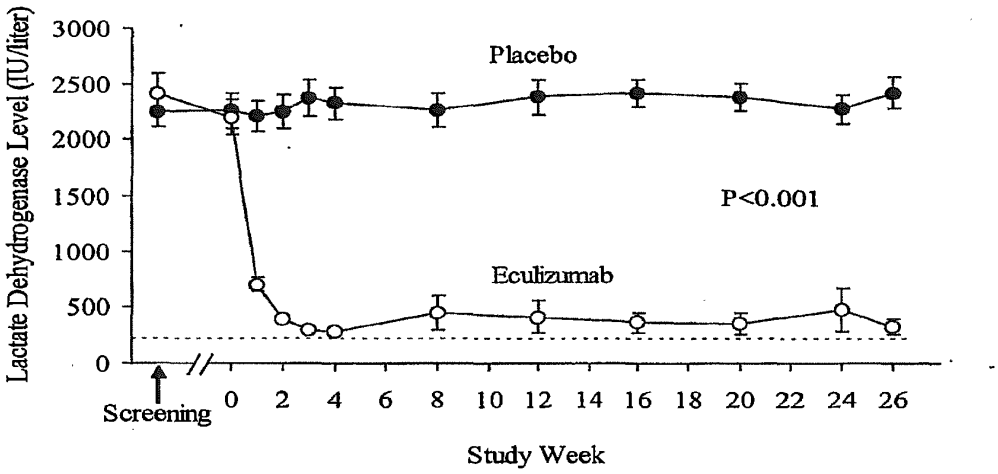


FIGURE 1A

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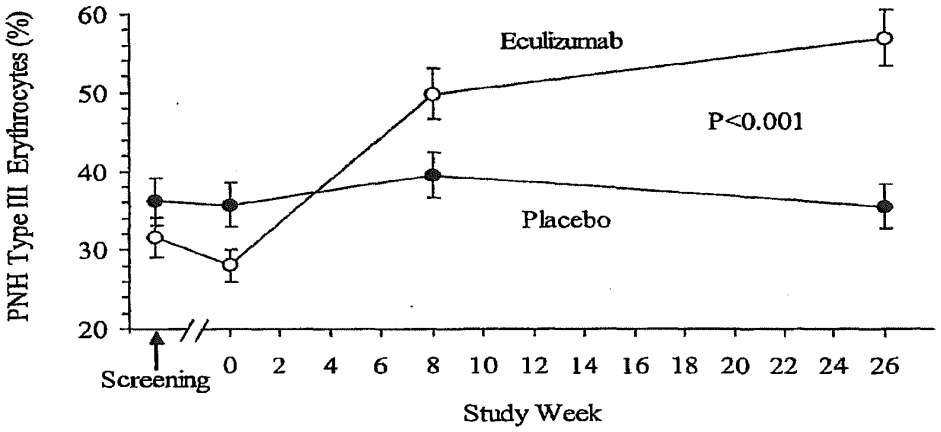


FIGURE 1B

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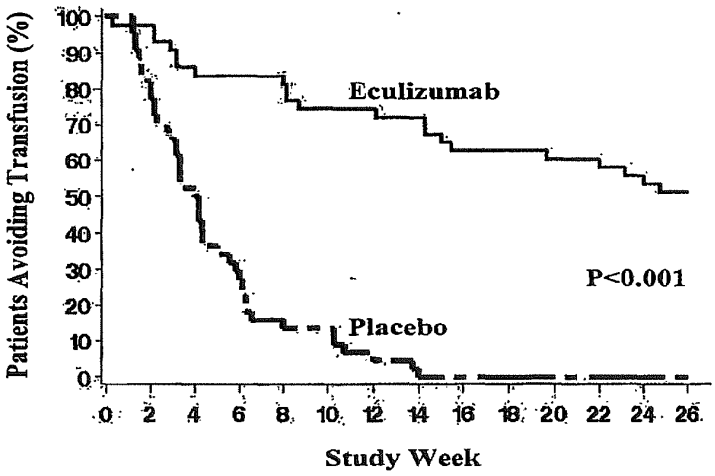


FIGURE 2

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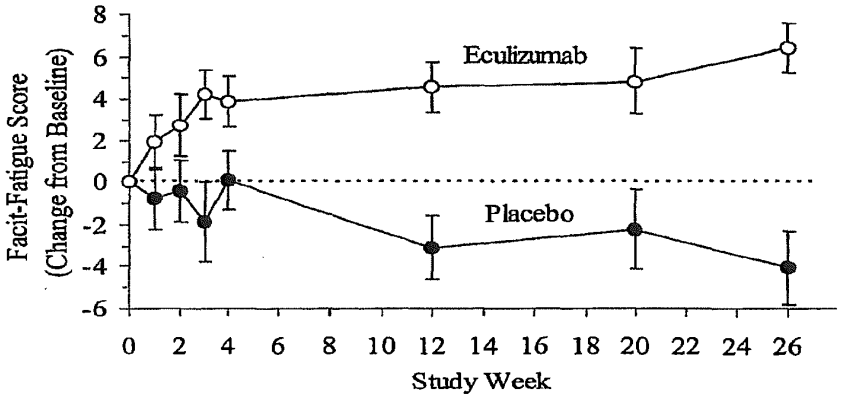


FIGURE 3

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Form 1002 - 1: Public inventor(s)

Designation of inventor 123

User reference: P2913 EP/3
Application No:

Public

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	The applicant has acquired the right to the European patent:	Under agreement: 02 May 2006

Signature(s)

Place:	**München**
Date:	**24 October 2016**
Signed by:	**/Dr. Joachim Wachenfeld/**
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Representative name:	**Dr. Joachim Wachenfeld**
Capacity:	**(Representative)**

New EP-Divisional Patent Application
based on EP 11 00 1632.6
Alexion Pharmaceuticals, Inc.
Our Ref.: P2913 EP/3

TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

BACKGROUND

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematologic disease that results from clonal expansion of hematopoietic stem cells with somatic mutations in the X-linked gene called PIG-A.[1,2] Mutations in PIG-A lead to an early block in the synthesis of glycosylphosphatidylinositol (GPI)-anchors, which are required to tether many proteins to the cell surface. Consequently, PNH blood cells have a partial (type II) or complete (type III) deficiency of GPI-anchored proteins.

Intravascular hemolysis is a prominent feature of PNH and a direct result of the absence of the GPI-anchored complement regulatory protein CD59.[3,4] Under normal circumstances, CD59 blocks the formation of the terminal complement complex (also called the membrane attack complex) on the cell surface, thereby preventing erythrocyte lysis and platelet activation.[5-8] Excessive or persistent intravascular hemolysis in PNH patients not only results in anemia (normal ranges of hemoglobin are 14-18 g/dL for men and 12-16 g/dL for women, and persons with lower levels are considered to be anemic), but also hemoglobinuria and clinical sequelae related to the release of the erythrocyte contents into the circulation: fatigue, thrombosis, abdominal pain, dysphagia, erectile dysfunction, and pulmonary hypertension.[9,10,21,22] Indeed, impaired quality of life in PNH is disproportionate to the degree of anemia. Many PNH patients depend on blood transfusions to maintain adequate erythrocyte hemoglobin levels. There have been no therapies that effectively reduce intravascular hemolysis and improve the associated clinical morbidities in PNH.

Eculizumab is a humanized monoclonal antibody directed against the terminal complement protein C5.[11] In a preliminary, 12-week, open-label clinical study in 11 PNH patients, eculizumab was shown to reduce intravascular hemolysis and transfusion requirements.[12] However, this unblinded study involved a small number of patients with no control arm and without protocol-driven transfusion standards.

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SUMMARY

The present pivotal, phase III study, Transfusion Reduction Efficacy and Safety Clinical Investigation, Randomized, Multi-Center, Double-Blind, Placebo-Controlled, Using Eculizumab in Paroxysmal Nocturnal Hemoglobinuria (TRIUMPH), evaluated the effect of eculizumab on the stabilization of hemoglobin levels and transfusion requirements during 6 months of treatment in a cohort of 87 transfusion-dependent PNH patients. Measures of intravascular hemolysis and quality of life were also assessed. This is the first placebo controlled study of a PNH patient population to control hemolysis and to differentiate between the effects due to hemolysis and the effects due to anemia.

It has been surprisingly discovered that certain aspects of quality of life were unexpectedly improved by the treatment of PNH patients with eculizumab. Furthermore, these improvements in the quality of life were independent of transfusion. The improved aspects include, e.g., global health status, physical functioning, emotional functioning, cognitive functioning, role functioning, social functioning, fatigue, pain, dyspnea, appetite loss and insomnia. Improvement was also seen in nausea and vomiting, diarrhea, constipation, and financial difficulties but did not reach the level of statistical significance. Because the treated patients remained anemic throughout their treatment, it was unexpected that all of these improvements would have been seen because they were previously thought to be a result of the patient being anemic. Although not wishing to be bound by any theory, it appears that some of the symptoms are likely due, at least in part, to hemolysis and release of hemoglobin into the bloodstream and do not result solely from the patient being anemic. The treatment with eculizumab decreases the amount of lysis thereby limiting hemoglobin release into the bloodstream, thereby apparently resulting in the improvements seen in the treated patients' quality of life. The results presented herein indicate that any treatment that decreases hemolysis in a patient will result in an improvement in the quality of life of said patient.

In certain aspects, the application provides a method to improve at least one aspect of the quality of life of a patient suffering from paroxysmal nocturnal hemoglobinuria, said method comprising administering to said patient in need thereof a compound which inhibits complement or inhibits formation of C5b-9.

In certain aspects, the application provides a method to improve at least one aspect of the quality of life of a patient suffering from paroxysmal nocturnal hemoglobinuria, said method comprising administering to said patient in need thereof a compound which inhibits

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intravascular hemolysis. In certain embodiments, said method results in a greater than 30% reduction in LDH in said patient.

In certain aspects, the application provides a method to improve at least one aspect of the quality of life of an anemic patient whose anemia results at least in part from hemolysis, said method comprising administering to said patient in need thereof a compound which inhibits intravascular hemolysis, wherein said patient remains anemic. In certain embodiments, said method results in a greater than 30% reduction in LDH in said patient.

In certain aspects, the application provides a method of prolonging the health-adjusted life expectancy of a patient comprising administering to said patient in need thereof a compound which inhibits formation of C5b-9. In certain embodiments, said patient is anemic. In certain embodiments, said patient remains anemic following treatment. In certain embodiments, said patient has a hemoglobin level less than i) 14 g/dL if a man or ii) 12 g/dL if a woman. In certain embodiments, said patient has a hemoglobin level less than i) 13 g/dL if a man or ii) 11 g/dL if a woman. In certain embodiments, said patient has a hemoglobin level less than i) 12 g/dL if a man or ii) 10 g/dL if a woman. In certain embodiments, said patient suffers from paroxysmal nocturnal hemoglobinuria.

In certain aspects, the application provides a pharmaceutical composition comprising an antibody that binds C5 or an active antibody fragment thereof. In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof is eculizumab. In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof is pexelizumab. In certain embodiments, the pharmaceutical formulations of the application may be administered to a subject, particularly a subject having PNH.

In certain aspects, the application provides a method of treating a patient suffering from paroxysmal nocturnal hemoglobinuria by administering a pharmaceutical composition comprising an antibody that binds C5 or an active antibody fragment thereof. In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof is eculizumab. In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof is pexelizumab. In certain embodiments, the pharmaceutical formulations of the application may be administered to a subject, particularly a subject having PNH.

In certain aspects, the application provides kits comprising a pharmaceutical composition of the application. In some embodiments, the kit further comprises at least one component of a closed sterile system. Components of the closed sterile system

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include, but are not limited to, needles, syringes, catheter based syringes, needle based injection devices, needle-less injection devices, filters, tubing, valves and cannulas. In a related embodiment, the kit comprise components for the removal of a preservative from the composition. Such components include filters, syringes, vials, containers, tubing, etc.

In certain embodiments, said quality of life is measured by a FACIT-Fatigue score. In certain embodiments, the FACIT-Fatigue score increases by at least 3 points. In certain embodiments, the FACIT-Fatigue score increases by ≥ 4 points.

In certain embodiments, said quality of life is measured by an EORTC QLQ-C30 score. In certain embodiments, said EORTC QLQ-C30 score improves by $\geq$ 10% of the pretreatment score. In certain embodiments, said aspect of the quality of life as measured by an EORTC QLQ-C30 score is selected from the group consisting of a) global health status, b) physical functioning, c) emotional functioning, d) cognitive functioning, e) role functioning, f) social functioning, g) fatigue, h) pain, i) dyspnea, j) appetite loss, and k) insomnia. In certain embodiments, said aspect of quality of life is fatigue.

In certain embodiments, said 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, said compound is a steroid that suppresses complement.

In certain embodiments, said compound is selected from the group consisting of antibodies, active antibody fragments, soluble complement inhibitory compounds, proteins, soluble complement inhibitors with a lipid tail, protein fragments, peptides, small organic compounds, 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, said compound is an antibody or an active antibody fragment. In certain embodiments, said antibody or active antibody fragment is selected from the group consisting of a) polyclonal antibodies, b) monoclonal antibodies, c) single chain antibodies, d) chimeric antibodies, e) humanized antibodies, f) Fabs, g) F(ab')s, h) $F(ab')_2$s, i) Fvs, j) diabodies, and k) human antibodies.

In certain embodiments, said antibody or an active antibody fragment thereof binds C5. In certain embodiments, said antibody or active antibody fragment blocks C5 cleavage. In certain embodiments, said antibody or active antibody fragment inhibits the formation of C5b-9. In certain embodiments, said antibody is eculizumab. In certain embodiments, said antibody or active antibody fragment is administered for at least 6

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months. In certain embodiments, said patient has aplastic anemia or myelodysplastic syndrome.

