information. Between 1983 and 1987, information on births of Hispanic parentage was available for 23 States and the District of Columbia. In 1988, this information became available for Alabama, Connecticut, Kentucky, Massachusetts, Montana, North Carolina, and Washington, increasing the number of States reporting information on births of Hispanic parentage to 30 States and the District of Columbia. In 1989 this information became available from an additional 17 States, increasing the number of Hispanic-reporting States to 47 and the District of Columbia. In 1989, only Louisiana, New Hampshire, and Oklahoma did not report Hispanic parentage on the birth certificate. With the inclusion of Oklahoma in 1989 and Louisiana in 1990 as Hispanic-reporting States, 99 percent of birth records included information on mother's origin. Hispanic origin of the mother was reported on the birth certificates of 49 States and the District of Columbia in 1991 and 1992; only New Hampshire did not provide this information. Starting in 1993 Hispanic origin of mother was reported by all 50 States and the District of Columbia.

Mortality File—The reporting area for an Hispanic-origin item on the death certificate expanded between 1985 and 1997. In 1985 mortality data by Hispanic origin of decedent were based on deaths to residents of the following 17 States and the District of Columbia whose data on the death certificate were at least 90 percent complete on a place-of-occurrence basis and of comparable format: Arizona, Arkansas, California, Colorado, Georgia, Hawaii, Illinois, Indiana, Kansas, Mississippi, Nebraska, New York, North Dakota, Ohio, Texas, Utah, and Wyoming. In 1986 New Jersey began reporting Hispanic origin of decedent, increasing the number of reporting States to 18 and the District of Columbia in 1986 and 1987. In 1988 Alabama, Kentucky, Maine, Montana, North Carolina, Oregon, Rhode Island, and Washington were added to the reporting area, increasing the number of States to 26 and the District of Columbia. In 1989 an additional 18 States were added, increasing the Hispanic reporting area to 44 States and the District of Columbia; only Connecticut, Louisiana, Maryland, New Hampshire, Oklahoma, and Virginia were not included in the reporting area. Starting with 1990 data in this book, the criterion was changed to include States whose data were at least 80 percent complete. In 1990 Maryland, Virginia, and Connecticut, in 1991 Louisiana, and in 1993 New Hampshire were added, increasing the reporting area for Hispanic origin of decedent to 47 States and the District of Columbia in 1990, 48 States and the District of Columbia in 1991 and 1992, and 49 States and the District of Columbia in 1993–96. Only Oklahoma did not provide this information in 1993–96. Starting in 1997 Hispanic origin of decedent was reported by all 50 States and the District of Columbia. Based on data from the U.S. Census Bureau, the 1990 reporting area encompassed 99.6 percent of the U.S. Hispanic population. In 1990 more than 96 percent of death records included information on Hispanic origin of decedent.

See related *Race.*

HIV—See *Human immunodeficiency virus (HIV) disease.*

Home health care—Home health care as defined by the National Home and Hospice Care Survey is care provided by a home health care agency to individuals and families in their place of residence for promoting, maintaining, or restoring health or for minimizing the effects of disability and illness including terminal illness.

Home visit—Starting in 1997, the National Health Interview Survey has been collecting information on home visits received during the past 12 months. Respondents are asked "During the past 12 months, did you receive care at home from a nurse or other health care professional? What was the total number of home visits received?" These data are combined with data on visits to doctors' offices, clinics, and emergency departments to provide a summary measure of health care visits. See related *Emergency department or emergency room visit; Health care contact.*

Hospice care—Hospice care as defined by the National Home and Hospice Care Survey is a program of palliative and supportive care services providing physical, psychological, social, and spiritual care for dying persons, their families, and other loved ones by a hospice program or agency. Hospice services are available in home and inpatient settings.

Hospital—According to the American Hospital Association, hospitals are licensed institutions with at least six beds whose primary function is to provide diagnostic and therapeutic patient services for medical conditions by an organized physician staff and that have continuous nursing services

Samsung Bioepis Exhibit No. 1002
Page 4933

under the supervision of registered nurses. The World Health Organization considers an establishment to be a hospital if it is permanently staffed by at least one physician, can offer inpatient accommodation, and can provide active medical and nursing care. Hospitals may be classified by type of service, ownership, size in terms of number of beds, and length of stay. In the National Hospital Ambulatory Medical Care Survey, hospitals include all those with an average length of stay for all patients of less than 30 days (short-stay) or hospitals whose specialty is general (medical or surgical) or children's general. Federal hospitals and hospital units of institutions and hospitals with fewer than six beds staffed for patient use are excluded. See related *Average length of stay; Bed; Days of care; Emergency department; Inpatient; Outpatient department.*

Community hospitals—Community hospitals based on the American Hospital Association definition include all non-Federal short-term general and special hospitals whose facilities and services are available to the public. Special hospitals include obstetrics and gynecology; eye, ear, nose, and throat; rehabilitation; orthopedic; and other specialty services. Short-term general and special childrens hospitals are also considered to be community hospitals. A hospital may include a nursing-home-type unit and still be classified as short-term, provided that the majority of its patients are admitted to units where the average length of stay is less than 30 days. Hospital units of institutions such as prisons and college infirmaries that are not open to the public and are contained within a nonhospital facility are not included in the category of community hospitals. Traditionally the definition included all non-Federal short-stay hospitals except facilities for the mentally retarded. In a revised definition the following additional sites were excluded: hospital units of institutions, and alcoholism and chemical dependency facilities.

Federal hospitals—Federal hospitals are operated by the Federal Government.

For-profit hospitals—For-profit hospitals are operated for profit by individuals, partnerships, or corporations.

General hospitals—General hospitals provide diagnostic, treatment, and surgical services for patients with a variety of medical conditions. According to the World Health Organization, these hospitals provide medical and nursing care for more than one category of medical discipline (e.g., general medicine, specialized medicine, general surgery, specialized surgery, and obstetrics). Excluded are hospitals, usually in rural areas, that provide a more limited range of care.

Nonprofit hospitals—Nonprofit hospitals are controlled by nonprofit organizations, including religious organizations, fraternal societies, and others.

Psychiatric hospitals—Psychiatric hospitals are ones whose major type of service is psychiatric care. See related *Mental health organization.*

Registered hospitals—Registered hospitals are registered with the American Hospital Association. About 98 percent of hospitals are registered.

Short-stay hospitals—Short-stay hospitals in the National Hospital Discharge Survey, are those in which the average length of stay is less than 30 days. The National Health Interview Survey defines a short-stay hospitals as any hospital or hospital department in which the type of service provided is general; maternity; eye, ear, nose, and throat; children's; or osteopathic.

Specialty hospitals—Specialty hospitals such as psychiatric, tuberculosis, chronic disease, rehabilitation, maternity, and alcoholic or narcotic, provide a particular type of service to the majority of their patients.

Hospital-based physician—See *Physician.*

Hospital days—See *Days of care.*

Hospital utilization—Estimates of hospital utilization (such as hospital discharge rate, days of care rate, and average length of stay) presented in *Health, United States* are based on data from three different sources—the National Health Interview Survey (NHIS), the National Hospital Discharge Survey (NHDS), and the American Hospital Association. Estimates of hospital utilization from the NHIS and NHDS may differ because NHIS data are based on household interviews of the civilian noninstitutionalized population whereas NHDS data are based on hospital discharge records of all persons. Starting in 1997, hospital utilization data from the NHIS are for all hospital discharges whereas estimates for prior years excluded hospitalizations for delivery and newborns. NHDS includes hospital discharge records for all persons discharged alive or

Samsung Bioepis Exhibit No. 1002
Page 4934

Table VIII. Codes for industries, according to the 2002 North American Industry Classification System (NAICS)

Industry	Code numbers
Goods producing	
Natural resources and mining:	
Agriculture, forestry, fishing, and hunting	11
Mining	21
Construction	23
Manufacturing	31–33
Service providing	
Trade, transportation, and utilities:	
Wholesale trade	42
Retail trade	44–45
Transportation and warehousing	48–49
Utilities	22
Information	51
Financial activities:	
Finance and insurance	52
Real estate and rental and leasing	53
Professional and business services:	
Professional, scientific, and technical services	54
Management of companies and enterprises	55
Administrative and support and waste management and remediation services	56
Education and health services:	
Educational services	61
Health care and social assistance	62
Leisure and hospitality:	
Arts, entertainment, and recreation	71
Accommodation and food services	72
Other services, except public administration	81
Public administration	92

deceased and institutionalized persons and excludes data for newborn infants. Differences in hospital utilization estimated by these two surveys are particularly evident for children and the elderly. For children, NHIS estimates are higher than NHDS estimates because NHIS includes data for newborns. For the elderly NHDS estimates are higher than NHIS estimates because NHIS includes data for institutionalized persons and persons who died while hospitalized. Estimates for average length of stay between the three sources presented in *Health, United States* differ because of different methods for counting days of care. See related *Average length of stay; Days of care; Discharge; Appendix I, National Health Interview Survey, National Hospital Discharge Survey.*

Human immunodeficiency virus (HIV) disease—HIV disease is a serious disease caused by a cytopathic retrovirus that is the cause of Acquired Immunity Syndrome (AIDS). It is also called AIDS-related virus, human T-cell leukemia virus type III, human T-cell lymphotrophic virus type III, and lymphadenopathy-associated virus. Mortality and morbidity coding for HIV disease are similar and have evolved over time.

Mortality coding—Starting with data year 1999 and the introduction of the Tenth Revision of the *International Classification of Diseases* (ICD–10), the title for this cause of death was changed to "HIV disease" from "HIV infection" and the ICD codes changed to B20–B24. Beginning with data for 1987, NCHS introduced category numbers *042–*044 for classifying and coding HIV infection as a cause of death in ICD–9. The asterisk before the category numbers indicates that these codes were not part of the original ICD–9. HIV infection was formerly referred to as human T-cell lymphotropic virus-III/lymphadenopathy-associated virus (HTLV-III/LAV) infection. Before 1987 deaths involving HIV infection were classified to Deficiency of cell-mediated immunity (ICD–9 279.1) contained in the title "All other diseases"; to Pneumocystosis (ICD–9 136.3) contained in the title "All other infectious and parasitic diseases"; to "Malignant neoplasms, including neoplasms of lymphatic and hematopoietic tissues"; and to a number of other causes. Therefore, before 1987, death statistics for HIV infection are not strictly comparable with data for 1987 and later years and are not shown in this report.