In certain embodiments, said antibody that binds C5 or an active antibody fragment thereof is administered in a single unit dosage form. In certain embodiments, the single unit dosage form is a 300 mg unit dosage form. In certain embodiments, the single unit dosage form is lyophilized. In certain embodiments, the single unit dosage form is a sterile solution. In certain embodiments, the single unit dosage form is a preservative free formulation. In certain embodiments, the 300 mg single-use dosage form comprises 30 ml of a 10 mg/ml sterile, preservative free solution.

In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises an altered constant region, wherein said antibody or antigen-binding fragment exhibits decreased effector function relative to an anti-CDCP1 antibody with a native constant region. In certain embodiments, decreased effector function comprises one or more properties of the following group: a) decreased antibody-dependent cell-mediated cytotoxicity (ADCC), and b) decreased complement dependent cytotoxicity (CDC), compared to an anti-CDCP1 antibody with a native constant region. In certain embodiments, the altered constant region comprises a G2/G4 construct in place of the G1 domain.

In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region comprises one or more CDR regions having an amino acid sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7, and wherein the light chain variable region comprises one or more CDR regions having an amino acid sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10. In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region consists of SEQ ID NO: 1 and the light chain variable region consists of SEQ ID NO: 3. In certain embodiments, the pharmaceutical composition comprises eculizumab. In certain embodiments, the pharmaceutical composition comprises pexelizumab. In certain embodiments, the antibody that binds C5 or an active antibody 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.

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In certain embodiments, said patient is anemic. In certain embodiments, said patient remains anemic following treatment. In certain embodiments, said patient has a hemoglobin level less than i) 14 g/dL if a man or ii) 12 g/dL if a woman. In certain embodiments, said patient has a hemoglobin level less than i) 13 g/dL if a man or ii) 11 g/dL if a woman. In certain embodiments, said patient has a hemoglobin level less than i) 12 g/dL if a man or ii) 10 g/dL if a woman.

In certain embodiments, said health-adjusted life expectancy is measured according to a unit selected from the group consisting of Years of potential life lost, Disability-free life expectancy, Health-adjusted life year, Quality adjusted life year, Healthy years equivalents, Healthy days gained, Episode-free day, Q-TWiST, Health Utilities Index, or Years of healthy life.

In certain embodiments, the health-adjusted life expectancy in a subject is prolonged by at least one day. In certain embodiments, the health-adjusted life expectancy in a subject is prolonged by at least week. In certain embodiments, the health-adjusted life expectancy in a subject is prolonged by at least one month. In certain embodiments, the health-adjusted life expectancy in a subject is prolonged by at least one year.

In certain embodiments, the pharmaceutical composition is in a single unit dosage form. In certain embodiments, the single unit dosage form is a 300 mg unit dosage form. In certain embodiments, the pharmaceutical composition is lyophilized. In certain embodiments, the pharmaceutical composition is a sterile solution. In certain embodiments, the pharmaceutical composition is a preservative free formulation. In certain embodiments, the pharmaceutical composition comprises a 300 mg single-use formulation of 30 ml of a 10 mg/ml sterile, preservative free solution. In certain embodiments, the pharmaceutical composition comprises an antibody that binds C5 or an active antibody fragment thereof.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-B show that eculizumab treatment decreases intravascular hemolysis and increases PNH type III erythrocytes. Figure 1A shows the degree of intravascular hemolysis in PNH patients, demonstrated by mean lactate dehydrogenase (LDH) levels. Figure 1B shows the mean proportion of PNH type III erythrocytes assessed for placebo- and eculizumab-treated patients.

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Figure 2 shows the effect of eculizumab treatment on transfusion requirements in PNH patients. This is a Kaplan-Meier plot of time to first transfusion for eculizumab- and placebo-treated patients from baseline through week 26. The P value is from the log rank analysis.

Figure 3 shows the effect of eculizumab on fatigue assessed by the FACIT-Fatigue Instrument. Quality of Life scores were assessed using the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) instrument. Values for change from baseline to 26 weeks represent least-square means. A positive change indicates an improvement and a negative change indicates deterioration in the FACIT-Fatigue measures of quality of life.

DETAILED DESCRIPTION

I. Definitions

The term "derived from" means "obtained from" or "produced by" or "descending from".

The term "genetically altered antibodies" means antibodies wherein the amino acid sequence has been varied from that of a native antibody. Because of the relevance of recombinant DNA techniques to this application, one need not be confined to the sequences of amino acids found in natural antibodies; antibodies can be redesigned to obtain desired characteristics. The possible variations are many and 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 in the constant region will, in general, be made in order to improve or alter characteristics, such as complement fixation, interaction with membranes and other effector functions. Changes in the variable region will be made in order to improve the antigen binding characteristics.

The term "an antigen-binding fragment of an antibody" refers to any portion of an antibody that retains the binding utility to the antigen. An exemplary antigen-binding fragment of an antibody is the heavy chain and/or light chain CDR, or the heavy and/or light chain variable region.

The term "homologous," in the context of two nucleic acids or polypeptides refers to two or more sequences or subsequences that have at least about 85%, at least 90%, at least 95%, or higher nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using the following sequence comparison

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method and/or by visual inspection. In certain embodiments, the "homolog" exists over a region of the sequences that is about 50 residues in length, at least about 100 residues, at least about 150 residues, or over the full length of the two sequences to be compared.

Methods of determining percent identity are known in the art. "Percent (%) sequence identity" with respect to a specified subject sequence, or a specified portion thereof, may be defined as the percentage of nucleotides or amino acids in the candidate derivative sequence identical with the nucleotides or amino acids in the subject sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent sequence identity, as generated by the program WU-BLAST-2.0a19 (Altschul et al., J. Mol. Biol. 215:403-410 (1997); http://blast.wustl.edu/blast/README.htm- l) with search parameters set to default values. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched. A "% identity value" is determined by the number of matching identical nucleotides or amino acids divided by the sequence length for which the percent identity is being reported.

II. Overview

The present disclosure relates to a method of treating paroxysmal nocturnal hemoglobinuria ("PNH"), more specifically to improving certain aspects of quality of life which are impaired in PNH patients, 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

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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.

III The Complement System

The complement system, useful complement inhibitors, and use of these inhibitors to treat PNH and other patients are more fully described in PCT Patent Application PCT/US2005/003225 filed February 3, 2005 and published as International Publication Number WO 2005/074607 A2 on August 18, 2005, the contents of which are incorporated herein by reference in their entirety.

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. 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

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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).

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.

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.

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.

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.

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

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(Yamamoto and Gewurz, 1978; Vogt et al., 1989) 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.

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.

IV Measures of Quality of Life

Various measurements exist to assess quality of life and the effect of medical interventions on quality of life for example the Mini-Mental State Examination (MMSE), the Short Test of Mental Status, the European Organization for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire, the FACIT questionnaires and subscales including fatigue and anemia, the Likert Scale, and Borg Scale (Tombaugh, et al., J. Am. Geriatr. Soc. 40:922, 1992; Cummings, JAMA. 269(18):2420, 1993; Crum, et al., JAMA. 269(18):2386, 1993; Folstein, et al., J. Psychiat. Res. 12:189,1975; Kokmen, et al., Mayo Clin. Proc. 62:281, 1987; Tang-Wai, et al., Arch. Neurol. 60:1777, 2003; Tamburini, Ann.

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Oncol. 12(Suppl. 3):S7, 2001; Webster et al., Health and Quality of Life Outcomes. 1:79, 2003, www.hqlo.com/content/I/I/79; Grant, et al., Chest. 116:1208, 1999; and www.qolid.org). Any of these measurements may be used to assess the change in quality of life due to administration of a compound which inhibits complement or inhibits formation of C5b-9.

In certain embodiments, improvement in quality of life due to administration of a compound which inhibits complement or inhibits formation of C5b-9 is measured by the Functional Assessment of Chronic Illness Therapy (FACIT) Measurement System. In certain embodiments, improvement in quality of life is measured by: a) full scales; b) stand-alone subscales; and c) symptom indices.

In certain embodiments, improvement in quality of life due to administration of a compound which inhibits complement or inhibits formation of C5b-9 is measured by a European Organization for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire. In certain embodiments, the EORTC questionnaire is the QLQ-C30.

In certain embodiments, improvement in quality of life is measured by the health-adjusted life expectancy (HALE) index as described in Wilkins, R. and Adams, OB., Am J Public Health, 73:1073-1080 (1983). Health-adjusted life expectancy is an average of the quality-adjusted life years (QALY) for a given population and can be used to evaluate the therapeutic value of a medical intervention. Quality-adjusted life years is a health index that weighs each year of life on a scale from 1 to 0 (Weinstein MC and Stason WB, N Engl J Med, 296:716-721 (1977)). Perfect health is rated as 1, death is rated as 0, and disability and pain are rated based on severity. QALY is determined by multiplying the number of years at each health status.

In certain embodiments, improvement in quality of life is measured by the following instruments: Years of potential life lost, Disability-free life expectancy, Health-adjusted life year, Quality adjusted life year, Healthy years equivalents, Healthy days gained, Episode-free day, Q-TWiST, Health Utilities Index, and Years of healthy life. These measurements account for both changes in mortality as well as changes in morbidity and disability. Any of these measurements may be used to assess the change in quality of life due to administration of a compound which inhibits complement or inhibits formation of C5b-9.

In one embodiment, the disclosed methods improve the quality of life of a patient for at least one day, at least one week, at least two weeks, at least three weeks, at least one

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month, at least two months, at least three months, at least 6 months, at least one year, at least 18 months, at least two years, at least 30 months, or at least three years, or the duration of treatment.

In certain embodiments, the symptoms used to measure quality of life are scaled for intensity. In certain embodiments, the symptoms are scaled for frequency. In certain embodiments, the symptoms are scaled for intensity and frequency.

In certain aspects, the application provides a method for prolonging the health-adjusted life expectancy of a subject comprising administering to the subject a compound which inhibits complement or inhibits formation of C5b-9. The above measurements account for both changes in mortality as well as changes in morbidity and disability. Any of these measurements may be used to assess the change in quality-adjusted life expectancy due to administration of a compound which inhibits complement or inhibits formation of C5b-9.

In one embodiment, the disclosed methods prolong the health-adjusted life expectancy in a subject by at least one day, at least one week, at least two weeks, at least three weeks, at least one month, at least two months, at least three months, at least 6 months, at least one year, at least 18 months, at least two years, at least 30 months, or at least three years as measured by the health-adjusted life expectancy (HALE) index as described in Wilkins et al. Am J Public Health, 73:1073-1080 (1983). Health-adjusted life expectancy is an average of the quality-adjusted life years (QALY) for a given population and can be used to evaluate the therapeutic value of a medical intervention. Quality-adjusted life years is a health index that weighs each year of life on a scale from 1 to 0 (Weinstein et al., N Engl J Med, 296:716-721 (1977)). Perfect health is rated as 1, death is rated as 0, and disability and pain are rated based on severity. QALY is determined by multiplying the number of years at each health status.

V Inhibitors of the Complement Cascade

In certain embodiments, 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. 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, a serine protease inhibitor, or a protein. These agents include, but are not limited to, small organic molecules, RNA

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aptamers including ARC187 (which is commercially available from Archemix Corp., Cambridge, Mass.), L-RNA aptamers, Spiegelmers, antisense compounds, 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.

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. In certain embodiments, a complement inhibitor 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. Those of skill in the art recognize the above as some, but not all, of the known methods of inhibiting complement and its activation.

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 antibody that binds C5. 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.

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.

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

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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.

An antibody capable of inhibiting complement such as an antibody that binds C5 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 antibody that binds C5) 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.

Specific antibodies capable of inhibiting complement, such as an antibody that binds C5, 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 antibody that binds C5 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

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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 antibody that binds C5) if cleavage is interfered with by an antibody capable of inhibiting complement such as an antibody that binds C5.

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 antibody that binds C5 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 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.

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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, 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.

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" 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.

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

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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.

"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 antibody that binds C5 of the application. Alternatively, the target binding region is derived from a protein that binds C5.

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).

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)).

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

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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.

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.

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.

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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.

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.

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 antibody that binds C5 to bind C5).

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

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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).

VI Methods of Treatment

Methods of the application may be used to treat paroxysmal nocturnal hemoglobinuria associated symptoms. Methods of the application may be used to treat anemia associated symptoms. Treatment of paroxysmal nocturnal hemoglobinuria and/or anemia may be administered by standard means. Treatments of the application may be used in combination with other treatments of the application or known treatments for paroxysmal nocturnal hemoglobinuria and/or anemia. Treatments of the application may be co-administered with other treatments that treat symptoms of paroxysmal nocturnal hemoglobinuria and/or anemia.

VII Pharmaceutical Formulations and Uses

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

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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.

In certain embodiments, the pharmaceutical composition is in a single unit dosage form. In certain embodiments, the single unit dosage form is a 300 mg unit dosage form. In certain embodiments, the pharmaceutical composition is lyophilized. In certain embodiments, the pharmaceutical composition is a sterile solution. In certain embodiments, the pharmaceutical composition is a preservative free formulation. In certain embodiments, the pharmaceutical composition comprises a 300 mg single-use formulation of 30 ml of a 10 mg/ml sterile, preservative free solution. In certain embodiments, the antibody is administered according to the following protocol:

600 mg via 25 to 45 minute IV infusion every 7 ± 2 days for the first 4 weeks, followed by 900 mg for the fifth dose 7 ± 2 days later, then 900 mg every 14 ± 2 days thereafter. Antibody is administered via IV infusion over 25 to 45 minute.

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. In certain embodiments, complement inhibitors such as eculizumab may be administered via IV infusion and diluted to a final concentration of 5 mg/ml prior to administration.