Morbidity coding—The National Hospital Discharge Survey codes diagnosis data using the *International Classification of Diseases, Ninth Revision, Clinical Modification* (ICD–9–CM). Discharges with diagnosis of HIV as shown in *Health, United States* have at least one HIV diagnosis listed on the face sheet of the medical record and are not limited to the first-listed diagnosis. During 1984 and 1985 only data for AIDS (ICD–9–CM 279.19) were included. In 1986–94 discharges with the following diagnoses were included: Acquired immunodeficiency syndrome (AIDS), Human immunodeficiency virus (HIV) infection and associated conditions, and Positive serological or viral culture findings for HIV (ICD–9–CM 042–044, 279.19, and 795.8). Beginning in 1995 discharges with the following diagnoses were included: Human immunodeficiency virus (HIV) disease and Asymptomatic human immunodeficiency virus (HIV) infection status (ICD–9–CM 042 and V08).

See related *Acquired immunodeficiency syndrome (AIDS); Cause of death; International Classification of*

Samsung Bioepis Exhibit No. 1002
Page 4935

Diseases (ICD); International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM).

ICD; ICD codes—See *Cause of death; International Classification of Diseases (ICD).*

Illicit drug use—Illicit drug use refers to use and misuse of illegal and controlled drugs.

Monitoring the Future Study—In this school-based survey of secondary school students, information on marijuana use is collected using self-completed questionnaires. The information is based on the following questions: "On how many occasions (if any) have you used marijuana in the last 30 days?" and "On how many occasions (if any) have you used hashish in the last 30 days?" Questions on cocaine use include the following: "On how many occasions (if any) have you take 'crack' (cocaine in chunk or rock form) during the last 30 days?" and "On how many occasions (if any) have you taken cocaine in any other form during the last 30 days?"

National Survey on Drug Use & Health (NSDUH)—Information on illicit drug use is collected for all persons 12 years of age and over. Information on any illicit drug use, including marijuana or hashish, cocaine, heroin, hallucinogens, and nonmedical use of prescription drugs is based on the following question: "During the past 30 days, on how many days did you use (specific illicit drug)?" See related *Substance use.*

Incidence—Incidence is the number of cases of disease having their onset during a prescribed period of time. It is often expressed as a rate (e.g., the incidence of measles per 1,000 children 5–15 years of age during a specified year). Incidence is a measure of morbidity or other events that occur within a specified period of time. See related *Prevalence.*

Income—See *Family income.*

Individual practice association (IPA)—See *Health maintenance organization (HMO).*

Industry of employment—Industries are classified according to the 2002 North American Industry Classification System (NAICS) for the presentation of health data in Health, United States, starting with data year 2003. The NAICS classification system groups establishments into industries based on their production or supply function—establishments using similar raw material inputs, capital equipment, and labor are classified in the same industry. This approach creates homogeneous categories well suited for economic analysis. NAICS uses a six-digit hierarchical coding system to classify all economic activity into 20 industry sectors. The first two digits of the six-digit code designate the highest level of aggregation, with 20 such two-digit industry sectors (table VIII). Five sectors are primarily goods-producing sectors, and 15 are entirely services-providing sectors. NAICS allows for the classification of 1,170 industries.

NAICS replaces the Standard Industrial Classification (SIC) system, originally designed in the 1930s and revised and updated periodically to reflect changes in the U.S. economy. The last SIC revision was in 1987. The SIC system focused on the manufacturing sector of the economy and provided significantly less detail for the now dominant service sector, including newly developed industries in information services, health care delivery, and high-tech manufacturing. Although some titles in SIC and NAICS are similar, there is little comparability between the two systems because industry groupings are defined differently.

Starting with *Health United States, 2005*, health data by industry from the Bureau of Labor Statistics' Census of Fatal Occupational Injuries (CFOI) and Survey of Occupational Injuries and Illnesses (SOII) data systems are classified using the NAICS system and replace trends in occupational health data based on the SIC system in previous editions of *Health, United States.* With 2003 data in which industries are classified by NAICS system, CFOI and SOII start a new trend, and the estimates of deaths, injuries, and illnesses by industry should not be compared with earlier estimates that used the SIC.

Infant death—An infant death is the death of a live-born child before his or her first birthday. Age at death may be further classified according to neonatal and postneonatal. Neonatal deaths are those that occur before the 28th day of life; postneonatal deaths are those that occur between 28 and 365 days of age. See related *Rate: Death and related rates.*

Injury—The International Classification of External Causes of Injuries (ICECI) Coordination and Maintenance Group defines injury as a (suspected) bodily lesion resulting from acute overexposure to energy (this can be mechanical, thermal, electrical, chemical, or radiant) interacting with the body in amounts or rates that exceed the threshold of physiological

Samsung Bioepis Exhibit No. 1002
Page 4936

Table IX. Codes for diagnostic categories from the *International Classification of Diseases, Ninth Revision, Clinical Modification*

Diagnostic category	*Code numbers*
Females with delivery	V27
Human immunodeficiency virus (HIV) (1984–85)	279.19
(1986–94)	042–044, 279.19, 795.8
(Beginning in 1995)	042, V08
Malignant neoplasms	140–208
Large intestine and rectum	153–154, 197.5
Trachea, bronchus, and lung	162, 197.0, 197.3
Breast	174–175, 198.81
Prostate	185
Diabetes	250
Alcohol and drug	291–292, 303–305
Serious mental illness	295–298
Diseases of the nervous system and sense organs	320–389
Diseases of the circulatory system	390–459
Diseases of heart	391–392.0, 393–398, 402, 404, 410–416, 420–429
Ischemic heart disease	410–414
Acute myocardial infarction	410
Congestive heart failure	428.0
Cerebrovascular diseases	430–438
Diseases of the respiratory system	460–519
Pneumonia	466.1, 480–487.0
Asthma	493
Hyperplasia of prostate	600
Decubitus ulcers	707.0
Diseases of the musculoskeletal system and connective tissue	710–739
Osteoarthritis	715
Intervertebral disc disorders	722
Injuries and poisoning	800–999
Fracture, all sites	800–829
Fracture of neck of femur (hip)	820

tolerance. In some cases an injury results from an insufficiency of any of the vital elements. Acute poisonings and toxic effects, including overdoses of substances and wrong substances given or taken in error are included, as are adverse effects and complications of therapeutic, surgical, and medical care. Psychological harm is excluded. Injuries can be intentional or unintentional (i.e., accidental). External causes of nonfatal injuries in NCHS data systems are coded to the *International Classification of Diseases, Ninth Revision, Clinical Modification* Supplementary Classification of External Causes of Injury and Poisoning, often referred to as "E-Codes." See table VII for a list of injury categories and codes. See related *Diagnosis; Injury-related visit.* See ICECI Coordination and Maintenance Group (2004). *International Classification of External Causes of Injuries* (ICECI), version 1.2. Consumer Safety Institute, Amsterdam and AIHW National Injury Surveillance Unit, Adelaide, found at www.iceci.org.

Injury-related visit—In the National Hospital Ambulatory Medical Care Survey an emergency department visit was considered injury related if, on the Patient Record Form (PRF), the checkbox for injury was indicated. In addition, injury visits were identified if the physician's diagnosis was injury related (ICD–9–CM code of 800–999), an external cause-of-injury code was present (ICD–9–CM E800–E999), or the patient's reason for visit code was injury-related. See related *Emergency department or emergency room visit; External cause of injury; Injury.*

Inpatient—An inpatient is a person who is formally admitted to the inpatient service of a hospital for observation, care, diagnosis, or treatment. See related *Admission; Average length of stay; Days of care; Discharge; Hospital.*

Inpatient care—See *Hospital utilization; Mental health service type.*

Inpatient days—See *Days of care.*

Instrumental activities of daily living (IADL)—Instrumental activities of daily living are activities related to independent living and include preparing meals, managing money, shopping for groceries or personal items, performing light or

Samsung Bioepis Exhibit No. 1002
Page 4937

Table X. Codes for procedure categories from the *International Classification of Diseases, Ninth Revision, Clinical Modification*

Procedure category	*Code numbers*
Operations on vessels of heart	36
Removal of coronary artery obstruction and insertion of stent(s)	36.0
Insertion of coronary artery stent(s)[1]	36.06, 36.07
Coronary artery bypass graft	36.1
Cardiac catheterization	37.21–37.23
Insertion, replacement, removal, and revision of pacemaker leads or device	37.7–37.8
Incision, excision, and occlusion of vessels	38
Diagnostic procedures on small intestine	45.1
Diagnostic procedures on large intestine	45.2
Cholecystectomy	51.2
Laparoscopic cholecystectomy	51.23
Repair of hernia	53
Lysis of peritoneal adhesions	54.5
Transurethral prostatectomy	60.2
Total abdominal hysterectomy	68.4
Vaginal hysterectomy	68.5
Dilation and curettage of uterus	69.0
Forceps, vacuum, and breech delivery	72
Other procedures inducing or assisting delivery	73
Cesarean section and removal of fetus	74
Reduction of fracture and dislocation	79
Excision or destruction of intervertebral disc	80.5
Joint replacement of lower extremity	81.5
Total hip replacement	81.51
Partial hip replacement	81.52
Total knee replacement	81.54
Diagnostic Radiology	87
Computerized axial tomography	87.03, 87.41, 87.71, 88.01, 88.38
Angiocardiography using contrast material	88.5
Diagnostic ultrasound	88.7

[1]The procedure code for insertion of coronary artery stents (36.06) first appears in the 1996 data. A second procedure code for the insertion of drug-eluting stents (36.07) first appears in the 2003 data.

heavy housework, and using a telephone. In the National Health Interview Survey (NHIS) respondents are asked whether they or family members 18 years of age and over need the help of another person for handling routine IADL needs because of a physical, mental, or emotional problem. Persons are considered to have an IADL limitation in the NHIS if any causal condition is chronic.

In the Medicare Current Beneficiary Survey, if a sample person had any difficulty performing an activity by him or herself and without special equipment, or did not perform the activity at all because of health problems, the person was categorized as having a limitation in that activity. The limitation may have been temporary or chronic at the time of the interview. Sample persons in the community answered health status and functioning questions themselves, if able to do so. For sample persons in a long-term care facility, a proxy such as a nurse answered questions about the sample person's health status and functioning. See related *Activities of daily living (ADL); Limitation of activity.*

Insured—See *Health insurance coverage.*

Intermediate care facilities—See *Nursing home.*

International Classification of Diseases (ICD)—The ICD provides the ground rules for coding and classifying cause-of-death data. The ICD is developed collaboratively by the World Health Organization (WHO) and 10 international centers, one of which is housed at NCHS. The purpose of the ICD is to promote international comparability in the collection, classification, processing, and presentation of

Samsung Bioepis Exhibit No. 1002
Page 4938

health statistics. Since 1900, the ICD has been modified about once every 10 years, except for the 20-year interval between ICD–9 and ICD–10 (see table IV). The purpose of the revisions is to stay abreast with advances in medical science. New revisions usually introduce major disruptions in time series of mortality statistics (see tables V and VI). For more information, see www.cdc.gov/nchs/about/major/dvs/icd10des.htm. See related *Cause of death; Comparability ratio; International Classification of Diseases, Ninth Revision, Clinical Modification (ICD–9–CM).*

International Classification of Diseases, Ninth Revision, Clinical Modification (ICD–9–CM)—The ICD–9–CM is based on and is compatible with the World Health Organization's *International Classification of Diseases, Ninth Revision* (ICD–9). The United States currently uses ICD–9–CM to code morbidity diagnoses and inpatient procedures. ICD–9–CM consists of three volumes. Volumes 1 and 2 contain the diagnosis tabular list and index. Volume 3 contains the procedure classification (tabular and index combined).

ICD–9–CM is divided into 17 chapters and 2 supplemental classifications. The chapters are arranged primarily by body system. In addition, there are chapters for Infectious and parasitic diseases; Neoplasms; Endocrine, nutritional, and metabolic diseases; Mental disorders; Complications of pregnancy, childbirth and puerperium; Certain conditions originating in the perinatal period; Congenital anomalies; and Symptoms, signs and ill-defined conditions. The two supplemental classifications are for factors influencing health status and contact with health services ("V Codes"), and external causes of injury and poisoning ("E Codes").

In *Health, United States* morbidity data are classified using ICD–9–CM. Diagnostic categories and codes for ICD–9–CM are shown in table IX; ICD–9–CM procedure categories and codes are shown in table X. For additional information about ICD–9–CM, see www.cdc.gov/nchs/icd9.htm. See related *International Classification of Diseases (ICD).*

Late fetal death rate—See *Rate: Death and related rates.*

Leading causes of death—See *Cause-of-death ranking.*

Length of stay—See *Average length of stay.*

Life expectancy—Life expectancy is the average number of years of life remaining to a person at a particular age and is based on a given set of age-specific death rates, generally the mortality conditions existing in the period mentioned. Life expectancy may be determined by race, sex, or other characteristics using age-specific death rates for the population with that characteristic. See related *Rate: Death and related rates.*

Limitation of activity—Limitation of activity may be defined different ways, depending on the conceptual framework. In the National Health Interview Survey limitation of activity refers to a long-term reduction in a person's capacity to perform the usual kind or amount of activities associated with his or her age group as a result of a chronic condition. Limitation of activity is assessed by asking persons a series of questions about limitations in their or household members' ability to perform activities usual for their age group because of a physical, mental, or emotional problem. Persons are asked about limitations in activities of daily living, instrumental activities of daily living, play, school, work, difficulty walking or remembering, and any other activity limitations. For reported limitations, the causal health conditions are determined, and persons are considered limited if one or more of these conditions is chronic. Children under 18 years of age who receive special education or early intervention services are considered to have a limitation of activity. See related *Activities of daily living; Condition; Instrumental activities of daily living.*

Live-birth order—In the National Vital Statistics System, this item from the birth certificate refers to the total number of live births the mother has had, including the present birth as recorded on the birth certificate. Fetal deaths are excluded.

Long-term care facility—A long-term care facility is a residence that provides a specific level of personal or medical care or supervision to residents. In the Medicare Current Beneficiary Survey, a residence is considered a long-term care facility if it has three or more long-term care beds and provides personal care services to residents, continuous supervision of residents, or long-term care services throughout the facility or in a separately identifiable unit. Types of long-term care facilities include licensed nursing homes, skilled nursing homes, intermediate care facilities, retirement homes (that provide services), domiciliary or personal care facilities, distinct long-term care units in a hospital complex, mental health facilities and centers, assisted and foster care homes, and institutions for the mentally retarded and developmentally disabled. See related *Nursing home.*

Samsung Bioepis Exhibit No. 1002
Page 4939

Low birthweight—See *Birthweight*.

Mammography—Mammography is an x-ray image of the breast used to detect irregularities in breast tissue. In the National Health Interview Survey, questions concerning use of mammography were asked on an intermittent schedule, and question content differed slightly across years. In 1987 and 1990, women were asked to report when they had their last mammogram. In 1991, women were asked whether they had a mammogram in the past 2 years. In 1993 and 1994, women were asked whether they had a mammogram within the past year, between 1 and 2 years ago, or over 2 years ago. In 1998, women were asked whether they had a mammogram a year ago or less, more than 1 year but not more than 2 years, or more than 2 years ago. In 1999, women were asked when they had their most recent mammogram in days, weeks, months, or years. In 1999, 10 percent of women in the sample responded "2 years ago," and in this analysis these women were coded as "within the past 2 years" although a response of "2 years ago" may include women whose last mammogram was more than 2 but less than 3 years ago. Thus, estimates for 1999 are overestimated to some degree in comparison with estimates in previous years. In 2000 and 2003, women were asked when they had their most recent mammogram (give month and year). Women who did not respond were given a followup question that used the 1999 wording, and women who did not answer the followup question were asked a second followup question that used the 1998 wording. In 2000 and 2003, 2 percent of women in the sample answered "2 years ago" using the 1999 wording, and they were coded as "within the past 2 years." Thus, estimates for 2000 and 2003 may be slightly overestimated in comparison with estimates for years prior to 1999.

Managed care—Managed care is a term originally used to refer to the prepaid health care sector (health maintenance organizations or HMOs) where care is provided under a fixed budget and costs are therein capable of being managed. Increasingly, the term is being used to include preferred provider organizations (PPOs) and even forms of indemnity insurance coverage (or fee-for-service insurance) that incorporate preadmission certification and other utilization controls. See related *Health maintenance organization (HMO)*; *Preferred provider organization (PPO)*.

Marital status—Marital status is classified through self-reporting into the categories married and unmarried. The term "married" encompasses all married people including those separated from their spouses. Unmarried includes those who are single (never married), divorced, or widowed. The abortion surveillance program classified separated people as unmarried before 1978.

Birth File—In 1970, 39 States and the District of Columbia (DC) and in 1975, 38 States and DC included a direct question about mother's marital status on the birth certificate. Since 1980, national estimates of births to unmarried women have been based on two methods for determining marital status, a direct question in the birth registration process and inferential procedures. In 1980–96 marital status was reported on the birth certificates of 41–45 States and DC; with the addition of California in 1997, 46 States and DC; and in 1998–2001, 48 States and DC. In 1997, all but four States (Connecticut, Michigan, Nevada, and New York), and in 1998, all but two States (Michigan and New York) included a direct question about mother's marital status on their birth certificates. In 1998–2001, marital status was imputed as married on those 0.03–0.05 percent of birth records with missing information in the 48 States and DC where this information was obtained by a direct question.

For States lacking a direct question, marital status was inferred. Before 1980 the incidence of births to unmarried women in States with no direct question on marital status was assumed to be the same as the incidence in reporting States in the same geographic division. Starting in 1980 for States without a direct question, marital status was inferred by comparing the parents' and child's surnames. Inferential procedures in current use depend on the presence of a paternity acknowledgment or missing information on the father. Changes in reporting procedures by some States in 1995 and 1997 had little effect on national totals, but they did affect trends for age groups and some State trends. Details of the changes in reporting procedures are described in Ventura SJ, Bachrach CA. Nonmarital Childbearing in the United States, 1940–99. National vital statistics reports; vol 48 no 16. Hyattsville, MD: National Center for Health Statistics. 2000, available at www.cdc.gov/nchs/births.htm.

Maternal age—See *Age*.

Samsung Bioepis Exhibit No. 1002
Page 4940

Maternal death—Maternal death is defined by the World Health Organization as the death of a woman while pregnant or within 42 days of termination of pregnancy, irrespective of the duration and site of the pregnancy, from any cause related to or aggravated by the pregnancy or its management, but not from accidental or incidental causes. A maternal death is one for which the certifying physician has designated a maternal condition as the underlying cause of death. Maternal conditions are those assigned to pregnancy, childbirth, and the puerperium, ICD–10 codes A34, O00–O95, O98–O99 (see table V). Changes have been made in the classification and coding of maternal deaths between ICD–9 and ICD–10, effective with mortality data for 1999. ICD–10 changes pertain to indirect maternal causes and timing of death relative to pregnancy. If only indirect maternal causes of death (i.e., a previously existing disease or a disease that developed during pregnancy that was not due to direct obstetric causes but was aggravated by physiologic effects of pregnancy) are reported in Part I of the death certificate and pregnancy is reported in either Part I or Part II, ICD–10 classifies this as a maternal death. ICD–9 only classified the death as maternal if pregnancy was reported in Part I. Some State death certificates include a separate question regarding pregnancy status. A positive response to the question is interpreted as "pregnant" being reported in Part II of the cause-of-death section of the death certificate. If the medical certifier did not specify when death occurred relative to the pregnancy, it is assumed that the pregnancy terminated 42 days or less prior to death. Under ICD–10, a new category has been added for deaths from maternal causes that occurred more than 42 days after delivery or termination of pregnancy (O96–O97). In 1999, there were 15 such deaths, and, in 2000, there were eight. See related *Rate: Death and related rates.*

Maternal education—See *Education.*

Maternal mortality rate—See *Rate: Death and related rates.*

Medicaid—Medicaid was authorized by Title XIX of the Social Security Act in 1965 as a jointly funded cooperative venture between the Federal and State Governments to assist States in the provision of adequate medical care to eligible needy persons. Within broad Federal guidelines, each of the States establishes its own eligibility standards; determines the type, amount, duration, and scope of services; sets the rate of payment for services; and administers its own program.