Administration of the antibodies capable of inhibiting complement such as an antibody that binds C5 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

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desired and practicable for the particular antibody capable of inhibiting complement to be administered. Antibodies capable of inhibiting complement such as an antibody that binds C5 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 antibody that binds C5 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 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.

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.

For the treatment of hemolytic diseases such as PNH by systemic administration of an antibody capable of inhibiting complement such as an antibody that binds C5 (as opposed to local administration), administration of a large initial dose is specific, i.e., a

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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 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. 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 application.

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

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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 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).

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)]

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

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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.

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 hemolytic diseases such as PNH. 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 hemolytic diseases treatments known in the art.

In certain aspects, the application provides kits comprising a pharmaceutical composition of the application. In some embodiments, the kit further comprises at least one component of a closed sterile system. Components of the closed sterile system include, but are not limited to, needles, syringes, catheter based syringes, needle based injection devices, needle-less injection devices, filters, tubing, valves and cannulas. In a related embodiment, the kit comprise components for the removal of a preservative from the composition. Such components include filters, syringes, vials, containers, tubing, etc.

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EXEMPLIFICATION

METHODS

Patient Selection

The TRIUMPH trial consisted of a 2-week screening period, an observation period of up to 3 months duration, and a 26-week treatment period.

During the screening period, patients were evaluated with respect to inclusion and exclusion criteria. Men and women, 18 years or older, diagnosed as having PNH with a type III erythrocyte population of ≥10%, and who had received at least 4 transfusions in the previous 12 months were eligible. Concomitant administration of erythropoietin, immunosuppressants, corticosteroids, coumadin, low molecular weight heparin, iron supplements, and folic acid were not reasons for exclusion, provided the doses were steady prior to the first visit and throughout the duration of the study. Because of the increased frequency of neisserial infections in individuals genetically deficient in terminal complement proteins, all patients were vaccinated against *Neisseria meningitides*. Patients were to avoid conception. The protocol was approved by an Investigational Review Board at each clinical site and written informed consent was obtained from all patients enrolled.

Patients transfused with a mean pre-transfusion hemoglobin level >10.5 g/dL over the previous 12 months, and those who showed evidence of having a suppressed immune response, complement deficiency, or active bacterial infection, including any history of meningococcal disease, were excluded from the study. Patients were also not eligible if they had previously received a bone marrow transplant or if they had participated in another trial or received another investigational drug within 30 days of the first visit. An individualized transfusion algorithm was calculated for each patient based on their prior 12-month transfusion history; the written algorithm documented the number of packed red blood cell (PRBC) units transfused for given hemoglobin values and served as a prospectively determined guide for transfusion during observation and treatment periods.

Each patient considered eligible entered an observation period of up to 13 weeks in order to confirm their PBRC transfusion dependence. At least one transfusion --termed the "qualifying" transfusion-- during the 13-week observation period at a hemoglobin value at or below 9 g/dL with symptoms, or at or below 7 g/dL with or without symptoms, in accordance with the transfusion algorithm indicated for each patient, was a requirement to

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proceed to randomization. The hemoglobin value at which each individual's qualifying transfusion was administered, was defined as the hemoglobin "set point" for that individual for the purpose of the primary efficacy variable. A platelet count ≥100,000/mL and a LDH level ≥1.5 times the upper limit of the normal range were also required either at screening or during the observation period for eligibility.

Study Design

Patients were randomly assigned on a one-on-one basis to receive either placebo or eculizumab (Soliris™, Alexion Pharmaceuticals, Inc.) within 10 days of the qualifying transfusion. Study medication was dosed in a blinded fashion as follows: 600 mg eculizumab for patients randomly assigned to active drug, or placebo for those patients randomly assigned to placebo, respectively via IV infusion every 7±1 days for 4 doses; followed by 900 mg eculizumab, or placebo, respectively, via IV infusion 7±1 day later; followed by a maintenance dose of 900 mg eculizumab, or placebo, respectively, via IV infusion every 14±2 days for a total of 26 weeks of treatment.

Measures of Clinical Efficacy

There were two co-primary endpoints in the study: (1) stabilization of hemoglobin levels, defined as a hemoglobin value maintained above the individual hemoglobin set point in the absence of transfusions for the entire 26-week treatment period, and (2) reduction in units of PRBCs transfused during the 26-week treatment phase of the study. The trigger for transfusion during the study period remained unchanged for each patient, as compared with their care before entry into the study: patients received blood transfusions when they had symptoms resulting from anemia and reached their individualized, pre-determined "set point". Pre-specified secondary endpoints included transfusion avoidance, hemolysis as measured by LDH area under the curve from baseline to 26 weeks, and QoL changes as measured from baseline to 26 weeks using the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) instrument.[13] Pre-specified exploratory analyses included assessment of the EORTC QLQ-C30 instrument,[14] the change in LDH from baseline through week 26, and thrombosis. Other pre-specified measurements included pharmacokinetics, pharmacodynamics, and immunogenicity of eculizumab. Time to first transfusion during the 26-week treatment phase and the proportion of PNH type III blood cells were also assessed.

Safety Assessments

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Treatment-emergent adverse events, clinical laboratory tests (e.g., serum chemical analyses and complete blood counts), electrocardiogram data, and vital signs were assessed. Adverse events were defined using the MedDRA preferred terms and tabulated as incidence rates per treatment group.

Statistical Analysis

For co-primary endpoints, analyses were performed according to the intention to treat using the data from all patients who were randomized and received study drug; stabilization of hemoglobin levels was analyzed using the Fisher's exact test and total PRBC units transfused were analyzed with the Wilcoxon's rank sum test. For comparison of treatment effect on transfusion avoidance, the Fisher's exact test was used on the incidence and the log rank test was used for time to first transfusion. For LDH area under the curve the Wilcoxon's rank sum test was used.

Quality of life measure of fatigue was assessed using the scoring guidelines for the FACIT-Fatigue instrument.[15] Assessment of quality of life measures based on the EORTC QLQ-C30 instrument was conducted in accordance with the appropriate scoring guidelines.[16] The changes of FACIT-Fatigue and EORTC QLQ-C30 scores from baseline through 26 weeks were analyzed using a mixed model, with baseline as covariate, treatment and time as fixed effects, and patient as a random effect. Changes in LDH levels and PNH type III erythrocytes from baseline through 26 weeks were analyzed using the same mixed model. Two-sided tests were used for all analyses. The adverse events and long-term safety checklist were tabulated separately and compared between treatments using the Fisher's exact test. A p-value ≤ 0.05 was considered to be statistically significant.

RESULTS

Characteristics of Patients

A total of 115 PNH patients were screened. Six patients did not meet the inclusion/exclusion criteria during the screening period. Twenty-one other patients did not receive a qualifying transfusion and were not randomized into the treatment phase. One patient who did not meet the inclusion criteria was inadvertently randomized, but did not receive study medication. Thus 87 hemolytic PNH patients (35 men and 52 women) were enrolled and randomized to receive either eculizumab (N=43) or placebo (N=44), exceeding the original target of 75 randomized patients.

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Patient characteristics were similar in the eculizumab- and placebo-treated cohorts: median age, 41 (range 20-85) and 35 (range 18-78) years; median duration of PNH, 4.2 (range 0.8 to 29.7) and 9.2 (range 0.4 to 38.3) years; patients with history of aplastic anemia, 4 and 11; history of myelodysplastic syndrome, 1 and 0; and history of thrombosis, 9 (16 events) and 8 (11 events). Stable usage of concomitant medications at baseline in the eculizumab- and placebo-treated groups included the following: erythropoietin, 3 patients and 0 patients; cyclosporine, 1 and 1; anticoagulants (coumarins or heparins) 21 and 11; and steroids (glucocorticoids or androgenic steroids), 12 and 12, respectively.

Of the 87 patients randomized, 85 completed the trial. Two patients who did not complete the trial had been randomized to the eculizumab arm: one patient discontinued due to the inconvenience of travel to the study site and the second patient became pregnant. Ten patients in the placebo-treatment group discontinued infusions, in all cases due to perceived lack of efficacy, but they remained in the study for monitoring purposes.

Pharmacokinetics/Pharmacodynamics

In 42 of the 43 eculizumab-treated patients the levels of drug during the maintenance period (900 mg every 2 weeks±2 days) were sufficient to completely block serum hemolytic activity (mean trough value at week 26 of 101.8 μg/mL). A single patient did not sustain therapeutic trough levels of eculizumab and demonstrated a breakthrough in complement blockade during the last few days of each dosing interval. These breakthroughs were clinically manageable and quickly resolved following the next dose.

Hemolytic Efficacy Variables

The 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 (Figure 1A). The median LDH area under the curve during the 26-week study period was reduced 85.8% in eculizumab- relative to placebo-treated patients ($P<0.001$). The mean LDH level decreased from 2199.7±157.7 IU/L at baseline to 327.3±67.6 IU/L by 26 weeks in eculizumab-treated patients while levels in placebo-treated patients remained consistently elevated with values of 2259.0±158.5 IU/L at baseline and 2418.9±140.3 IU/L at 26 weeks ($P<0.001$, for eculizumab versus placebo). A second biochemical measure of hemolysis, serum aspartate aminotransferase (AST), also showed a statistically significant improvement following eculizumab- versus placebo-treatment (data not shown). Haptoglobin levels were statistically significantly increased in eculizumab- as compared to placebo-treated patients

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but mean levels of haptoglobin were still below normal levels in eculizumab-treated patients (data not shown).

Figure 1A shows the degree of intravascular hemolysis in PNH patients, demonstrated by mean lactate dehydrogenase (LDH) levels (±standard error) from baseline (study week 0) to week 26 for both eculizumab- and placebo-treated patient populations. Screening occurred up to 3 months prior to study week 0. The upper limit of the normal range (103-223 IU/L) for LDH is indicated by a dashed line. LDH was reduced to a mean level just above the upper limit of normal at week 26 for eculizumab-treated patients; 15 of 41 patients who completed the study demonstrated LDH levels within the normal range. All placebo-treated patients remained at least 5 times above the upper limit of normal at week 26. The P value is based on a mixed model analysis from baseline through week 26. Figure 1B shows the mean proportion (±standard error) of PNH type III erythrocytes assessed for placebo- and eculizumab-treated patients. The screening visit occurred up to 3 months prior to study week 0. The P value is based on a mixed model analysis from baseline through week 26.

A corollary to the reduction in intravascular hemolysis during eculizumab treatment was an observed increase in the PNH type III erythrocyte population (Figure 1B). The mean proportions of type III erythrocytes increased from 28.1±2.0% at baseline to 56.9±3.6% by week 26 for eculizumab-treated patients while proportions in the placebo group remained constant with mean values of 35.7±2.8% before treatment to 35.5±2.8% at 26 weeks ($P<0.001$, for eculizumab versus placebo). By contrast, the proportions of PNH type III granulocytes and monocytes did not change significantly between the treatment groups during the treatment period and were greater than 90% at week 26.

CLINICAL EFFICACY

Co-primary endpoints

The co-primary efficacy endpoints in the TRIUMPH trial were stabilization of hemoglobin levels and reduction in PRBC units transfused. At the end of the treatment period, 48.8% of eculizumab-treated patients had maintained levels of hemoglobin above the pre-specified set-point (median set-point value of 7.7 g/dL for both treatment groups) in the absence of transfusions, whereas stabilization of hemoglobin did not occur in any of the patients in the placebo group ($P<0.001$; Table 1). By week 26, the median of PRBC units transfused per patient was 0 in the eculizumab group and 10.0 in the placebo cohort

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(P<0.001), while the mean of PRBC units transfused was 3.0 and 11.0 in the eculizumab and placebo cohorts, respectively. In the 6-month period prior to the study, the median of PRBC units transfused per patient was 9.0 in the eculizumab cohort and 8.5 in placebo patients while the mean of PRBC units transfused was 9.6±0.6 and 9.7±0.7, respectively. Mean hemoglobin levels were similar between the treatment groups at baseline (10.0±1.8 g/dL in eculizumab-treated patients and 9.7±1.8 g/dL in placebo-treated patients) and did not substantially change by week 26 (10.1±2.5 g/dL and 8.9±2.2 g/dL in eculizumab and placebo cohorts, respectively).

The median time to first transfusion was not reached during the study period in eculizumab-treated patients (it was greater than 26 weeks) while the placebo group reached the median time to first transfusion in only 4 weeks (P<0.001; Figure 2). Transfusion avoidance was achieved in 51.2% and 0% of the eculizumab and placebo cohorts, respectively (P<0.001). By the end of the 26-week treatment period, the total PRBC units transfused were 131 in eculizumab-treated patients versus 482 in the placebo group (Table 1). By contrast, in the 6-month period prior to the study, total PRBC units transfused in the eculizumab- and placebo-cohorts were 413 and 417, respectively.

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Table 1

Stabilization of Hemoglobin Levels and Reduction

in Transfusion Requirements during Eculizumab Treatment

	PRE-TREATMENT HISTORY*		TREATMENT PERIOD		
	Placebo	Eculizumab	Placebo	Eculizumab	P Value
†Stabilization of Hemoglobin Levels in the absence of transfusions (percent of patients)	NA	NA	0	48.8	<0.001‡
†Units Transfused per Patient					
Median	8.5	9.0	10	0	<0.001§
Mean±SE	9.7±0.7	9.6±0.6	11.0±0.83	3.0±0.67	
Total Units Transfused per Group	417	413	482	131	

*Twelve month historical transfusion data normalized to 6 months

†Co-primary endpoints

‡Base on 2-sided Fisher's exact test

§Based on Wilcoxon's rank sum test

NA, not applicable

Improvements in quality of life measures

Assessments of quality of life in PNH patients during eculizumab treatment were performed using two different instruments, FACIT-Fatigue and the EORTC QLQ-C30. Eculizumab-treated patients showed a mean increase (improvement) in the FACIT-Fatigue score of 6.4±1.2 points from baseline to 26-weeks while the mean score in placebo patients decreased by 4.0±1.7 points, a total difference between the treatment groups of 10.4 points (Figure 3). Mixed model analysis of covariance demonstrated a statistically significant difference between treatment groups (P<0.001).