Medicaid is the largest program providing medical and health-related services to America's poorest people. However, Medicaid does not provide medical assistance to all poor persons. Under the broadest provisions of the Federal statute, Medicaid does *no* provide health care services even for very poor childless adults under age 65 years unless they are disabled. Except as noted, all States must provide Medicaid coverage to the following:

- Individuals who meet the requirements for the Aid to Families with Dependent Children (AFDC) program that were in effect in their State on July 16, 1996, or, at State option, more liberal criteria (with some exceptions).
- Children under age 6 whose family income is at or below 133 percent of the Federal poverty level.
- Pregnant women whose family income is below 133 percent of the Federal poverty level (services to these women are limited to those related to pregnancy, complications of pregnancy, delivery, and postpartum care).
- Supplemental Security Income (SSI) recipients in most States (some States use more restrictive Medicaid eligibility requirements that predate SSI).
- Recipients of adoption or foster care assistance under Title IV of the Social Security Act.
- Special protected groups (typically individuals who lose their cash assistance because of earnings from work or from increased Social Security benefits but who may keep Medicaid for a period of time).
- All children under age 19 in families with incomes at or below the Federal poverty level.
- Certain Medicare beneficiaries (low income is only one test for Medicaid eligibility for those within these groups; their resources also are tested against threshold levels, as determined by each State within Federal guidelines).

States also have the option of providing Medicaid coverage for other groups.

Medicaid operates as a vendor payment program. States may pay health care providers directly on a fee-for-service basis, or States may pay for Medicaid services through various prepayment arrangements, such as health maintenance organizations (HMOs) or other forms of managed care. Within Federally imposed upper limits and specific restrictions, each State for the most part has broad discretion in determining the payment methodology and payment rate for services. Thus, the Medicaid program varies considerably from State to

Samsung Bioepis Exhibit No. 1002
Page 4941

State, as well as within each State over time. See related *Health expenditures, national; Health insurance coverage; Health maintenance organization (HMO); Managed care; Appendix I, Medicaid Data System.*

Medical specialties—See *Physician specialty.*

Medical vendor payments—Under the Medicaid program, medical vendor payments are payments (expenditures) to medical vendors from the State through a fiscal agent or to a health insurance plan. Adjustments are made for Indian Health Service payments to Medicaid, cost settlements, third party recoupments, refunds, voided checks, and other financial settlements that cannot be related to specific provided claims. Excluded are payments made for medical care under the emergency assistance provisions, payments made from State medical assistance funds that are not federally matchable, disproportionate share hospital payments, cost sharing or enrollment fees collected from recipients or a third party, and administration and training costs.

Medicare—This is a nationwide health insurance program providing health insurance protection to people 65 years of age and over, people entitled to Social Security disability payments for 2 years or more, and people with end-stage renal disease, regardless of income. The program was enacted July 30, 1965, as Title XVIII, *Health Insurance for the Aged of the Social Security Act,* and became effective on July 1, 1966. From its inception, it has included two separate but coordinated programs: hospital insurance (Part A) and supplementary medical insurance (Part B). In 1999, additional choices were allowed for delivering Medicare Part A and Part B benefits. "Medicare+Choice" (Part C) is an expanded set of options for the delivery of health care under Medicare, created in the Balanced Budget Act passed by Congress in 1997. The term "Medicare+Choice" refers to options other than original Medicare. While all Medicare beneficiaries can receive their benefits through the original fee-for-service (FFS) program, most beneficiaries enrolled in both Part A and Part B can choose to participate in a Medicare+Choice plan instead. Organizations that seek to contract as Medicare+Choice plans must meet specific organizational, financial, and other requirements. Most Medicare+Choice plans are coordinated care plans, which include health maintenance organizations (HMOs), provider-sponsored organizations (PSOs), preferred provider organizations (PPOs), and other certified coordinated care plans and entities that meet the standards set forth in the law. The Medicare+Choice program also includes Medical savings account (MSA) plans, which provide benefits after a single high deductible is met, and private, unrestricted FFS plans, which allow beneficiaries to select certain private providers. These programs are available in only a limited number of States. For those providers who agree to accept the plan's payment terms and conditions, this option does not place the providers at risk, nor does it vary payment rates based on utilization. Only the coordinated care plans are considered managed care plans. Except for MSA plans, all Medicare+Choice plans are required to provide at least the current Medicare benefit package, excluding hospice services. Plans may offer additional covered services and are required to do so (or return excess payments) if plan costs are lower than the Medicare payments received by the plan.

In the National Health Interview Survey (NHIS), the category "Medicare HMO" is defined as persons who are age 65 years or over and who responded "Yes" when asked whether they were under a Medicare managed care arrangement such as an HMO. This is a subset of Medicare Part C. Respondents who stated they had Medicare coverage but did not answer yes to the "managed care arrangement such as an HMO" are included in the Medicare fee-for-service category. "Medicare fee-for-service" is defined as Medicare Part A or Part B. The majority of these people had coverage from another source, primarily employer-sponsored retiree health insurance. Some tables in *Health, United States* further classify persons 65 years of age and over with Medicare fee-for service into Medicare fee-for-service only and Private (including Medicare fee-for-service) coverage.

See related *Fee-for-service health insurance; Health insurance coverage; Health maintenance organization (HMO); Managed care; Appendix I, Medicare Administrative Data.*

Mental health organization—The Center for Mental Health Services of the Substance Abuse and Mental Health Services Administration defines a mental health organization as an administratively distinct public or private agency or institution whose primary concern is provision of direct mental health services to the mentally ill or emotionally disturbed. Excluded are private office-based practices of psychiatrists, psychologists, and other mental health providers; psychiatric services of all types of hospitals or outpatient clinics operated by Federal agencies other than the Department of Veterans Affairs (e.g., Public Health Service, Indian Health Service, Department of Defense, and Bureau of Prisons); general

Samsung Bioepis Exhibit No. 1002
Page 4942

Table XI. National Drug Code (NDC) therapeutic class analgesic drug recodes

Narcotic analgesics	*Nonnarcotic analgesics*	*Nonsteroidal anti-inflammatory drugs (NSAIDs)*
Alfentanil Hydrochloride	Acetaminophen	Bromfenac Sodium
Alphaprodine	Acetylsalicylic Acid	Celecoxib
Bupernorphine	Aminobenzoic Acid	Diclofenac Potassium
Butorphanol	Aspirin	Diclofenac Sodium
Codeine	Auranofin	Difunisal
Dihydrocodeine	Aurothioglucose	Etodolac
Fentanyl	Butalbital	Fenoprofen
Hydrocodone Bitartrate	Capsaicin	Flurbiprofen Sodium
Hydromorphone	Carbaspirin Calcium	Ibuprofen
Levorphanol	Choline Salicylate	Indomethacin
Meperidine	Etanercept	Ketoprofen
Meperidine HCl	Fluprednisolone	Ketorolac Tromethamine
Methadone	Gold Sodium Thiomalate	Meclofenamate
Morphine	Gold Sodium Thiosulfate	Meclofenamic Acid
Morphine Sulfate	Hyaluronic Acid	Mefenamic Acid
Nalbuphine	Leflunomide	Meloxicam
Opium	Magnesium Salicylate	Nabumetone
Oxycodone	Menthol	Naproxen
Oxycodone HCl	Methotrexate	Oxaprozin
Pentazocine	Methylprednisolone	Piroxicam
Propoxyphene	Methylsulfonylmethane	Rofecoxib
Remifentanyl	Oxyphenbutazone	Sulindac
	Phenyl Salicylate	Suprofen
	Phenylbutazone	Tolmetin
	Prednisolone	Valdecoxib
	Salicylamide	
	Salsalate	
	Sodium Hyaluronate	
	Sodium Salicylate	
	Sodium Thiosalicylate	
	Tramadol	
	Triamcinilone	
	Zomepirac	

NOTE: Drugs originally classified as National Drug Code (NDC) therapeutic category 1720 (general analgesics); 1721 (narcotic analgesics); 1722 (non-narcotic analgesics); 1724 (antiarthritics); 1727 (NSAIDs); 1726 (antipyretics); and 1729 (menstrual products) were recoded into the three mutually exclusive categories shown above. NDC codes for the analgesic categories 1723 (antimigraine) and 1725 (antigout) were not recoded.

hospitals that have no separate psychiatric services but admit psychiatric patients to nonpsychiatric units; and psychiatric services of schools, colleges, halfway houses, community residential organizations, local and county jails, State prisons, and other human services providers. The major types of mental health organizations are described below.

Freestanding psychiatric outpatient clinics—These clinics provide only outpatient mental health services on either a regular or emergency basis. A psychiatrist generally assumes the medical responsibility for services.

Psychiatric hospitals—These hospitals (public or private) primarily provide 24-hour inpatient care and treatment in a hospital setting to persons with mental illnesses. Psychiatric hospitals may be under State, county, private for profit, or private nonprofit auspices.

General hospital psychiatric services—These are organizations that provide psychiatric services with assigned staff for 24-hour inpatient or residential care and/or less than 24-hour outpatient care in a separate ward, unit, floor, or wing of the hospital.

Samsung Bioepis Exhibit No. 1002
Page 4943

Department of Veterans Affairs medical centers—These are hospitals are hospitals operated by the Department of Veterans Affairs (formerly Veterans Administration) and include Department of Veterans Affairs general hospital psychiatric services (including large neuropsychiatric units) and Department of Veterans Affairs psychiatric outpatient clinics.

Residential treatment centers for emotionally disturbed children—These centers must meet all of the following criteria: (a) provide 24-hour residential services; (b) are not licensed as a psychiatric hospital and have the primary purpose of providing individually planned mental health treatment services in conjunction with residential care; (c) include a clinical program directed by a psychiatrist, psychologist, social worker, or psychiatric nurse with a graduate degree; (d) serve children and youth primarily under the age of 18; and (e) have the primary diagnosis as mental illness, classified as other than mental retardation, developmental disability, or substance-related disorders, according to DSM-II/ICDA-8 or DSM-IIIR/ICD-9-CM codes, for the majority of admissions.