For the EORTC instrument, improvements were observed with eculizumab-treatment in each subscale. Statistically significant improvements with eculizumab-compared with placebo-treated groups were observed in the following quality of life

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subscales (Table 2): global health status (P<0.001), physical functioning (P<0.001), emotional functioning (P=0.008), cognitive functioning (P=0.002), role functioning (P<0.001), social functioning (P=0.003), fatigue (P<0.001), pain (P=0.002), dyspnea (P<0.001), appetite loss (P<0.001), and insomnia (P=0.014). The improvements with eculizumab treatment in the other scales, including nausea and vomiting, diarrhea, constipation, and financial difficulties, did not reach statistical significance.

Table 2

Effect of Eculizumab Treatment on

Quality of Life Assessed by the EORTC QLQ-C30 Instrument

	Mean Change from Baseline to Week 26*			
	Placebo	Eculizumab	Absolute Difference	P Value†
Global Health Status	-8.5	10.9	19.4	<0.001
Functional				
Role	-6.9	17.9	24.8	<0.001
Social	2.0	16.7	14.6	=0.003
Cognitive	-6.1	7.9	14.0	=0.002
Physical	-3.5	9.4	13.0	<0.001
Emotional	-3.7	7.5	11.2	=0.008
Symptoms/Single Items				
Fatigue	10.0	-16.9	27.0	<0.001
Pain	5.3	-12.3	17.6	=0.002
Dyspnea	8.9	-7.9	16.9	<0.001
Appetite Loss	3.3	-10.3	13.6	<0.001
Insomnia	4.9	-7.9	12.8	=0.014

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Financial Difficulties	0.0	-10.3	10.3	=0.186
Constipation	0.0	-6.3	6.3	=0.199
Nausea/vomiting	2.8	-0.4	3.2	=0.056
Diarrhea	5.7	4.8	0.9	=0.147

*A positive change indicates an improvement in global health status and functional scales and a negative change indicates an improvement in symptom and single item scales.

†Based on a mixed analysis-of-covariance model with visit as a fixed effect, patient as a random effect and baseline as a covariate.

Relationship between FACIT-Fatigue quality of life and intravascular hemolysis

In order to determine if there was a treatment independent relationship between the FACIT-Fatigue quality of life instrument and intravascular hemolysis, an analysis was performed whereby the mean LDH level (through the 26 week study period) for each TRIUMPH patient was analyzed as a function of the patient's respective mean change in FACIT-Fatigue score from baseline (through the 26 week study period) (see Table 3). For this analysis, mean levels of LDH were divided into 4 groups that included: normal levels, 1-2 times the upper limit of normal (ULN), 2-10 times the upper limit of normal, and greater than 10 times the upper limit of normal. The analysis demonstrated that patients who maintained normal LDH levels throughout the study experienced significant improvements in fatigue when compared to patients who had incrementally higher levels of LDH throughout the study (p=0.0048). These data establish a clear link between increasing intravascular hemolysis as measured by LDH levels and decreased quality of life as measured by the FACIT-Fatigue quality of life instrument.

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Table 3

Relationship between FACIT-Fatigue and Intravascular Hemolysis

		Change of FACIT from Baseline		
Treatment Group	LDH Category	< 4	>= 4	P Value
Combined	Normal	4 (30.77%)	9 (69.23%)	.0048
	1 - 2 X ULN	16 (61.54%)	10 (38.46%)	
	2 - 10 X ULN	18 (72.00%)	7 (28.00%)	
	> 10 X ULN	16 (80.00%)	4 (20.00%)	

Safety

There were no deaths in the study. Serious adverse events (SAEs) were reported for 13 patients, of which 4 occurred in the eculizumab-treated cohort and 9 were in the placebo-treated cohort (see Table 4). All patients recovered without sequelae.

The most common AEs reported for eculizumab-treated patients were headache, nasopharyngitis, back pain, and upper respiratory tract infection. Headache and back pain occurred more commonly in the eculizumab-treatment group compared with the placebo group. However, the increase in headaches was limited to the first 2 weeks of therapy and was mild to moderate. There were no statistically significant differences in incidents rates between treatment groups for any AEs reported.

One episode of thrombosis (Budd-Chiari) occurred in a placebo-treated patient. There were no thromboses in eculizumab-treated patients.

Only one patient showed a detectable level of anti-eculizumab antibodies in the eculizumab-treated cohort; this response was weak (did not titrate), occurred at only one time point and did not result in a disruption of complement blockade.

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Table 4

Adverse Event Reporting

SERIOUS ADVERSE EVENTS*	**Placebo Patients n (percent)**	**Eculizumab Patients n (percent)**
Total	9 (20.5)	4 (9.3)
Eculizumab treatment emergent		
Exacerbation of PNH	3 (6.8)	1 (2.3)
Renal colic	0	1 (2.3)
Lumbar sacral disc prolapse	0	1 (2.3)
Alpha streptococcal bacteremia	0	1 (2.3)
Central line infection and UTI	1 (2.3)	0 (0)
Upper respiratory tract infection	1 (2.3)	0 (0)
Probable viral infection	1 (2.3)	0 (0)
Neutropenia	1 (2.3)	0 (0)
Cellulitis / folliculitis / neutropenia	1 (2.3)	0 (0)
Anemia and pyrexia	1 (2.3)	0 (0)
MOST FREQUENT ADVERSE EVENTS*†		
Headache§	12 (27.3)	19‡ (44.2)
Nasopharyngitis	8 (18.2)	10 (23.3)
Upper respiratory tract infection	10 (22.7)	6 (14)
Back pain	4 (9.1)	8 (18.6)
Nausea	5 (11.4)	7 (16.3)
Cough	4 (9.1)	5 (11.6)
Diarrhea	5 (11.4)	4 (9.3)
Arthralgia	5 (11.4)	3 (7.0)
Abdominal pain	5 (11.4)	2 (4.7)
Dizziness	5 (11.4)	2 (4.7)
Vomiting	5 (11.4)	2 (4.7)
Fatigue	1 (2.3)	5 (11.6)

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Viral infection	5 (11.4)	1 (2.3)

*By preferred terms

†Occurring in 10% or more of patients

‡Sixteen of 19 patients experienced headache within 48 hours of infusion

§Following the first 2 weeks of dosing, 20.9% of eculizumab- and 22.7% of placebo-treated patients experienced headache

DISCUSSION

Chronic intravascular hemolysis with periods of acute exacerbation are the classical manifestations of PNH, frequently resulting in anemia, the need for transfusions to sustain hemoglobin levels, and deterioration in quality of life. In the phase III pivotal TRIUMPH study, we examined the effect of terminal complement inhibition with eculizumab on hemoglobin levels and transfusion requirements in patients with PNH. Forty-nine percent of patients treated with eculizumab over the 6-month period demonstrated stabilization of hemoglobin in the absence of transfusions compared to no patients in the placebo arm of the trial. Over 50% of eculizumab-treated patients were transfusion independent during the entire study compared to none in the placebo arm, and the overall mean transfusion rate was reduced by 73%. Moreover, even in patients who did not achieve transfusion independence, eculizumab treatment was associated with a 44% reduction in the rate of transfusion (data now shown).

Lactate dehydrogenase, a biochemical marker of hemolysis in PNH,[9] was immediately and consistently decreased in all eculizumab-treated patients, while patients in the placebo cohort continued to hemolyze with levels of LDH exceeding 5 times the upper limit of the normal range in all patients at the study end. Levels of LDH were reduced into the normal range in approximately one-third of eculizumab-treated patients, while the remainder stabilized at a level just above the upper limit of normal suggesting residual low level hemolysis in some patients. Levels of haptoglobin, a more sensitive marker of the presence of cell free hemoglobin in the circulation, were undetectable in most patients. Low level hemolysis in a subset of eculizumab-treated patients is possibly due to an inherent decrease in survival of these cells or C3b-mediated, extravascular clearance of PNH erythrocytes through the reticuloendothelial system.[17]

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Before eculizumab treatment, hemoglobin levels in study patients were artificially maintained by frequent transfusion. Therefore, stabilization of hemoglobin levels with a concomitant cessation of or reduction in transfusions represents a net increase in endogenous hemoglobin levels. Our data suggest that resolution of hemolysis with eculizumab results in a new steady state hemoglobin level determined by a balance between the extent of the underlying bone marrow dysfunction, the number of PNH erythrocytes that are preserved by eculizumab therapy and the new level (if any) of transfusion requirement.

Patients with PNH generally experience markedly impaired quality of life characterized by fatigue, anemia, thrombosis, and pulmonary hypertension as well as smooth muscle dystonia including abdominal pain, dysphagia, and erectile dysfunction.[9,10,18] These symptoms have been attributed to both excessive intravascular hemolysis and downstream scavenging of nitric oxide by cell free hemoglobin in plasma. The reduction of intravascular hemolysis in eculizumab-treated patients in the current study was associated with significant improvements in the fatigue component of quality of life relative to placebo-treated patients as assessed via the FACIT-Fatigue instrument. Further, eculizumab therapy was associated with a median increase of 6.4 points over baseline values established before treatment. It has previously been demonstrated that an increase of 3 or more points from baseline represents a clinically important difference in this quality of life instrument.[19] Patients who received eculizumab also experienced a significant improvement in most domains of the EORTC QLQ-30 relative to the placebo-treated cohort including global health status, physical functioning, emotional functioning, cognitive functioning, role functioning, social functioning, fatigue, pain, dyspnea, appetite loss, and insomnia. Improvement in the fatigue component of the EORTC QLQ-30 provides support for the improvement demonstrated in the FACIT-Fatigue instrument during eculizumab therapy. Importantly, these improvements in quality of life in the eculizumab-treated patients occurred despite similar levels of erythrocyte hemoglobin in the two treatment groups, further supporting the contribution of hemolysis per se, as opposed to anemia, in mediating the poor quality of life in PNH patients. Clinical assessment of additional life quality-related symptoms of PNH, including abdominal pain, dysphagia, and erectile dysfunction, have also been reported to improve during eculizumab therapy.[20]

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Eculizumab was safe and well-tolerated. There were no deaths in the study and only a single thrombotic event which occurred in a placebo patient in a site (the hepatic veins) which is typical of the thrombosis in PNH. The relative brief duration of this study was not sufficient to address the relevant issue of a possible protection from thrombosis by terminal complement inhibition with eculizumab.

Adverse events were generally mild with headache occurring at increased frequency in the eculizumab-treated patients; however, this increased frequency did not persist following the first two doses of therapy. There were 4 SAEs in the eculizumab treatment group and 9 SAEs in the placebo group. There was no evidence of increased infection risk in eculizumab-treated patients during the study period. One eculizumab-treated patient showed a low level of anti-eculizumab antibodies at one time point during the study which did not persist and did not result in a disruption of complement blockade. There were no AEs associated with eculizumab withdrawal in the 2 eculizumab-treated patients who did not complete the trial. Additional safety assessments, as well as efficacy measures, are being examined in an ongoing multi-center, open-label Phase III safety trial of eculizumab (SHEPHERD) in approximately 95 patients with PNH.

Results from the current randomized, double-blind, placebo-controlled, global study show that terminal complement inhibition with eculizumab appears to be a safe and effective therapy for patients with the rare disorder PNH. Treatment with eculizumab reduced intravascular hemolysis, and stabilized hemoglobin levels despite a reduction of transfusions, to the point where most PNH patients were rendered transfusion independent. Substantial and clinically meaningful improvements in fatigue and other key quality of life parameters were also demonstrated. All of the 85 patients who completed the study elected to receive eculizumab in an open-label extension study and all currently remain on drug. The results of the TRIUMPH study indicate that terminal complement inhibition with eculizumab safely and effectively addresses an important consequence of the underlying genetic defect in PNH hematopoietic stem cells by providing a therapeutic replacement for the terminal complement inhibitor deficiency.

The present invention provides among other things treatment with an inhibitor of complement. Many variations of the invention will become apparent to those skilled in the art upon review of this specification. The full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

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All publications and patents mentioned herein, including those items listed below, 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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SEQUENCES

SEQ ID NO: 1 – Eculizumab V_H

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SGSTEYTENFKDRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARYFFGSSPNWYF
DVWGQGTLVTVSSA

SEQ ID NO: 2 – Eculizumab Heavy chain

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GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVE
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WYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLP
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SEQ ID NO: 3 – Eculizumab V_L

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SEQ ID NO: 4 – Eculizumab Light chain

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LSSPVTKSFNRGEC

SEQ ID NO: 5 – Eculizumab CDRH1

NYWIQ

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SEQ ID NO: 6 – Eculizumab CDRH2
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SEQ ID NO: 7 – Eculizumab CDRH3
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SEQ ID NO: 8 – Eculizumab CDRL1
GASENIYGALN

SEQ ID NO: 9 – Eculizumab CDRL2
GATNLAD

SEQ ID NO: 10 – Eculizumab CDRL3
QNVLNTPLT

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The invention furthermore comprises the following items:

1. A method to improve at least one aspect of the quality of life of a patient suffering from paroxysmal nocturnal hemoglobinuria, said method comprising administering to said patient in need thereof a compound which inhibits complement or inhibits formation of C5b-9.

2. The method of item 1 wherein said quality of life is measured by a FACIT-Fatigue score.

3. The method of item 2 wherein the FACIT-Fatigue score increases by at least 3 points.

4. The method of item 2 wherein the FACIT-Fatigue score increases by $\geq$ 4 points.

5. The method of item 1 wherein said quality of life is measured by an EORTC QLQ-C30 score.

6. The method of item 5 wherein said EORTC QLQ-C30 score improves by $\geq$ 10% of the pretreatment score.

7. The method of item 5 wherein said aspect of the quality of life as measured by an EORTC QLQ-C30 score is selected from the group consisting of a) global health status, b) physical functioning, c) emotional functioning, d) cognitive functioning, e) role functioning, f) social functioning, g) fatigue, h) pain, i) dyspnea, j) appetite loss, and k) insomnia.

8. The method of item 7 wherein said aspect of quality of life is fatigue.

9. The method of item 1 wherein said 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.

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10. The method of item 1 wherein said compound is a steroid that suppresses complement.

11. The method of item 1 wherein said compound is selected from the group consisting of antibodies, active antibody fragments, soluble complement inhibitory compounds, proteins, soluble complement inhibitors with a lipid tail, protein fragments, peptides, small organic compounds, 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.

12. The method of item 11 wherein said compound is an antibody or an active antibody fragment.

13. The method of item 12 wherein said antibody or active antibody fragment is selected from the group consisting of a) polyclonal antibodies, b) monoclonal antibodies, c) single chain antibodies, d) chimeric antibodies, e) humanized antibodies, f) Fabs, g) F(ab')s, h) $F(ab')_2$s, i) Fvs, j) diabodies, and k) human antibodies.

14. The method of item 12 wherein said antibody or active antibody fragment blocks C5 cleavage.

15. The method of item 12 wherein said antibody or active antibody fragment inhibits the formation of C5b-9.

16. The method of item 12 wherein said antibody is eculizumab.

17. The method of item 12 wherein said antibody or active antibody fragment is administered for at least 6 months.

18. The method of item 1 wherein said patient has aplastic anemia or myelodysplastic syndrome.

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19. The method of item 1 wherein said patient is anemic

20. The method of item 19 wherein said patient remains anemic following treatment.

21. The method of item 19 wherein said patient has a hemoglobin level less than i) 14 g/dL if a man or ii) 12 g/dL if a woman.

22. The method of item 19 wherein said patient has a hemoglobin level less than i) 13 g/dL if a man or ii) 11 g/dL if a woman.

23. The method of item 19 wherein said patient has a hemoglobin level less than i) 12 g/dL if a man or ii) 10 g/dL if a woman.

24. A method to improve at least one aspect of the quality of life of a patient suffering from paroxysmal nocturnal hemoglobinuria, said method comprising administering to said patient in need thereof a compound which inhibits intravascular hemolysis.

25. The method of item 24 wherein said method results in a greater than 30% reduction in LDH in said patient.

26. The method of item 24 wherein said quality of life is measured by a FACIT-Fatigue score.

27. The method of item 26 wherein the FACIT-Fatigue score increases by at least 3 points.

28. The method of item 26 wherein the FACIT-Fatigue score increases by $\geq$ 4 points.

29. The method of item 24 wherein said quality of life is measured by an EORTC QLQ-C30 score.

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30. The method of item 29 wherein said EORTC QLQ-C30 score improves by $\geq$ 10% of the pretreatment score.

31. The method of item 29 wherein said aspect of the quality of life is selected from the group consisting of a) global health status, b) physical functioning, c) emotional functioning, d) cognitive functioning, e) role functioning, f) social functioning, g) fatigue, h) pain, i) dyspnea, j) appetite loss, and k) insomnia.

32. The method of item 31 wherein said aspect of quality of life is fatigue.

33. The method of item 24 wherein said 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.

34. The method of item 24 wherein said compound is a steroid that suppresses complement.

35. The method of item 24 wherein said compound is selected from the group consisting of antibodies, active antibody fragments, soluble complement inhibitory compounds, proteins, soluble complement inhibitors with a lipid tail, protein fragments, peptides, small organic compounds, 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.

36. The method of item 35 wherein said compound is an antibody or an active antibody fragment.

37. The method of item 36 wherein said antibody or active antibody fragment is selected from the group consisting of a) polyclonal antibodies, b) monoclonal antibodies, c) single chain antibodies, d) chimeric antibodies, e) humanized antibodies, f) Fabs, g) F(ab')s, h) $F(ab')_2$s, i) Fvs, j) diabodies, and k) human antibodies.

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38. The method of item 36 wherein said antibody or active antibody fragment blocks C5 cleavage.

39. The method of item 36 wherein said antibody or active antibody fragment is administered for at least 6 months.

40. The method of item 24 wherein said compound inhibits complement or inhibits the formation of C5b-9.

41. The method of item 40 wherein said compound is an antibody or active antibody fragment.

42. The method of item 41 wherein said antibody is eculizumab.

43. The method of item 24 wherein said patient has aplastic anemia or myelodysplastic syndrome.

44. A method to improve at least one aspect of the quality of life of an anemic patient whose anemia results at least in part from hemolysis, said method comprising administering to said patient in need thereof a compound which inhibits intravascular hemolysis, wherein said patient remains anemic.

45. The method of item 44 wherein said method results in a greater than 30% reduction in LDH in said patient.

46. The method of item 44 wherein said patient has a hemoglobin level less than i) 14 g/dL if a man or ii) 12 g/dL if a woman.

47. The method of item 44 wherein said patient has a hemoglobin level less than i) 13 g/dL if a man or ii) 11 g/dL if a woman.

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48. The method of item 44 wherein said patient has a hemoglobin level less than i) 12 g/dL if a man or ii) 10 g/dL if a woman.

49. The method of item 44 wherein said patient suffers from paroxysmal nocturnal hemoglobinuria.

50. The method of item 44 wherein said quality of life is measured by a FACIT-Fatigue score.

51. The method of item 50 wherein the FACIT-Fatigue score increases by at least 3 points.

52. The method of item 50 wherein the FACIT-Fatigue score increases by $\geq$ 4 points.

53. The method of item 44 wherein said quality of life is measured by an EORTC QLQ-C30 score.

54. The method of item 53 wherein said EORTC QLQ-C30 score improves by $\geq$ 10% of the pretreatment score.

55. The method of item 53 wherein said aspect of the quality of life is selected from the group consisting of a) global health status, b) physical functioning, c) emotional functioning, d) cognitive functioning, e) role functioning, f) social functioning, g) fatigue, h) pain, i) dyspnea, j) appetite loss, and k) insomnia.

56. The method of item 55 wherein said aspect of quality of life is fatigue.

57. The method of item 44 wherein said 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.

58. The method of item 44 wherein said compound is a steroid that suppresses complement.

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59. The method of item 44 wherein said compound is selected from the group consisting of antibodies, active antibody fragments, soluble complement inhibitory compounds, proteins, soluble complement inhibitors with a lipid tail, protein fragments, peptides, small organic compounds, 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.

60. The method of item 59 wherein said compound is an antibody or an active antibody fragment.

61. The method of item 60 wherein said antibody or active antibody fragment is selected from the group consisting of a) polyclonal antibodies, b) monoclonal antibodies, c) single chain antibodies, d) chimeric antibodies, e) humanized antibodies, f) Fabs, g) F(ab')s, h) $F(ab')_2$s, i) Fvs, j) diabodies, and k) human antibodies.

62. The method of item 60 wherein said antibody or active antibody fragment blocks C5 cleavage.

63. The method of item 60 wherein said antibody or active antibody fragment is administered for at least 6 months.

64. The method of item 44 wherein said compound inhibits complement or inhibits the formation of C5b-9.

65. The method of item 64 wherein said compound is an antibody or an active antibody fragment.

66. The method of item 65 wherein said antibody is eculizumab.

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67. The method of item 44 wherein said patient has aplastic anemia or myelodysplastic syndrome.

68. A method of prolonging the health-adjusted life expectancy of a patient comprising administering to said patient in need thereof a compound which inhibits formation of C5b-9.

69. The method of item 68 wherein said patient is anemic.

70. The method of item 69 wherein said patient remains anemic following treatment.

71. The method of item 68 wherein said patient has a hemoglobin level less than i) 14 g/dL if a man or ii) 12 g/dL if a woman.

72. The method of item 68 wherein said patient has a hemoglobin level less than i) 13 g/dL if a man or ii) 11 g/dL if a woman.

73. The method of item 68 wherein said patient has a hemoglobin level less than i) 12 g/dL if a man or ii) 10 g/dL if a woman.

74. The method of item 68 wherein said patient suffers from paroxysmal nocturnal hemoglobinuria.

75. The method of item 68 wherein said health-adjusted life expectancy is measured according to a unit selected from the group consisting of Years of potential life lost, Disability-free life expectancy, Health-adjusted life year, Quality adjusted life year, Healthy years equivalents, Healthy days gained, Episode-free day, Q-TWiST, Health Utilities Index, or Years of healthy life.

76. The method of item 75 wherein the health-adjusted life expectancy in a subject is prolonged by at least one day.

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77. The method of item 75 wherein the health-adjusted life expectancy in a subject is prolonged by at least week.

78. The method of item 75 wherein the health-adjusted life expectancy in a subject is prolonged by at least one month.

79. The method of item 75 wherein the health-adjusted life expectancy in a subject is prolonged by at least one year.

80. The method of item 68 wherein said 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.

81. The method of item 68 wherein said compound is a steroid that suppresses complement.

82. The method of item 68 wherein said compound is selected from the group consisting of antibodies, active antibody fragments, soluble complement inhibitory compounds, proteins, soluble complement inhibitors with a lipid tail, protein fragments, peptides, small organic compounds, 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.

83. The method of item 82 wherein said compound is an antibody or an active antibody fragment.

84. The method of item 83 wherein said antibody or active antibody fragment is selected from the group consisting of a) polyclonal antibodies, b) monoclonal antibodies, c) single chain antibodies, d) chimeric antibodies, e) humanized antibodies, f) Fabs, g) F(ab')s, h) $F(ab')_2$s, i) Fvs, j) diabodies, and k) human antibodies.

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85. The method of item 83 wherein said antibody or active antibody fragment blocks C5 cleavage.

86. The method of item 83 wherein said antibody or active antibody fragment is administered for at least 6 months.

87. The method of item 68 wherein said compound inhibits complement or inhibits the formation of C5b-9.

88. The method of item 87 wherein said compound is an antibody or an active antibody fragment.

89. The method of item 88 wherein said antibody is eculizumab.

90. The method of item 68 wherein said patient has aplastic anemia or myelodysplastic syndrome.

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SEQUENCE LISTING

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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 Ser Thr Lys Gly Pro
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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
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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
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Page 3872

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Cys Val Glu Cys Pro Pro Cys Pro Ala Pro Pro Val Ala Gly Pro Ser
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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
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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
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Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu
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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
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Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala
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Samsung Bioepis Exhibit No. 1002
Page 3873

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Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Gly Ala Ser
        35                  40                  45

Glu Asn Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys
    50                  55                  60

Ala Pro Lys Leu Leu Ile Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
65                  70                  75                  80

Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
                85                  90                  95

Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Asn
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Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Gly Ala Ser
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Samsung Bioepis Exhibit No. 1002
Page 3874

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Glu Asn Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys
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Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
                85                  90                  95

Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Asn
            100                 105                 110

Val Leu Asn Thr Pro Leu Thr Phe Gly Gln Gly Thr Lys Val Glu Ile
        115                 120                 125

Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp
    130                 135                 140

Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn
145                 150                 155                 160

Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu
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Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp
            180                 185                 190

Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr
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Samsung Bioepis Exhibit No. 1002
Page 3875

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Samsung Bioepis Exhibit No. 1002
Page 3876

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Samsung Bioepis Exhibit No. 1002
Page 3877

CLAIMS

1. An antibody that binds C5 comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain comprising SEQ ID NOS: 8, 9 and 10.

2. The antibody of claim 1 comprising a light chain consisting of SEQ ID NO: 4.

3. A pharmaceutical composition comprising the antibody of claim 1 or 2.

Europäisches Patentamt
European Patent Office
Office européen des brevets

Acknowledgement of receipt

We hereby acknowledge receipt of your request for grant of a European patent as follows:

Submission number	4744651
Application number	EP16195355.9
File No. to be used for priority declarations	EP16195355
Date of receipt	24 October 2016
Your reference	P2913 EP/3
Applicant	Alexion Pharmaceuticals, Inc.
Country	US
Title	Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement
Documents submitted	package-data.xml application-body.xml SPECEPO-1.pdf\P2913 EP_3 Specification.pdf (61 p.) OTHER-1.pdf\P2913 EP_3 Additional sheet.pdf (1 p.) ep-request.xml ep-request.pdf (5 p.) SEQLTXT.txt\P2913 EP_3 Sequence listing.txt f1002-1.pdf (1 p.)
Submitted by	CN=Jasmin Soukenka 47563
Method of submission	Online
Date and time receipt generated	24 October 2016, 16:59 (CEST)
Message Digest	52:37:E5:FA:8E:E5:CE:B1:1E:88:CC:7C:2B:36:42:73:3A:24:67:35

Correction by the EPO of errors in debit instructions filed by eOLF
Errors in debit instructions filed by eOLF that are caused by the editing of Form 1038E entries or the continued use of outdated software (all forms) may be corrected automatically by the EPO, leaving the payment date unchanged (see decision T 152/82, OJ EPO 1984, 301 and point 6.3 ff ADA, Supplement to OJ EPO 10/2007).

/European Patent Office/

ABSTRACT

Eculizumab, a humanized monoclonal antibody against C5 that inhibits terminal complement activation, showed activity in a preliminary 12-week open-label trial in a small cohort of patients with paroxysmal nocturnal hemoglobinuria (PNH). The present study examined whether chronic eculizumab therapy could reduce intravascular hemolysis, stabilize hemoglobin levels, reduce transfusion requirements, and improve quality of life in a double-blind, randomized, placebo-controlled, multi-center global Phase III trial. It has been found that eculizumab stabilized hemoglobin levels, decreased the need for transfusions, and improved quality of life in PNH patients via reduced intravascular hemolysis. Chronic eculizumab treatment appears to be a safe and effective therapy for PNH.