Multiservice mental health organizations—These organizations provide services in both 24-hour and less than 24-hour settings and are not classifiable as a psychiatric hospital, general hospital, or residential treatment center for emotionally disturbed children. (The classification of a psychiatric or general hospital or residential treatment center for emotionally disturbed children takes precedence over a multiservice classification, even if two or more services are offered.)

Partial care organizations—These organizations provide a program of ambulatory mental health services or rehabilitation, habitation, or education programs.

See related *Addition; Mental health service type.*

Mental health service type—This term refers to the following types of mental health services:

24-hour mental health care, formerly called inpatient care, provides care in a mental health hospital setting.

Less than 24-hour care, formerly called outpatient or partial care treatment, provides mental health services on an ambulatory basis.

Residential treatment care provides overnight mental health care in conjunction with an intensive treatment program in a setting other than a hospital. Facilities may offer care to emotionally disturbed children or mentally ill adults.

See related *Addition; Mental health organization.*

Metropolitan statistical area (MSA)—The Office of Management and Budget (OMB) defines metropolitan areas according to published standards that are applied to Census Bureau data. An MSA is a county or group of contiguous counties that contains at least one urbanized area of 50,000 or more population. In addition to the county or counties that contain all or part of the urbanized area, an MSA may contain other counties that are metropolitan in character and that are economically and socially integrated with the main city. In New England, cities and towns, rather than counties, are used to define MSAs. Counties that are not within an MSA are considered to be nonmetropolitan.

For National Health Interview Survey (NHIS) data before 1995, metropolitan population is based on MSAs as defined by OMB in 1983 using the 1980 census. Starting with the 1995 NHIS, metropolitan population is based on MSAs as defined by OMB in 1993 using the 1990 census. For additional information about metropolitan statistical areas see www.census.gov/population/www/estimates/metrodef.html.
See related *Urbanization.*

Micropolitan statistical area—The Office of Management and Budget (OMB) defines micropolitan areas based on published standards that are applied to Census Bureau data. A micropolitan statistical area is a nonmetropolitan county or group of contiguous nonmetropolitan counties that contains an urban cluster of 10,000 to 49,999 persons. A micropolitan statistical area may include surrounding counties if there are strong economic ties between the counties, based on commuting patterns. In New England, cities and towns, rather than counties, are used to define micropolitan statistical areas. Nonmetropolitan counties that are not classified as part of a micropolitan statistical area are considered nonmicropolitan. For additional information about micropolitan statistical areas see www.census.gov/population/www/estimates/metrodef.html.
See related *Urbanization.*

Multiservice mental health organizations—See *Mental health organization.*

Samsung Bioepis Exhibit No. 1002
Page 4944

National Drug Code (NDC) Directory therapeutic class—The NDC system was originally established as an essential part of an out-of-hospital drug reimbursement program under Medicare. The NDC serves as a universal product identifier for human drugs. The current edition of the NDC is limited to prescription drugs and a few selected over-the-counter (OTC) products. The directory consists of prescription and selected OTC insulin, domestic, and foreign drug products that are in commercial distribution in the United States. The products have been listed in accordance with the Drug Listing Act and applicable Code of Federal Regulations for submitting drug product information to the Food and Drug Administation (FDA). NDC therapeutic class codes are used to identify each of 20 major drug classes to which the drug entry may belong, adapted from Standard Drug Classifications in the NDC Directory, 1995. The two-digit categories are general and represent all subcategories (e.g., Antimicrobial agents), and the specific four-digit categories represent the breakouts of the general category (e.g., Penicillin). The general two-digit codes include medications that do not fit into any of the subcategories (four-digit codes). Starting in 1995, the NDC four-digit classes were changed to include more classes than the previous classification in 1985. Therefore some drugs switched from a general two-digit class into a more specific four-digit class. In addition, drugs may be approved for several different therapeutic classes. Some drugs receive approval for additional therapeutic uses after their initial approval, so the same drug can change classes because of new uses.

Numerous drug products have many uses or indications. In an effort to categorize the vast number of the broad analgesic or pain-relief individual products in the marketplace into manageable and nonoverlapping categories, all four-digit categories within the analgesic two-digit therapeutic class were recoded by staff of the FDA's Center for Drug Center for Drug Evaluation and Research. Thus, the codes presented in *Health, United States* do not match the published NDC codes for analgesic therapeutic categories. The NDC contains the following four-digit analgesic therapeutic categories: 1720—general analgesic, 1721—narcotic analgesic, 1722—nonnarcotic analgesic, 1724—antiarthritics, 1723—antimigraine/headache, 1726—central pain syndrome, 1727—nonsteroidal anti-inflammatory drugs (NSAID), 1728—antipyretic, and 1729—menstrual products. These categories were collapsed into broader and mutually exclusive categories of narcotic analgesics, nonnarcotic analgesics, and NSAIDs. Under the NDC system, aspirin is coded as an NSAID because of its anti-inflammatory properties, but also as an analgesic, an antiarthritic, and an antipyretic. In this report aspirin has been recoded into the nonnarcotic analgesic category. Aspirin was not included as an NSAID because of its common use for cardiac therapy and its many other indications.

Table XI shows how generic analgesic drugs were reclassified for *Health, United States.* Analgesic drugs were reclassified based on the product's main ingredients or indication of use. For example, Robitussin AC contains several ingredients, one of which is codeine, a narcotic. However, its main use is not for pain but for cough suppression, and it is therefore categorized as a cough and cold product as opposed to a narcotic analgesic product. Another example is methotrexate, which is used for treating certain neoplastic diseases and severe psoriasis in some formulations, but it is also used to treat rheumatoid arthritis and therefore appears in the list of nonnarcotic analgesic drugs, which include previously defined antiarthritic drugs in table XI.

Neonatal mortality rate—See *Rate: Death and related rates.*

Nonpatient revenues—Nonpatient revenues are those revenues received for which no direct patient care services are rendered. The most widely recognized source of nonpatient revenues is philanthropy. Philanthropic support may be direct from individuals or may be obtained through philanthropic fundraising organizations, foundations, or corporations. Philanthropic revenues may be designated for direct patient care use or may be contained in an endowment fund where only the current income may be tapped.

Nonprofit hospitals—See *Hospital.*

North American Industry Classification System (NAICS)—See *Industry of employment.*

Notifiable disease—A notifiable disease is one that, when diagnosed, health providers are required, usually by law, to report to State or local public health officials. Notifiable diseases are those of public interest by reason of their contagiousness, severity, or frequency.

Nurse supply estimates—Nurse supply estimates are based on a model developed by Health Resources and Services Administration's (HRSA's) Bureau of Health Professions to meet the requirements of Section 951, P.L. 94–63. The model estimates the following for each State: (a) population of

Samsung Bioepis Exhibit No. 1002
Page 4945

nurses currently licensed to practice; (b) supply of full-time and part-time practicing nurses (or available to practice); and (c) full-time equivalent supply of nurses practicing full-time plus one-half of those practicing part-time (or available on that basis). The three estimates are divided into three levels of highest educational preparation—associate degree or diploma, baccalaureate, and master's and doctorate. Among the factors considered are new graduates, changes in educational status, nursing employment rates, age, migration patterns, death rates, and licensure phenomena. The base data for the model are derived from the National Sample Surveys of Registered Nurses, conducted by the Division of Nursing, Bureau of Health Professions, HRSA. Other data sources include National League for Nursing for data on nursing education and National Council of State Boards of Nursing for data on licensure. For further information, see HRSA's Division of Nursing Web site at www.bhpr.hrsa.gov/nursing.

Nursing care—The following definition of nursing care applies to data collected in National Nursing Home Surveys through 1977. Nursing care is provision of any of the following services: application of dressings or bandages; bowel and bladder retraining; catheterization; enema; full bed bath; hypodermic, intramuscular, or intravenous injection; irrigation; nasal feeding; oxygen therapy; and temperature-pulse-respiration or blood pressure measurement. See related *Nursing home*.

Nursing care homes—See *Nursing home*.

Nursing home—In the Online Survey Certification and Reporting database, a nursing home is a facility that is certified and meets the Center for Medicare & Medicaid Services' long-term care requirements for Medicare and Medicaid eligibility.

In the National Master Facility Inventory (NMFI), which provided the sampling frame for 1973–74, 1977, and 1985 National Nursing Home Surveys, a nursing home was an establishment with three or more beds that provided nursing or personal care services to the aged, infirm, or chronically ill. The 1977 National Nursing Home Survey included personal care homes and domiciliary care homes, whereas the National Nursing Home Surveys of 1973–74, 1985, 1995, 1997, and 1999 excluded them. The following definitions of nursing home types applied to facilities listed in the NFMI:

Nursing care homes—These homes employ one or more full-time registered or licensed practical nurses and provide nursing care to at least one-half the residents.

Personal care homes with nursing—These homes have fewer than one-half the residents receiving nursing care. In addition, such homes employ one or more registered or licensed practical nurses or provided administration of medications and treatments in accordance with physicians' orders, supervision of self-administered medications, or three or more personal services.

Personal care homes without nursing—These homes have no residents who receive nursing care. These homes provide administration of medications and treatments in accordance with physicians' orders, supervise self-administered medications, or provide three or more personal services.

Domiciliary care homes—These homes primarily provide supervisory care and one or two personal services.

The following definitions of certification levels apply to data collected in National Nursing Home Surveys of 1973–74, 1977, and 1985:

Skilled nursing facilities—These facilities provide the most intensive nursing care available outside a hospital. Facilities certified by Medicare provide posthospital care to eligible Medicare enrollees. Facilities certified by Medicaid as skilled nursing facilities provide skilled nursing services on a daily basis to individuals eligible for Medicaid benefits.

Intermediate care facilities—These facilities are certified by the Medicaid program to provide health-related services on a regular basis to Medicaid eligibles who do not require hospital or skilled nursing facility care but do require institutional care above the level of room and board.

Not certified facilities—These facilities are not certified as providers of care by Medicare or Medicaid.

Beginning with the 1995 National Nursing Home Surveys, nursing homes have been defined as facilities that routinely provide nursing care services and have three or more beds set up for residents. Facilities may be certified by Medicare or Medicaid or not certified but licensed by the State as a

Samsung Bioepis Exhibit No. 1002
Page 4946

nursing home. The facilities may be freestanding or a distinct unit of a larger facility.