Samsung Bioepis Exhibit No. 1002
Page 3881

VOSSIUS & PARTNER
Patentanwälte Rechtsanwälte mbB

EPO - Munich
50
20. Dez. 2016

VOSSIUS & PARTNER · POB 86 07 67 · 81634 München · Germany

European Patent Office

80298 M U N I C H

1. EP-Patent Application 07 75 3249.7
   Alexion Pharmaceuticals, Inc.
   Our Ref.: P2913 EP

2. EP-Patent 2359834
   (EP 11 00 1632.6)
   Alexion Pharmaceuticals, Inc.
   Our Ref.: P2913 EP/1

3. EP-Patent Application 16 17 2335.8
   Alexion Pharmaceuticals, Inc.
   Our Ref.: P2913 EP/2

4. EP-Patent Application 16 19 5355.9
   Alexion Pharmaceuticals, Inc.
   Our Ref.: P2913 EP/3

5. EP-Patent 2380907
   (EP 11 00 5615.7)
   Alexion Pharmaceuticals, Inc.
   Our Ref.: R1249 EP/1

6. EP-Patent Application 16 20 1244.7
   Alexion Pharmaceuticals, Inc.
   Our Ref.: R1249 EP/3

München, December 20, 2016
WA/FLG/SOJ

The address of the applicant/patentee of the above-identified European patent applications/patents has changed and now reads as follows:

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Wolfgang von Meibom (Of Counsel)

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5) European Patent Attorney
6) Registered U.S. Patent Attorney
7) Korean Patent Attorney
8) Abogada (Spain)
9) mit Zweigstelle in Berlin
10) Solicitor (England & Wales)
11) kein European Trademark Attorney
12) auch Patentanwalt - Deutschland

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Switzerland: HR Firmennummer CH-270.9.001.271-9

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Samsung Bioepis Exhibit No. 1002
Page 3882

It is respectfully requested that the change of address be recorded in the European Patent Register and that EPO Form 2544 be issued promptly.

Dr. Joachim Wachenfeld
European Patent Attorney

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

Vossius & Partner
Patentanwälte Rechtsanwälte mbB
Siebertstrasse 3
81675 München
ALLEMAGNE

Date
02.01.17

Reference	Application No./Patent No.
P2913 EP/3	16195355.9 - 1466
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

C o m m u n i c a t i o n

concerning the registration of amendments relating to

☐ a transfer (R. 22 and 85 EPC)

☑ entries pertaining to the applicant / the proprietor (R. 143(1)(f) EPC)

As requested, the entries pertaining to the applicant of the above-mentioned European patent application / to the proprietor of the above-mentioned European patent have been amended to the following:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IS IT LI LT LU LV MC MT NL PL PT RO SE SI SK TR
Alexion Pharmaceuticals, Inc.
100 College Street
New Haven, CT 06510/US

The registration of the changes has taken effect on 20.12.16.

In the case of a published application / a patent, the change will be recorded in the Register of European Patents and published in the European Patent Bulletin (Section I.12 / II.12).

Your attention is drawn to the fact that, in the case of the registration of a transfer, any automatic debit order only ceases to be effective from the date of its express revocation (cf. point 14(c) of the Arrangements for the automatic debiting procedure, supplementary publication 3 - OJ EPO 2015).

Receiving Section / For the Examining Division / For the Opposition Division / For the Legal Division *)

Europäisches Patentamt • European Patent Office • Office européen des brevets

*) See note.

Samsung Bioepis Exhibit No. 1002
Page 3884

Note
This communication is issued by/for the department with whom responsibility lies. The Legal Division is responsible for the registration of transfers, changes of name (Articles 71, 72 and 74 EPC and Rules 22 and 85 EPC) as well as for the rectification of the designation of the inventor (Rule 21 EPC) (see Decision of the President of the EPO, OJ EPO 2013, 600). In all other cases, the Receiving Section, the Examining Division or the Opposition Division is responsible, depending on the stage in proceedings.

Samsung Bioepis Exhibit No. 1002
Page 3885

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European Patent Office
Office européen des brevets

Payment of fees and expenses

European Patent Office
Treasury and Accounting
80298 München
Germany
Fax +49(0)89 2399-4465

Alexion Pharmaceuticals, Inc.

Please complete in typescript only

Name of payer

VOSSIUS & PARTNER (No. 31)
Patentanwälte Rechtsanwälte mbB

Address

Siebertstraße 3
81675 München
Germany

Payer's reference

P2913 EP/3

Mode of payment

☐ Bank transfer to Commerzbank AG[1]
IBAN: DE20 7008 0000 0333 8800 00
BIC: DRESDEFF700

EPO - Munich
100

16. Feb. 2017

☒ Debit from deposit account with the EPO is requested[2]

Deposit account No. 2800.0321

Patent application/patent No. (please use a separate form for each application)

EP 16 19 5355.9

PCT

Code		Amount/EUR
001	Filing fee – EP direct[3]	
501	Additional fee (more than 35 pages)	
020	Filing fee – entry EP phase[3] (Rule 159(1)(c) EPC)	
520	Additional fee (more than 35 pages) - entry EP phase	
	Additional fee for divisional applications (Rule 38(4)EPC)[9]	
002	Fee for a European search	
055	Additional copy of docs cited in search report	
015	Claims fee(s) (Rules 45(1), 162(1)EPC)[5]	
005	Designation fee(s)[4]	
006	Examination fee	
122	Fee for further processing (non-fee related cases)	
123	Fee for further processing (late payment of a fee)	
007	Fee for grant and printing or fee for grant incl. fee for publication[6]	
008	Additional printing fee for 36th and each subsequent page[7]	
016	Claims fee according to Rule 71(4) EPC[5]	
121	Fee for further processing (late performance of acts R. 71(3))	
022	Registering of transfer	

Code		Amount/EUR
010	Opposition fee	
011	Fee for appeal	
029	Certified copy of application; priority document	
033	Renewal fee for the 3rd year	470,00
034	Renewal fee for the 4th year	585,00
035	Renewal fee for the 5th year	820,00
036	Renewal fee for the 6th year	1.050,00
	Validation for[8]	
037	Renewal fee for the 7th year	1.165,00
038	Renewal fee for the 8th year	1.280,00
039	Renewal fee for the 9th year	1.395,00
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040	Renewal fee for the 10th year	1.575,00

Notes 1 - 9 see overleaf.

Total 8.340,00

Signature (handwritten, no block letters)

Dr. Joachim Wachenfeld
European Patent Attorney

Place, Date 16. FEB. 2017

München, February 24, 2017, WA/FLG/SOJ

EPO 1010 11.15

Samsung Bioepis Exhibit No. 1002
Page 3886

Application No.: 16195355.9

The search has started on 03.03.17 (see Notice from the European Patent Office dated 03/2013, OJ EPO 2013, 153).

Receiving Section

Information on Search Strategy - Pilot phase (see OJ 2015, A86)
The type of information contained in this sheet may change during the pilot for improving the usefulness of this new service.

Application Number
EP 16 19 5355

TITLE: TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

APPLICANT: Alexion Pharmaceuticals, Inc.

IPC CLASSIFICATION: A61K31/7105, A61K38/00, A61K39/395, A61P7/06, C07K16/18, A61K9/00

EXAMINER: Collura, Alessandra

CONSULTED DATABASES: WPI, EMBASE, MEDLINE, REG, CA

CLASSIFICATION SYMBOLS DEFINING EXTENT OF THE SEARCH:

IPC:

CPC:

FI/F-TERMS:

KEYWORDS OR OTHER ELEMENTS FEATURING THE INVENTION:
antibody that binds to C5 having the indicated heavy and light chain

EPO FORM P04A42

Samsung Bioepis Exhibit No. 1002
Page 3888

Europäisches Patentamt
European Patent Office
Office européen des brevets

EUROPEAN SEARCH REPORT

Application Number
EP 16 19 5355

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	US 2005/191298 A1 (BELL LEONARD [US] ET AL) 1 September 2005 (2005-09-01) * the whole document * * paragraphs [0036], [0037], [0082] * -----	1-3	INV. A61K31/7105 A61K38/00 A61K39/395 A61P7/06
X	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, WILEY-BLACKWELL PUBLISHING LTD, GB, vol. 121, no. Suppl.1, 1 May 2003 (2003-05-01), page 87, XP009144758, ISSN: 0007-1048 * the whole document * -----	1-3	C07K16/18 A61K9/00
X,D	HILLMEN P ET AL: "EFFECT OF ECULIZUMAB ON HEMOLYSIS AND TRANSFUSION REQUIREMENTS IN PATIENTS WITH PAROXYSMAL NOCTRURNAL HEMAGLOBINURIA", NEW ENGLAND JOURNAL OF MEDICINE, MASSACHUSETTS MEDICAL SOCIETY, BOSTON, MA, US, vol. 350, no. 6, 5 February 2004 (2004-02-05), pages 552-559, XP009057948, ISSN: 1533-4406, DOI: DOI:10.1056/NEJMOA031688 * abstract * -----	1-3	TECHNICAL FIELDS SEARCHED (IPC) A61K

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	6 March 2017	Collura, Alessandra

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

1

EPO FORM 1503 03.82 (P04C01)

Samsung Bioepis Exhibit No. 1002
Page 3889

EUROPEAN SEARCH REPORT

Application Number
EP 16 19 5355

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	HARDING J: "Eculizumab", DRUGS OF THE FUTURE, PROUS SCIENCE, ES, vol. 29, no. 7, 1 July 2004 (2004-07-01), pages 673-676, XP008113000, ISSN: 0377-8282, DOI: DOI:10.1358/DOF.2004.029.07.819330 * the whole document * * page 674, left-hand column, paragraph 3 *	1-3	
X	HILL ANITA: "Eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria", CLINICAL ADVANCES IN HEMATOLOGY & ONCOLOGY, MILLENNIUM MEDICAL PUB., NEW YORK, NY, US, vol. 3, no. 11, 1 November 2005 (2005-11-01), pages 849-850, XP001538426, ISSN: 1543-0790 * the whole document *	1-3	
X	DATABASE REGISTRY [Online] CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; 14 February 1999 (1999-02-14), XP002767828, retrieved from STN accession no. 219685-50-4 Database accession no. 219685-50-4 * abstract *	1,2	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	6 March 2017	Collura, Alessandra

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 16 19 5355

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.

06-03-2017

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2005191298 A1	01-09-2005	AU 2005209854 A1	18-08-2005
		CA 2554632 A1	18-08-2005
		CN 1938047 A	28-03-2007
		CN 103446584 A	18-12-2013
		CN 103977402 A	13-08-2014
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		EP 2910253 A1	26-08-2015
		ES 2389093 T3	23-10-2012
		HK 1094950 A1	01-02-2013
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		NZ 549328 A	24-12-2010
		PL 1720571 T3	30-11-2012
		PT 1720571 E	29-08-2012
		SI 1720571 T1	30-10-2012
		US 2005169921 A1	04-08-2005
		US 2005191298 A1	01-09-2005
		US 2010068202 A1	18-03-2010
		US 2011086040 A1	14-04-2011
		US 2012308559 A1	06-12-2012
		WO 2005074607 A2	18-08-2005

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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The examination is being carried out on the **following application documents**

Description, Pages

1-55 as originally filed

Sequence listings, SEQ ID NO

1-10 as originally filed

Claims, Numbers

1-3 as originally filed

Drawings, Sheets

1/4-4/4 as originally filed

Reference is made to the following documents; the numbering will be adhered to in the rest of the procedure.

D1 US 2005/191298 A1 (BELL LEONARD [US] ET AL) 1 September 2005 (2005-09-01)

D2 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, WILEY-BLACKWELL PUBLISHING LTD, GB,
vol. 121, no. Suppl.1, 1 May 2003 (2003-05-01), page 87, XP009144758,
ISSN: 0007-1048

D3 HILLMEN P ET AL: "EFFECT OF ECULIZUMAB ON HEMOLYSIS AND TRANSFUSION REQUIREMENTS IN PATIENTS WITH PAROXYSMAL NOCTRURNAL HEMAGLOBINURIA",
NEW ENGLAND JOURNAL OF MEDICINE, MASSACHUSETTS MEDICAL SOCIETY, BOSTON, MA, US,
vol. 350, no. 6, 5 February 2004 (2004-02-05), pages 552-559, XP009057948,
ISSN: 1533-4406, DOI: DOI:10.1056/NEJMOA031688

D4 HARDING J: "Eculizumab",
DRUGS OF THE FUTURE, PROUS SCIENCE, ES,
vol. 29, no. 7, 1 July 2004 (2004-07-01), pages 673-676, XP008113000,
ISSN: 0377-8282, DOI: DOI:10.1358/DOF.2004.029.07.819330

D5 HILL ANITA: "Eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria",
CLINICAL ADVANCES IN HEMATOLOGY & ONCOLOGY, MILLENNIUM MEDICAL PUB., NEW YORK, NY, US,
vol. 3, no. 11, 1 November 2005 (2005-11-01), pages 849-850, XP001538426,
ISSN: 1543-0790

D6 DATABASE REGISTRY [Online]
CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; 14 February 1999 (1999-02-14),
retrieved from STN accession no. 219685-50-4
Database accession no. 219685-50-4

Fro what concerns the most relevant passages of the above-mentioned documents, please see citations in the European Search Report, unless otherwise indicated.

1. Article 76(1) EPC

The present application does not meet the requirements of Article 76(1) EPC, because it relates to subject-matter which extends beyond the parent application as originally filed.

The applicant indicated page 5, last paragraph as basis for the claims of the application.

However, said paragraph puts into relation sequence 2 with sequence 4 and not with the specific sequences 8, 9 and 10. These last sequences are explicitly mentioned only in relation with specific sequences 5, 6 or 7.

2. Novelty

The present application does not meet the requirements of Article 54 EPC, because the subject-matter of the claims 1-3 is not novel.

The attention of the applicant is drawn to the fact that the present claims are directed to a product and are not limited in scope by any other features than those relating to the product itself.

The sequences indicated in the claims are told to be the sequences of the heavy and light chains of eculizuman or part of them.

Therefore, any document disclosing eculizumab published before the filing date of the present application obviously destroys the novelty of the present claims.

All documents cited in the search report refer to eculizumab and to the fact that it is an antibody which binds C5. D1-D5 also describe pharmaceutical compositions of the compound.

3. Inventive step

The present application does not meet the requirements of Article 56 EPC, because the subject-matter of the claims 1-3 is not inventive.

As a matter of fact, claims which are not novel cannot be considered to involve any exercise of an inventive activity.

4. Procedure

The applicant is invited to file new claims which take account of the above comments.

In order to facilitate the examination of the conformity of the amended application with the requirements of Article 123(2) EPC, the applicant should clearly identify the amendments carried out, irrespective of whether they concern amendments by addition, replacement or deletion, and to indicate the passages of the application as filed on which these amendments are based (see Guidelines E-II, 1).

The attention of the applicant is drawn to the fact that the application may not be amended in such a way that it contains subject-matter which extends beyond the content of the application as filed (Article 123(2) EPC).

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Office européen des brevets

European Patent Office
80298 MUNICH
GERMANY

Questions about this communication ?
Contact Customer Services at www.epo.org/contact

Vossius & Partner
Patentanwälte Rechtsanwälte mbB
Siebertstrasse 3
81675 München
ALLEMAGNE

Date
17.03.17

Reference	Application No./Patent No.
P2913 EP/3	16195355.9 - 1466
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Communication

The extended European search report is enclosed.

The extended European search report includes, pursuant to Rule 62 EPC, the European search report (R. 61 EPC) or the partial European search report/ declaration of no search (R. 63 EPC) and the European search opinion.

Copies of documents cited in the European search report are attached.

☑ 0 additional set(s) of copies of such documents is (are) enclosed as well.

The following have been approved:

☑ Abstract ☑ Title

☐ The Abstract was modified and the definitive text is attached to this communication.

The following figure(s) will be published together with the abstract:

Refund of search fee
If applicable under Article 9 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.

Should you wish to further prosecute this application in the examination phase, your attention is drawn to the provisions of Rule 70a EPC. An invitation to respond to the extended European search report will be issued once the date of publication of the European search report has been mentioned in the European Patent Bulletin (R. 69(1), R. 70(2) EPC).

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Vossius & Partner
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81675 München
ALLEMAGNE

Date
05.04.17

Reference	Application No./Patent No.
P2913 EP/3	16195355.9 - 1466
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Refund of fees

The following fee(s) was (were) paid in respect of the application 16195355.9:

Fee	Reference	Voucher No	Date	Currency	Amount
Search fee	002	01354109	24.11.16	EUR	1 300,00

The fee(s) was (were) paid by Vossius & Partner.

According to the present state of the file the refund will be made by:

CREDITING THE DEPOSIT ACCOUNT 28000321 Vossius & Partner.

Amount refundable:	Reference	Currency	Amount	Voucher No
	002	EUR	1.300,00	00739430

Reason for refund: Full use of earlier European search report with written opinion drawn up by the EPO (100 % of fee paid for current search refunded).

The Authorising Officer
Adams
(49)(89)23992668

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Samsung Bioepis Exhibit No. 1002
Page 3896

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Vossius & Partner
Patentanwälte Rechtsanwälte mbB
Siebertstrasse 3
81675 München
ALLEMAGNE

Date
20.04.17

Reference	Application No./Patent No.
P2913 EP/3	16195355.9 - 1466 / 3167888
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Notification of European publication number and information on the application of Article 67(3) EPC

You are hereby informed that the technical preparations for the publication of the above-mentioned European patent application have been completed.

The application will be published on 17.05.17 with the European search report.
The publication number is: 3167888.
The publication will be mentioned in European Patent Bulletin number 2017/20.
(**http://bulletin.european-patent-office.org**).

Provisional protection under Article 67(1) and (2) EPC in the individual contracting states becomes effective when the conditions referred to in Article 67(3) EPC have been fulfilled. Provisional protection in the individual extension and validation states is governed by the national laws of the countries concerned. For more details, see Chapter III of the EPO brochure "National Law relating to the EPC" (**www.epo.org/law-practice/legal-texts/national-law.html**), which also contains information about the extension and validation system. Further information can also be found in the Official Journal of the European Patent Office.

The title of the invention in the three official languages of the European Patent Office is worded as follows:

BEHANDLUNG VON PATIENTEN MIT PAROXYSMALER NÄCHTLICHER HÄMOGLOBINURIE MIT EINEM KOMPLEMENTINHIBITOR
TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
TRAITEMENT DE PATIENTS SOUFFRANT D'HÉMOGLOBINURIE PAROXYSTIQUE NOCTURNE PAR UN INHIBITEUR DE COMPLÉMENT

In all future communications to the EPO, please quote the application number as indicated above, i.e. including the final four figures (which identify the Directorate responsible for the subsequent procedure).

Remark
The publication can be downloaded, free of charge, from the EPO publication server **https://data.epo.org/publication-server/** or can be ordered from the Vienna sub-office upon payment of a fee (see Decision of the President of the EPO dated 12 July 2007 concerning the form of publication of European patent applications, European search reports and European patent specifications, Special edition No. 3, OJ EPO 2007, 97).

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Note concerning renewal fees
Under Article 86 EPC renewal fees shall be paid to the EPO for the European patent application. These fees shall be due in respect of the third and each subsequent year, calculated from the date of filing. Due account should be taken that the EPO will not send invoices or reminders to pay any fees in due time.

Accelerated prosecution of European patent applications
Applicants wishing to accelerate proceedings before the EPO may file a request according to the programme for accelerated prosecution of European patent applications - "PACE" (OJ EPO 2010, 352). EPO Form 1005 is available on the EPO website (www.epo.org) for this purpose.

Receiving Section



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Page 3898

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European Patent Office
80298 MUNICH
GERMANY

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Vossius & Partner
Patentanwälte Rechtsanwälte mbB
Siebertstrasse 3
81675 München
ALLEMAGNE

Date
22.05.17

Reference	Application No./Patent No.
P2913 EP/3	16195355.9 - 1466 / 3167888
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Communication pursuant to Rule 69 EPC - reminder concerning payment of the designation fee (Art. 79(2) EPC) and of the examination fee (Art. 94(1) EPC) - and invitation pursuant to Rule 70a(1) EPC

The date on which the European Patent Bulletin mentions the publication of the European search report for the above-mentioned European patent application is: 17.05.17.

Your attention is drawn to Article 79(2) and Rule 39(1) EPC as well as Article 94(1), Rule 70(1) and Rule 70a(1) EPC, according to which within **six months** of the above-mentioned publication date of the search report

- the designation fee must be paid,
- the request for examination must be filed and the examination fee must be paid,
- comments on the objections raised in the opinion accompanying the European search report and/or any amendments to the description, claims and drawings correcting any deficiencies noted in the opinion must be filed (R. 70a(1), 137(2) EPC; Guidelines for Examination in the EPO, B-XI, 8).

1. Payment of the designation fee and of the examination fee

The current designation fee for one or more Contracting States designated is: EUR 585,00

The current examination fee is: EUR 1635,00

If the written request for examination is filed in an admissible non-EPO language in accordance with Article 14(4) EPC, a reduction of the examination fee according to Rule 6(3) EPC applies.

For European patent applications filed on or after 01.04.2014, the reduction applies only if the applicant(s) has (have) declared to be an entity or a natural person within the meaning of Rule 6(4) EPC (Decision of the Administrative Council of 13 December 2013, OJ EPO 2014, A4).

If the designation fee and the examination fee are not paid within the period laid down in Rule 39(1) and Rule 70(1) EPC, the application **will be deemed to be withdrawn** (R. 39(2) and Art. 94(2) EPC).

Any extension or validation fees are also payable within the above-mentioned period.

Registered letter

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2. Comments on the opinion accompanying the European search report and filing of amendments

Should the reply to the invitation pursuant to Rule 70a(1) EPC be filed in an admissible non-EPO language, a translation is to be submitted within one month of its filing (R. 6(2) EPC).

If filing amendments, you must identify them and indicate the basis for them in the application as filed. Failure to meet either requirement may lead to a communication from the Examining Division requesting that you correct this deficiency (R. 137(4) EPC).

Should you not comply with this invitation within the time limit, the application **will be deemed to be withdrawn** in accordance with Rule 70a(3) EPC.

Note to users of the automatic debiting procedure
Unless the EPO receives prior instructions to the contrary, the examination fee and the designation fee will be debited on the last day of the period for payment. For further details see the Arrangements for the automatic debiting procedure, supplementary publication 3 - OJ EPO 2015.

Important information concerning fee amounts
Following any amendment to the Rules relating to Fees, the amount(s) mentioned in this communication may be different from the amount(s) **actually due on the date of payment.** The latest version of the Schedule of fees and expenses, published as a Supplement to the Official Journal of the EPO, is also available on the EPO website (**www.epo.org**) and can be found under **www.epo.org/schedule-of-fees**, which allows the viewing, downloading and searching for individual fee amounts, both current and previous.
Please note that procedural fees are usually adjusted every two years, on even years, with effect from 1 April.

Payments by cheque delivered or sent direct to the EPO are no longer accepted as from 1 April 2008 (see OJ EPO 2007, 626).

Note concerning renewal fees
Under Article 86 EPC renewal fees shall be paid to the EPO for the European patent application. These fees shall be due in respect of the third and each subsequent year, calculated from the date of filing. Due account should be taken that the EPO will not send invoices or reminders to pay any fees in due time.

Receiving Section



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SEQUENCES

SEQ ID NO: 1 – Eculizumab V_H

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DVWGQGTLVTVSSA

SEQ ID NO: 2 – Eculizumab Heavy chain

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DVWGQGTLVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVE
RKCCVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFN
WYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLP
SSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQ
PENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSL
SLSLGK

SEQ ID NO: 3 – Eculizumab V_L

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NWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
NVLNTPLTFGQGTKVEIKRT

SEQ ID NO: 4 – Eculizumab Light chain

~~MDMRVPAQLLGLLLLWLRGARC~~DIQMTQSPSSLSASVGDRVTITCGASENIYGAL
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SEQ ID NO: 5 – Eculizumab CDRH1

NYWIQ

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SEQUENCE LISTING

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<150> 60/783,070
<151> 2006-03-15

<160> 10

<170> BiSSAP 1.3.6

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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
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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 Ser Thr Lys Gly Pro

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145                 150                 155                 160
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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
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Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala
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    290                 295                 300
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305                 310                 315                 320
Lys Cys Lys Val Ser Asn Lys Gly Leu Pro Ser Ser Ile Glu Lys Thr
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Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu
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Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys
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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
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        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
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Page 3903

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<220>
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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
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<210> 5
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<212> PRT
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<220>
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Samsung Bioepis Exhibit No. 1002
Page 3904

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<220>
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1 5

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Page 3905

EPO - Munich
100
10. Juli 2017

VOSSIUS & PARTNER
Patentanwälte Rechtsanwälte mbB

VOSSIUS & PARTNER · POB 86 07 67 · 81634 München · Germany

European Patent Office

80298 M U N I C H

EP 16 19 5355.9 – 1466
Alexion Pharmaceuticals, Inc.
Vossius Ref.: P2913 EP/3

München, July 10, 2017
WA/FLG/SOJ

I. COMMUNICATION PURSUANT TO RULE 69 EPC

Payment of the examination fee and the designation fee for all contracting states is effected with the enclosed sheet of Payment of fees and expenses (EPO Form 1010). Examination is herewith requested.

II. REPLY TO THE EESR PURSUANT TO RULE 70a(1) EPC

This is in reply to the extended European search report issued in the above-mentioned case on March 17, 2017.

1. INADMISSIBLE BROADENING (Article 76(1) EPC)

1.1. The Examiner is of the view that the subject-matter of claim 1 cannot be clearly and unambiguously derived from the parent application as filed.

Patentanwälte - Deutschland
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European Trademark Attorneys
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1.2. Claim 1 is directed to an antibody comprising the complete heavy chain of eculizumab (i.e. SEQ ID NO: 2) and the three light chain CDRs of eculizumab (i.e. SEQ ID NOs 8, 9 and 10).

1.3. The basis for pending claim 1 in the parent application as filed can be found on page 9, last paragraph of the parent application as filed which reads:

> "In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof comprises a heavy chain variable region and a light chain variable region, wherein the heavy chain variable region comprises one or more CDR regions having an amino acid sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7, and wherein the light chain variable region comprises one or more CDR regions having an amino acid sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10. [...] In certain embodiments, the antibody that binds C5 or an active antibody 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."

On pages 44 and 45 of the apparent application as filed SEQ ID NOs NOs 1 to 10 are shown and it is described that all of them characterize the antibody eculizumab. Hence, in order arrive at claim 1 it is required to combine features of a broader embodiment and a narrower embodiment both characterizing the structure of eculizumab. As the Examining Division is aware, this is generally seen in the EPO as resulting in a so-called "intermediate generalisation".

In T 461/05 the Board of Appeal ruled that an intermediate generalisation has to be held distinct from a simple generalisation, since in the former case a definition of the invention in general terms forms part of the original disclosure. In accordance with the pertinent case law an intermediate generalisation can be accepted under Articles 123(2) and 76(1) EPC in the absence of any clearly recognisable functional or structural relationship among the features of the specific combination (see, for example, T 1067/97 and T 25/03), or if the extracted feature is not inextricably linked with those features (see T 714/00).