After October 1, 1990, long-term care facilities that met the Omnibus Budget Reconciliation Act of 1987 (OBRA 87) nursing home reform requirements that were formerly certified under the Medicaid program as skilled nursing, nursing home, or intermediate care facilities were reclassified as nursing facilities. The Medicare program continues to certify skilled nursing facilities, but not intermediate care facilities. State Medicaid programs can certify intermediate care facilities for the mentally retarded or developmentally disabled. Nursing facilities must also be certified to participate in the Medicare program to be certified for participation in Medicaid except those facilities that have obtained waivers. Thus most nursing home care is now provided in skilled care facilities.

See related *Long-term care facility; Nursing care; Resident.*

Nursing home expenditures—See *Health expenditures, national.*

Obesity—See *Body mass index (BMI).*

Occupancy rate—In American Hospital Association statistics, hospital occupancy rate is calculated as the average daily census divided by the number of hospital beds, cribs, and pediatric bassinets set up and staffed on the last day of the reporting period, expressed as a percent. Average daily census is calculated by dividing the total annual number of inpatients, excluding newborns, by 365 days to derive the number of inpatients receiving care on an average day during the annual reporting period. The occupancy rate for facilities other than hospitals is calculated as the number of residents at the facility reported on the day of the interview divided by the number of reported beds. In the Online Survey Certification and Reporting database, occupancy is determined as of the day of certification inspection as the total number of residents on that day divided by the total number of beds on that day.

Office—In the National Ambulatory Medical Care Survey, a physician's ambulatory practice (office) can be in any location other than in a hospital, nursing home, other extended care facility, patient's home, industrial clinic, college clinic, or family planning clinic. Offices in health maintenance organizations and private offices in hospitals are included. See related *Office visit; Outpatient visit; Physician.*

Office-based physician—See *Physician.*

Office visit—In the National Ambulatory Medical Care Survey, an office visit is any direct personal exchange between an ambulatory patient and a physician or members of his or her staff for the purposes of seeking care and rendering health services. See related *Outpatient visit.*

Operations—See *Procedure.*

Outpatient department—According to the National Hospital Ambulatory Medical Care Survey (NHAMCS), an outpatient department (OPD) is a hospital facility where nonurgent ambulatory medical care is provided. The following types of OPDs are excluded from the NHAMCS: ambulatory surgical centers, chemotherapy, employee health services, renal dialysis, methadone maintenance, and radiology. See related *Emergency department; Outpatient visit.*

Outpatient surgery—According to the American Hospital Association, outpatient surgery is a surgical operation, whether major or minor, performed on patients who do not remain in the hospital overnight. Outpatient surgery may be performed in inpatient operating suites, outpatient surgery suites, or procedure rooms within an outpatient care facility. A surgical operation involving more than one surgical procedure is considered one surgical operation. See related *Procedure.*

Outpatient visit—The American Hospital Association defines outpatient visits as visits for receipt of medical, dental, or other services at a hospital by patients who are not lodged in the hospital. Each appearance by an outpatient to each unit of the hospital is counted individually as an outpatient visit, including all clinic visits, referred visits, observation services, outpatient surgeries, and emergency department visits. In the National Hospital Ambulatory Medical Care Survey an outpatient department visit is a direct personal exchange between a patient and a physician or other health care provider working under the physician's supervision for the purpose of seeking care and receiving personal health services. See related *Emergency department or emergency room visit; Outpatient department.*

Overweight—See *Body mass index (BMI).*

Pap smear—A Pap smear (also known as a Papanicolaou smear or Pap test) is a microscopic examination of cells scraped from the cervix that is used to detect cancerous or precancerous conditions of the cervix or other medical

Samsung Bioepis Exhibit No. 1002
Page 4947

conditions. In the National Health Interview Survey questions concerning use of Pap smear were asked on an intermittent schedule, and the question content differed slightly across years. In 1987, women were asked to report when they had their most recent Pap smear in days, weeks, months, or years. Women who did not respond were asked a followup question, "Was it 3 years ago or less, between 3 and 5 years, or 5 years or more ago?" Pap smear data in the past 3 years were not available in 1990 and 1991. In 1993 and 1994, women were asked whether they had a Pap smear within the past year, between 1 and 3 years ago, or more than 3 years ago. In 1998 women were asked whether they had a Pap smear 1 year ago or less, more than 1 year but not more than 2 years, more than 2 years but not more than 3 years, more than 3 years but not more than 5 years, or more than 5 years ago. In 1999, women were asked when they had their most recent Pap smear in days, weeks, months, or years. In 1999, 4 percent of women in the sample responded "3 years ago." In this analysis, these women were coded as "within the past 3 years," although a response of "3 years ago" may include women whose last Pap smear was more than 3 but less than 4 years ago. Thus, estimates for 1999 are overestimated to some degree in comparison with estimates for previous years. In 2000 and 2003, women were asked when they had their most recent Pap smear (give month and year). Women who did not respond were given a followup question that used the 1999 wording and women who did not answer the followup question were asked a second followup question that used the 1998 wording. In 2000 and 2003 less than 1 percent of women in the sample answered "3 years ago" using the 1999 wording, and they were coded as "within the past 3 years." Thus, estimates for 2000 and 2003 may be slightly overestimated in comparison with estimates for years prior to 1999.

Partial care organization—See *Mental health organization.*

Partial care treatment—See *Mental health service type.*

Patient—See *Home health care; Hospice care; Inpatient; Office visit; Outpatient visit.*

Percent change—See *Average annual rate of change.*

Perinatal mortality rate; ratio—See *Rate: Death and related rates.*

Personal care homes with or without nursing—See *Nursing home.*

Personal health care expenditures—See *Health expenditures, national.*

Physical activity, leisure-time—Beginning in 1998 leisure-time physical activity is assessed in the National Health Interview Survey by asking adults a series of questions about how often they do vigorous or light/moderate physical activity of at least 10 minutes duration and for about how long these sessions generally last. Vigorous physical activity is described as causing heavy sweating or a large increase in breathing or heart rate and light/ moderate as causing light sweating or a slight to moderate increase in breathing or heart rate. Adults classified as inactive did not report any sessions of moderate or vigorous leisure-time physical activity of at least 10 minutes duration or reported they were unable to perform leisure-time physical activity. Adults classified with some leisure-time activity reported at least one session of light/moderate or vigorous activity but did not meet the requirement for regular leisure-time activity. Adults classified with regular leisure-time activity reported at least three sessions per week of vigorous leisure-time physical activity lasting at least 20 minutes in duration or at least five sessions per week of light or moderate physical activity lasting at least 30 minutes in duration.

Physician—Data on physician characteristics are obtained through physician self-report for the American Medical Association's (AMA) Physician Masterfile. The AMA tabulates data only for doctors of medicine (MDs), but some tables in *Health, United States* include data for both MDs and doctors of osteopathy (DOs).

Active (or professionally active) physicians—These physicians are currently engaged in patient care or other professional activity for a minimum of 20 hours per week. Other professional activity includes administration, medical teaching, research, and other activities, such as employment with insurance carriers, pharmaceutical companies, corporations, voluntary organizations, medical societies, and the like. Physicians who are retired, semiretired, working part-time, or not practicing are classified as inactive and are excluded. Also excluded are physicians with address unknown and physicians who did not provide information on type of practice or present employment (not classified).

Samsung Bioepis Exhibit No. 1002
Page 4948

Hospital-based physicians—These physicians are employed under contract with hospitals to provide direct patient care and include physicians in residency training (including clinical fellows) and full-time members of the hospital staff.

Office-based physicians—These physicians are engaged in seeing patients in solo practice, group practice, two-physician practice, other patient care employment, or inpatient services such as those provided by pathologists and radiologists.

Data for physicians are presented by type of education (doctors of medicine and doctors of osteopathy); place of education (U.S. medical graduates and international medical graduates); activity status (professionally active and inactive); area of specialty; and geographic area. See related *Office; Physician specialty.*

Physician specialty—A physician specialty is any specific branch of medicine in which a physician may concentrate. Data are based on physician self-reports of their primary area of specialty. Physician data are broadly categorized into two areas of practice: those who provide primary care, and those who provide specialty care.

Primary care generalists—These physicians are physicians who practice in the general fields of family medicine, general practice, internal medicine, obstetrics and gynecology, and pediatrics. They specifically exclude primary care specialists associated with these generalist fields.

Primary care specialists—These specialists practice in the primary care subspecialties of family medicine, internal medicine, obstetrics and gynecology, and pediatrics. Family medicine subspecialties include geriatric medicine and sports medicine. Internal medicine subspecialties include adolescent medicine, critical care medicine, diabetes, endocrinology, diabetes and metabolism, hematology, hepatology, hematology/oncology, cardiac electrophysiology, infectious diseases, clinical and laboratory immunology, geriatric medicine, sports medicine, nephrology, nutrition, medical oncology, pulmonary critical care medicine, and rheumatology. Obstetrics and gynecology subspecialties include gynecological oncology, gynecology, maternal and fetal medicine, obstetrics, critical care medicine, and reproductive endocrinology. Pediatric subspecialties include adolescent medicine, pediatric critical care medicine, pediatrics/internal medicine, neonatal-perinatal medicine, pediatric allergy, pediatric cardiology, pediatric endocrinology, pediatric infectious disease, pediatric pulmonology, medical toxicology (pediatrics), pediatric emergency medicine, pediatric gastroenterology, pediatric hematology/oncology, clinical and laboratory immunology (pediatrics), pediatric nephrology, pediatric rheumatology, and sports medicine (pediatrics).

Specialty care physicians—These physicians are sometimes called specialists, and include primary care subspecialists listed above in addition to all other physicians not included in the generalist definition. Specialty fields include allergy and immunology, aerospace medicine, anesthesiology, cardiovascular diseases, child and adolescent psychiatry, colon and rectal surgery, dermatology, diagnostic radiology, forensic pathology, gastroenterology, general surgery, medical genetics, neurology, nuclear medicine, neurological surgery, occupational medicine, ophthalmology, orthopedic surgery, otolaryngology, psychiatry, public health and general preventive medicine, physical medicine and rehabilitation, plastic surgery, anatomic and clinical pathology, pulmonary diseases, radiation oncology, thoracic surgery, urology, addiction medicine, critical care medicine, legal medicine, and clinical pharmacology.

See related *Physician.*

Point-of-service (POS) plan—This is a health plan that allows members to choose to receive services from a participating or non-participating network provider, usually with a financial disincentive for going outside the network. More of a product than an organization, POS plans can be offered by HMOs, PPOs, or self-insured employers. See related *Health maintenance organization (HMO); Managed care; Preferred provider organization (PPO).*

Population—The U.S. Census Bureau collects and publishes data on populations in the United States according to several different definitions. Various statistical systems then use the appropriate population for calculating rates. See also *Appendix I, Population Census and Population Estimates.*

Samsung Bioepis Exhibit No. 1002
Page 4949

Table XII. Current cigarette smoking by persons 18 years of age and over, according to race and Hispanic origin under the 1977 and 1997 Standards for Federal data on race and ethnicity: United States, average annual 1993–95

1997 Standards	Sample size	Percent	Standard error	1977 Standards	Sample size	Percent	Standard error
			Race				
White only	46,228	25.2	0.26	White	46,664	25.3	0.26
Black or African American only	7,208	26.6	0.64	Black	7,334	26.5	0.63
American Indian or Alaska Native only	416	32.9	2.53	American Indian or Alaska Native	460	33.9	2.38
Asian only	1,370	15.0	1.19	Asian or Pacific Islander	1,411	15.5	1.22
2 or more races total	786	34.5	2.00				
Black or African American; White	83	*21.7	6.05				
American Indian or Alaska Native; White	461	40.0	2.58				
			Race, any mention				
White, any mention	46,882	25.3	0.26				
Black or African American, any mention	7,382	26.6	0.63				
American Indian or Alaska Native, any mention	965	36.3	1.71				
Asian, any mention	1,458	15.7	1.20				
Native Hawaiian and Other Pacific Islander, any mention	53	*17.5	5.10				
			Hispanic origin and race				
Not Hispanic or Latino:				Non-Hispanic:			
White only	42,421	25.8	0.27	White	42,976	25.9	0.27
Black or African American only	7,053	26.7	0.65	Black	7,203	26.7	0.64
American Indian or Alaska Native only	358	33.5	2.69	American Indian or Alaska Native	407	35.4	2.53
Asian only	1,320	14.8	1.21	Asian or Pacific Islander	1,397	15.3	1.24
2 or more races total	687	35.6	2.15				
Hispanic or Latino	5,175	17.8	0.65	Hispanic	5,175	17.8	0.65

*Relative standard error 20–30 percent.

NOTES: The 1997 Standards for Federal data on race and ethnicity set five single-race groups (White, Black, American Indian or Alaska Native, Asian, and Native Hawaiian or Other Pacific Islander) and allow respondents to report one or more race groups. Estimates for single-race and multiple-race groups not shown above do not meet standards for statistical reliability or confidentiality (relative standard error greater than 30 percent). Race groups under the 1997 Standards were based on the question, "What is the group or groups which represents _____ race?" For persons who selected multiple groups, race groups under the 1977 Standards were based on the additional question, "Which of those groups would you say best represents _____ race?" Race-specific estimates in this table were calculated after excluding respondents of other and unknown race. Other published race-specific estimates are based on files in which such responses have been edited. Percents are age adjusted to the year 2000 standard using three age groups: Under 18 years, 18–44 years, and 45–64 years of age. See Appendix II, Age adjustment.

SOURCE: Centers for Disease Control and Prevention, National Center for Health Statistics. National Health Interview Survey.

Total population—This is the population of the United States, including all members of the Armed Forces living in foreign countries, Puerto Rico, Guam, and the U.S. Virgin Islands. Other Americans abroad (e.g., civilian Federal employees and dependents of members of the Armed Forces or other Federal employees) are not included.

Resident population—This population includes persons whose usual place of residence (i.e., the place where one usually lives and sleeps) is in one of the 50 States or the District of Columbia. It includes members of the Armed Forces stationed in the United States and their families. It excludes international military, naval, and diplomatic personnel and their families located in this country and residing in embassies or similar quarters. Also excluded are international workers and international students in this country and Americans living abroad. The resident population is the denominator for calculating birth and death rates and incidence of disease.

Civilian population—The civilian populatin is the resident population excluding members of the Armed Forces. However, families of members of the Armed Forces are included. This population is the denominator in rates calculated for the National Hospital Discharge Survey, the National Home and Hospice Care Survey, and the National Nursing Home Survey.

Civilian noninstitutionalized population—This is the civilian population not residing in institutions such as correctional institutions, detention homes, and training

Samsung Bioepis Exhibit No. 1002
Page 4950

Table XIII. Private health care coverage for persons under 65 years of age, according to race and Hispanic origin under the 1977 and 1997 Standards for Federal data on race and ethnicity: United States, average annual 1993–95

1997 Standards	Sample size	Percent	Standard error	1977 Standards	Sample size	Percent	Standard error
			Race				
White only	168,256	76.1	0.28	White	170,472	75.9	0.28
Black or African American only	30,048	53.5	0.63	Black	30,690	53.6	0.63
American Indian or Alaska Native only	2,003	44.2	1.97	American Indian or Alaska Native	2,316	43.5	1.85
Asian only	6,896	68.0	1.39	Asian or Pacific Islander	7,146	68.2	1.34
Native Hawaiian and Other Pacific Islander only	173	75.0	7.43				
2 or more races total	4,203	60.9	1.17				
Black or African American; White	686	59.5	3.21				
American Indian or Alaska Native; White	2,022	60.0	1.71				
Asian; White	590	71.9	3.39				
Native Hawaiian and Other Pacific Islander; White	56	59.2	10.65				
			Race, any mention				
White, any mention	171,817	75.8	0.28				
Black or African American, any mention	31,147	53.6	0.62				
American Indian or Alaska Native, any mention	4,365	52.4	1.40				
Asian, any mention	7,639	68.4	1.27				
Native Hawaiian and Other Pacific Islander, any mention	283	68.7	6.23				
			Hispanic origin and race				
Not Hispanic or Latino:				Non-Hispanic:			
White only	146,109	78.9	0.27	White	149,057	78.6	0.27
Black or African American only	29,250	53.9	0.64	Black	29,877	54.0	0.63
American Indian or Alaska Native only	1,620	45.2	2.15	American Indian or Alaska Native	1,859	44.6	2.05
Asian only	6,623	68.2	1.43	Asian or Pacific Islander	6,999	68.4	1.40
Native Hawaiian and Other Pacific Islander only	145	76.4	7.79				
2 or more races total	3,365	62.6	1.18				
Hispanic or Latino	31,040	48.8	0.74	Hispanic	31,040	48.8	0.74

NOTES: The 1997 Standards for Federal data on race and ethnicity set five single-race groups (White, Black, American Indian or Alaska Native, Asian, and Native Hawaiian or Other Pacific Islander) and allow respondents to report one or more race groups. Estimates for single-race and multiple-race groups not shown above do not meet standards for statistical reliability or confidentiality (relative standard error greater than 30 percent). Race groups under the 1997 Standards were based on the question, "What is the group or groups which represents _____ race?" For persons who selected multiple groups, race groups under the 1977 Standards were based on the additional question, "Which of those groups would you say best represents _____ race?" Race-specific estimates in this table were calculated after excluding respondents of other and unknown race. Other published race-specific estimates are based on files in which such responses have been edited. Percents are age adjusted to the year 2000 standard using three age groups: Under 18 years, 18–44 years, and 45–64 years of age. See Appendix II, Age adjustment.

SOURCE: Centers for Disease Control and Prevention, National Center for Health Statistics, National Health Interview Survey.

schools for juvenile delinquents; homes for aged and dependent persons (e.g., nursing homes and convalescent homes); homes for dependent and neglected children; homes and schools for mentally or physically handicapped persons; homes for unwed mothers; psychiatric, tuberculosis, and chronic disease hospitals; and residential treatment centers. Census Bureau estimates of the civilian noninstitutionalized population are used to calculate sample weights for the National Health Interview Survey, National Health and Nutrition Examination Survey, and National Survey of Family Growth, and as denominators in rates calculated for the National Ambulatory Medical Care Survey and the National Hospital Ambulatory Medical Care Survey.

Introduction of census 2000 population estimates—Health United States, 2003 marked the transition to the use of year 2000 resident population estimates based on the 2000 census for calculation of rates. Previously, 1991–2000 rates were based on post-1990 population estimates. Birth rates and death rates for 1991–99 were revised using intercensal population estimates based on the 2000 census. Rates for 2000 were revised using Census 2000 counts. Data systems and surveys that use civilian and civilian noninstitutionalized population

Samsung Bioepis Exhibit No. 1002
Page 4951

estimates as denominators for computation of rates for the period 1991–99 have not been updated with intercensal estimates based on the 2000 civilian and civilian noninstitutionalized populations. See *Appendix I, Population Census and Population Estimates.*

Postneonatal mortality rate—See *Rate: Death and related rates.*

Poverty level—Poverty statistics are based on definitions originally developed by the Social Security Administration. These include a set of money income thresholds that vary by family size and composition. Families or individuals with income below their appropriate thresholds are classified as below the poverty level. These thresholds are updated annually by the U.S. Census Bureau to reflect changes in the Consumer Price Index for all urban consumers (CPI-U). For example, the average poverty threshold for a family of four was $17,603 in 2000 and $13,359 in 1990. For more information, see *Consumer Income and Poverty 2003.* Series P-60. Washington, DC. U.S. Government Printing Office. 2003. Also see www.census.gov/hhes/www/poverty.html.