Moreover, the general "gold standard" (see G 2/10) for assessing compliance with Articles 123(2) and 76(1) EPC is that amendments to the claims can only be made within the limits of what a skilled person would derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of these documents as filed.

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1.4. In the present case the skilled person could be an immunologist. The structure of an antibody is part of the common general knowledge of an immunologist. As the Examining Division is aware, an antibody (monomer) is a "Y"-shaped molecular structure that consists of four polypeptide chains; two identical heavy chains and two identical light chains connected by disulfide bonds. Three variable loops each on the light and heavy chains are responsible for binding to the antigen. These loops are referred to as the complementarity determining regions (CDRs).

Thus, the skilled person knows that an antibody is composed of two different sequences, the light and heavy chain. These two chains are connected by disulfide bonds. The skilled person also knows that while the complete chains exhibit binding specificity for a particular antigen, the binding specificity is determined by the unique amino acid sequences found in the three hypervariable complementarity determining regions known as the CDRs.

Hence, the skilled person on the basis of the above-quoted last paragraph on page 9 of the parent application as filed arrives at the antibody of claim 1 comprising the heavy chain of eculizumab (i.e. SEQ ID NO: 2) and the three light chain CDRs of eculizumab (i.e. SEQ ID NOs 8, 9 and 10) without the requirement of extracting a feature being inextricably linked with other features. In line with T 714/00 this constitutes an allowable intermediate generalisation. It is of further note that since the light and heavy chain are separate there is no functional or structural relationship among these sequences that distracts a skilled person from directly and unambiguously deriving the antibody of claim 1. Thus, also in line with T 1067/97 and T 25/03 claim 1 constitutes an allowable intermediate generalisation.

In summary, it is our firm position that the claims find a clear and unambiguous basis in the parent application as filed as well as the application as filed.

2. NOVELTY AND INVENTIVE STEP (Articles 54 and 56 EPC)

2.1. The Examiner objects to the novelty of claims 1 to 3 in view of documents D1 to D5. The Examiner states that each of these documents describes eculizumab.

In view of the alleged lack of novelty the Examiner also contests inventive step of claims 1 to 3.

2.2. Because the use of eculizumab for the treatment of PNH is described in D1 to D5, the Examiner apparently assumes that eculizumab must have been publically available before the filing date of the present application.

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We respectfully note that this assumption is not correct because the mere naming of a drug such as eculizumab in a publication describing a confidential clinical trial does not place the biochemical structure in the prior art or in public use. As is evident from the enclosed declaration from the corresponding US proceedings by the inventor of the present application, founder and CEO of the Applicant Alexion Pharmaceuticals, Inc. Dr. Leonard Bell, prior to the March 15, 2007 effective filing date of the present application, the complete structure of eculizumab was not disclosed in the prior art. Also, eculizumab was never provided to a member of the public absent strict confidentiality and storage restrictions before March 15, 2007, as described in the enclosed declaration from Dr. Laural Boone that was submitted in the corresponding US proceedings..

Moreover, D1 to D5 fail to teach or suggest any part of the sequence of eculizumab, and in particular the unique, non-naturally occurring, protein-engineered full heavy chain of eculizumab (including the CH1-hinge-CH2-CH3 regions) of SEQ ID NO: 2. This engineered full heavy chain confers important and unexpected functional effects to eculizumab, as described in the declaration of Dr. Bell.

In more detail and as evidenced by the sequence alignment within the declaration, the heavy chain constant regions of eculizumab and a naturally-occurring IgG4 heavy chain differ in fourteen amino acid residues (i.e., four different residues in the CH1 domain, six different residues in the hinge region, and four different residues in the CH2 domain). Furthermore and as also discussed in the accompanying declaration, these structural differences confer eculizumab with advantageous functional features, including decreased effector function, reduced immunogenicity and increased half-life.

In sum, the complete structure of eculizumab as currently claimed – being an antibody characterized by the heavy of SEQ ID NOs 2 and the light chain CDRs of SEQ ID NOs 8, 9 and 10 – was not disclosed or otherwise made available to the public prior to the March 15, 2007 effective filing date of the present application. Therefore, prior to the effective filing date of the present application the complete structure of eculizumab as currently claimed in the present application was neither taught nor suggested in the cited prior art; nor was it obtainable by way of public availability.

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We therefore submit that the new claims are novel over D1 to D5. In view of the above-discussed unexpected and advantageous technical properties being associated with the complete structure of eculizumab, we submit that the claims are also inventive.

3. CORRECTION OF AN OBVIOUS ERROR (Rule 139 EPC)

Enclosed herewith is corrected page 44 of the application as filed and a corrected sequence listing, wherein an obvious error in the sense of Rule 139 EPC has been corrected in SEQ ID NOs 3 and 4. In more detail, the light chain of eculizumab as shown in SEQ ID NO: 4 as well as the corresponding shorter V_L sequence of eculizumab as shown in SEQ ID NO: 3 as originally filed erroneously comprise the N-terminal 22-amino acids long leader peptide which cannot be found in the mature light chain as comprised with the mature antibody. In the corrected SEQ ID NOs 3 and 4 the leader peptide has been removed.

We submit that the requested correction of SEQ ID NOs 3 and 4 complies with the prerequisites of Rule 139 EPC, because the skilled person in the field of antibodies immediately recognizes

(I) that SEQ ID NOs 3 and 4 are not correct, and

(II) that the correct SEQ ID NOs 3 and 4 are nothing else than SEQ ID NOs 3 and 4 without the N-terminal 22-amino acids long leader peptide; see Guidelines for Examination in the EPO, Part H – Chapter VI-2.2.

In this respect we refer the Examining Division to the enclosed declaration from the technical expert in the field of antibodies Dr. Bruce Andrien (Director, Early Assay Development, Alexion Pharmaceuticals, Inc.).

Regarding prerequisite (I), it can be taken from section 8 of the declaration that the skilled person immediately recognizes that SEQ ID NO: 4 is not correct, because (i) SEQ ID NO: 4 starts with methionine (M), (ii) M is followed by a stretch of 21 amino acids being mostly hydrophobic, and (iii) SEQ ID NO: 4 is with 236 amino acids considerable longer than typical mature antibody light chains of 211 to 217 amino acids. It was common textbook knowledge at the priority date in 2006 that the start codon of the genetic code (ATG) codes for the amino acid M and therefore most unprocessed protein sequences start with M. It

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was also commonly known that antibody light chains originally contain a leader peptide beginning with a methionine and followed by a hydrophobic core section (known as leader or signal petide) that is removed during protein processing and maturation. As regards the length of 211 to 217 amino acids of mature antibody light chains the declaration references the textbook Janeway et al., Immunology, 5th ed. (2001). The contents of textbook as well as handbooks, monographs, encyclopaedias and reference books establish the common general knowledge of the skilled person; see 8th ed. Of the Case Law book, section I.C.2.8.1, second paragraph. Hence, it is immediately recognisable for the skilled person, using common general knowledge, from the originally-filed application documents taken by themselves that SEQ ID NOs 3 and 4 are erroneous because they contain the signal peptide that is not present in the mature protein.

Prerequisite (II) is considered to be met if the correction is within the limits of what a skilled person derives directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the originally-filed application documents. As can be taken from sections 9 to 12 of the declaration of Dr. Andrien, the skilled person immediately recognizes that the correct SEQ ID NOs 3 and 4 are nothing else than SEQ ID NOs 3 and 4 without the N-terminal 22 amino acids long leader peptide, when considering the common general knowledge of the skilled person as being evidenced by the contents of the Kabat database in the last version as a textbook. The Kabat Database was first published as textbook in 1976 and the last edition in textbook form was published in 1991. Since then the Kabat database is accessible online. Since its establishment until today the Kabat database is extensively and foremost used by people skilled in the field of antibodies in order to derive structural and functional information on primary sequences of antibodies. As of its establishment, it was the most complete compilation of data in the antibody field. In the antibody field it had an equally high importance as the Maniatis and later Sambrook, "Molecular Cloning" textbook in the field of molecular cloning. It follows that the contents of the Kabat database and in particular the early contents as published in the format of a textbook are to be considered common general knowledge of the skilled person in the field of antibodies. Hence, it was common general knowledge of the skilled person at the priority date in 2006 that (i) leader peptides of kappa antibody light chains are usually 22 amino acids long

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and start with the amino acids "MDM" (see section 11 of the declaration), and (ii) that the mature form of kappa antibody light chains usually starts with the amino acids "DIQM" (see section 11 of the declaration), noting that also the leader peptide of eculizumab is 22 amino acids long and starts with the amino acids "MDM" and that also the mature antibody light chain of eculizumab starts with the amino acids "DIQM".

In summary, the requested correction of the antibody light chain of SEQ ID NO: 4 and consequently also the corresponding V_L sequence of SEQ ID NO: 3 are within the limits of what a skilled person can derive directly and unambiguously, using common general knowledge, and seen objectively and relative to the date of filing, from the whole of the documents as filed; see also G 3/89 and G 11/91. We therefore submit that our request for the correction of SEQ ID NOs 3 and 4 under Rule 139 EPC is fully justified.

4. REQUESTS

With the above amendments and explanations it is submitted that the applicant has met the requirements as set forth in the EESR. It is requested that the adaptation of the description of the present application be deferred until an allowable set of claims is agreed upon.

If, however, the Examining Division does not agree to the above, it is requested that either a Communication pursuant to Article 94(3) EPC or a summons to attend oral proceedings according to Article 116(1) EPC be issued. If deemed expedient an informal interview is requested. The undersigned is prepared to discuss minor amendments over the phone.

Dr. Joachim Wachenfeld
European Patent Attorney

Enclosures:
Bell Declaration
Andrien Declaration (including Exhibits)
Boone Declaration
EPO Form 1010
Corrected Page 44
Corrected sequence listing (also on CD-ROM)

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055	Additional copy of docs cited in search report		036	Renewal fee for the 6th year	
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008	Additional printing fee for 36th and each subsequent page[7]				
016	Claims fee according to Rule 71(4) EPC[5]				
121	Fee for further processing (late performance of acts R. 71(3))				
022	Registering of transfer				

Notes 1 - 9 see overleaf.

Total **2.220,00**

Signature (handwritten/in block letters)

Dr. Joachim Wachenfeld
European Patent Attorney

Place, Date

München, July 10, 2017, WA/FLG/SOJ

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Our Ref.: P2913 EP/3

Exhibit A

BRUCE A. ANDRIEN, Jr., Ph.D.

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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

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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.

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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.

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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)

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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)

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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).

"Characterizing Glycosylation Patterns in Recombinant IgG2/4 Monoclonal Antibodies by Combining Fluorescence HPLC, MALDI-ToF-MS, and LC-MS and MS/MS Data", Adam Lucka, Christine Nowak, Rekha Patel, and Bruce Andrien, 56th ASMS Conference, (2008).

"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).

"Improving Monoclonal Antibody and SCFv Protein Characterization by Obtaining Complementary MALDI-TOF-MS Spectra from a Single Tryptic Digest", Adam Lucka, Rekha Patel, and Bruce Andrien, 55th ASMS Conference, (2007).

"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.

"Disulfide Bond Confirmation of a Humanized Anti-C5 Complement Monoclonal Antibody and Its Single Chain Homolog by Peptide Mapping", Bruce A. Andrien, Adam W. Lucka, and Rekha Patel, 54th ASMS Conference, (2006).

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"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).

"Peptide and Protein Analysis and the Identification of Post-Translational Modifications Using Fast LC-MS with Rapid State Switching", Craig M. Whitehouse, Bruce A. Andrien, Jr., Staffan Renlund, Erol Gulcicek, Marketa Berkova, Lars Ronnstrand, and Rannar Sillard, 15th IMSC Barcelona (2000).

"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).

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"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).

"Combining Autoinjector and Nebulizer Hardware for Enhanced Automation in FIA-MS,", B. A. Andrien, J. G. Boyle, and M. Berkova, 48th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Long Beach, CA, (2000).

"Multipole Storage Assisted Dissociation for Enhancing Sequence and Structural Studies in LC-TOF-MS", C. M. Whitehouse, B. A. Andrien, and M. Berkova, 48th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Long Beach, CA, (2000).

"Evaluation of a New LC TOF-MS with Wide Dynamic Range for Full Chromatographic Compatibility", B. A. Andrien, E. E. Gulcicek, S. Shen, C. M. Whitehouse, and J. G. Boyle, Pittsburgh Conference Proceedings, (2000).

"High Performance at High Throughput Lessons from LC-API-TOF-MS", E. G. Gulcicek, B. A. Andrien S. Shen, C. M. Whitehouse, and J. G. Boyle, Pittsburgh Conference Proceedings, (2000).

"Increasing the Duty Cycle for Time-of-Flight by Trap-Pulse Mode", B. A. Andrien, C. M. Whitehouse, E. E. Gulcicek, 47th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Dallas, TX, (1999).

"Rapid API TOF State Switching with Fast LC-MS", C. M. Whitehouse, E. E. Gulcicek, B. A. Andrien, S. Shen, 47th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Dallas, TX, (1999).

"Obtaining High Mass Accuracy on LC-TOF-MS", E. E. Gulcicek, C. M. Whitehouse, B. A. Andrien, 47th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Dallas, TX, (1999).

"Dual Parallel Probes for Electrospray Sources", S. Shen, B. A. Andrien, M. Sansone, and C. M. Whitehouse, 47th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Dallas, TX, (1999).

"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).

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"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).

"Emerging Biomedical High Throughput Sample Inlet Systems Utilizing an API TOF MS Detector", E. E. Gulcicek, B. A. Andrien, J.F. Banks, J.G. Boyle, and C. M. Whitehouse, 45th ASMS Conference of Mass Spectrometry and Allied Topics Proceedings, Palm Springs, CA (1997).

"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).

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"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.

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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

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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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