National Health Interview Survey (NHIS) and *National Health and Nutrition Examination Survey (NHANES)*—Poverty level, for years prior to 1997, was based on family income and family size using Census Bureau poverty thresholds. In the NHIS, beginning in 1997 poverty status is based on family income, family size, number of children in the family, and for families with two or fewer adults, the age of the adults in the family. Poverty status in the NHANES is also based on family income and family size and composition. See related *Consumer Price Index (CPI); Family income; Appendix I, Current Population Survey; National Health Interview Survey; National Health and Nutrition Examination Survey.*

Preferred provider organization (PPO)—A PPO is a type of medical plan where coverage is provided to participants through a network of selected health care providers (such as hospitals and physicians). The enrollees may go outside the network, but they would pay a greater percentage of the cost of coverage than within the network. See related *Health maintenance organization (HMO); Managed care; Point-of-service (POS) plan.*

Prenatal care—Prenatal care is medical care provided to a pregnant woman to prevent complications and decrease the incidence of maternal and prenatal mortality. Information on when pregnancy care began is recorded on the birth certificate. Between 1970 and 1980, the reporting area for prenatal care expanded. In 1970, 39 States and the District of Columbia reported prenatal care on the birth certificate. Data were not available from Alabama, Alaska, Arkansas, Connecticut, Delaware, Georgia, Idaho, Massachusetts, New Mexico, Pennsylvania, and Virginia. In 1975 these data were available from three additional States—Connecticut, Delaware, and Georgia increasing the number of States reporting prenatal care to 42 and the District of Columbia. During 1980–2002 prenatal care information was available for the entire United States. In 2003, data on prenatal care was included for all States except Pennsylvania, and Washington both of which implemented the 2003 revision of the U.S. Standard Certificate of Live Birth. The prenatal care item on the 2003 certificate, "Date of first prenatal visit," is not comparable with the prenatal care item on the 1989 revision, "Month prenatal care began." In addition the 2003 revision recommends that information on prenatal care be gathered from prenatal care or medical records whereas the 1989 revision did not recommend a source for these data.

Prevalence—Prevalence is the number of cases of a disease, infected persons, or persons with some other attribute present during a particular interval of time. It is often expressed as a rate (e.g., the prevalence of diabetes per 1,000 persons during a year). See related *Incidence.*

Primary admission diagnosis—In the National Home and Hospice Care Survey the primary admission diagnosis is the first-listed diagnosis at admission on the patient's medical record as provided by the agency staff member most familiar with the care provided to the patient.

Primary care specialties—See *Physician specialty.*

Private expenditures—See *Health expenditures, national.*

Procedure—The National Hospital Discharge Survey (NHDS) used to classify a procedure as a surgical or nonsurgical operation, diagnostic procedure, or therapeutic procedure (such as respiratory therapy); however the distinction between types of procedures has become less meaningful because of the development of minimally invasive and noninvasive surgery. Thus the practice of classifying the type of procedure

Samsung Bioepis Exhibit No. 1002
Page 4952

has been discontinued. Procedures are coded according to the *International Classification of Diseases, Ninth Revision, Clinical Modification* (see table X). Up to four different procedures are coded in the NHDS. Procedures per hospital stay can be classified as any-listed—that is, if more than one procedure with the same code is performed it is counted only once— or all-listed where multiple occurrences of the same procedure would be counted the number of times it appears on the medical record up to the maximum of four available codes. For example, a triple coronary artery bypass graft would be counted once as "any-listed" but three times as "all-listed". All-listed procedures double-count the number of procedures of a given type that are performed, thus all listed procedure counts are greater than the number of hospital stays that occurred. Any-listed procedure counts approximate the number of hospital stays where a procedure was performed at any time during the stay. See related *Outpatient surgery*.

Proprietary hospitals—See *Hospital*.

Psychiatric hospitals—See *Hospital; Mental health organization*.

Public expenditures—See *Health expenditures, national*.

Public health activities—Public health activities may include any of the following essential services of public health—surveillance, investigations, education, community mobilization, workforce training, research, and personal care services delivered or funded by governmental agencies.

Race—In 1977, the Office of Management and Budget (OMB) issued Race and Ethnicity Standards for Federal Statistics and Administrative Reporting to promote comparability of data among Federal data systems. The 1977 Standards called for the Federal Government's data systems to classify individuals into the following four racial groups: American Indian or Alaska Native, Asian or Pacific Islander, black, and white. Depending on the data source, the classification by race was based on self-classification or on observation by an interviewer or other person filling out the questionnaire.

In 1997, new standards were announced for classification of individuals by race within the Federal Government's data systems (*Federal Register*, 62FR58781–58790). The 1997 Standards have five racial groups: American Indian or Alaska Native, Asian, Black or African American, Native Hawaiian or Other Pacific Islander, and White. These five categories are the minimum set for data on race in Federal statistics. The 1997 Standards also offer an opportunity for respondents to select more than one of the five groups, leading to many possible multiple-race categories. As with the single-race groups, data for the multiple-race groups are to be reported when estimates meet agency requirements for reliability and confidentiality. The 1997 Standards allow for observer or proxy identification of race but clearly state a preference for self-classification. The Federal Government considers race and Hispanic origin to be two separate and distinct concepts. Thus Hispanics may be of any race. Federal data systems were required to comply with the 1997 Standards by 2003.

National Health Interview Survey (NHIS)—Starting with *Health, United States, 2002*, race-specific estimates based on the NHIS were tabulated using the 1997 Standards for data year 1999 and beyond and are not strictly comparable with estimates for earlier years. The 1997 Standards specify five single-race categories plus multiple-race categories. Estimates for specific race groups are shown when they meet requirements for statistical reliability and confidentiality. The race categories "White only," "Black or African American only," "American Indian and Alaska Native only," "Asian only," and "Native Hawaiian and Other Pacific Islander only" include persons who reported only one racial group; the category "2 or more races" includes persons who reported more than one of the five racial groups in the 1997 Standards or one of the five racial groups and "Some other race." Prior to data year 1999, data were tabulated according to the 1977 Standards with four racial groups, and the category "Asian only" included Native Hawaiian and Other Pacific Islander. Estimates for single-race categories prior to 1999 included persons who reported one race or, if they reported more than one race, identified one race as best representing their race. Differences between estimates tabulated using the two Standards for data year 1999 are discussed in the footnotes for each NHIS table in the *Health, United States*, 2002, 2003, and 2004 editions.

Tables XII and XIII illustrate NHIS data tabulated by race and Hispanic origin according to the 1997 and 1977 Standards for two health statistics (cigarette smoking and private health insurance coverage). In these illustrations, three separate tabulations using the 1997 Standards are shown: 1) Race: mutually exclusive race groups, including several multiple-race combinations; 2) Race,

Samsung Bioepis Exhibit No. 1002
Page 4953

any mention: race groups that are not mutually exclusive because each race category includes all persons who mention that race; and 3) Hispanic origin and race: detailed race and Hispanic origin with a multiple-race total category. Where applicable, comparison tabulations by race and Hispanic origin are shown based on the 1977 Standards. Because there are more race groups with the 1997 Standards, the sample size of each race group under the 1997 Standards is slightly smaller than the sample size under the 1977 Standards. Only those few multiple-race groups with sufficient numbers of observations to meet standards of statistical reliability are shown. Tables XII and XIII also illustrate changes in labels and group categories in the 1997 Standards. The race designation of Black was changed to Black or African American, and the ethnicity designation of Hispanic was changed to Hispanic or Latino.

Data systems included in *Health, United States,* other than the NHIS, the National Survey of Drug Use & Health (NSDUH), and the National Health and Nutrition Examination Survey (NHANES), generally do not permit tabulation of estimates for the detailed race and ethnicity categories shown in tables XII and XIII, either because race data based on the 1997 standard categories are not yet available or because there are insufficient numbers of observations to meet statistical reliability or confidentiality requirements.

National Health and Nutrition Examination Survey (NHANES)—Starting with *Health, United States, 2003* race-specific estimates based on NHANES were tabulated using the 1997 Standards for data years 1999 and beyond. Prior to data year 1999, the 1977 Standards were used. Because of the differences between the two Standards, the race-specific estimates shown in trend tables based on the NHANES for 1999–2002 are not strictly comparable with estimates for earlier years. Each trend table based on the NHANES includes a footnote that discusses differences between estimates tabulated using the two Standards for survey years 1999–2002. Race in NHANES I and II was determined primarily by interviewer observation; starting with NHANES III, race was self-reported by survey participants.

The NHANES sample was designed to provide estimates specifically for persons of Mexican origin and not for all Hispanic-origin persons in the United States. Persons of Hispanic origin other than Mexicans were entered into the sample with different selection probabilities that are not nationally representative of the total U.S. Hispanic population. Estimates are shown for non-Hispanic white, non-Hispanic black, and Mexican. Although data were collected according to the 1997 Standards, there are insufficient numbers of observations to meet statistical reliability or confidentiality requirements for reporting estimates for additional race categories.

National Survey on Drug Use & Health (NSDUH)—Race-specific estimates based on NSDUH are tabulated using the 1997 Standards. Estimates in the NSDUH trend table begin with the data year 1999. Estimates for specific race groups are shown when they meet requirements for statistical reliability and confidentiality. The race categories "White only," "Black or African American only," "American Indian and Alaska Native only," "Asian only," and "Native Hawaiian and Other Pacific Islander only" include persons who reported only one racial group; and the category "2 or more races" includes persons who reported more than one of the five racial groups in the 1997 Standards or one of the five racial groups and "Some other race."

National Vital Statistics System—Most of the States in the Vital Statistics Cooperative Program are still revising their birth and death records to conform to the 1997 standards on race and ethnicity. During the transition to full implementation of the 1997 standards, vital statistics data will continue to be presented for the four major race groups—white, black or African American, American Indian or Alaska Native, and Asian or Pacific Islander—in accordance with 1977 standards.

Birth File—Information about the race and Hispanic ethnicity of the mother and father are provided by the mother at the time of birth and recorded on the birth certificate and fetal death record. Since 1980, birth rates, birth characteristics, and fetal death rates for live-born infants and fetal deaths are presented in this report according to race of mother. Before 1980 data were tabulated by race of newborn and fetus, taking into account the race of both parents. If the parents were of different races and one parent was white, the child was classified according to the race of the other parent. When neither parent was white, the child was classified according to father's race, with one exception: if either parent was Hawaiian, the child was classified Hawaiian.

Samsung Bioepis Exhibit No. 1002
Page 4954

Before 1964, if race was unknown, the birth was classified as white. Beginning in 1964 unknown race was classified according to information on the birth record. Beginning with the 2000 data year, the race and ethnicity data used for denominators (population) to calculate birth rates are collected in accordance with 1997 revised OMB standards for race and ethnicity. However, the numerators (births) will not be compatible with the denominators until all the States revise their birth certificates to reflect the new standards. In order to compute rates, it is necessary to bridge population data for multiple-race persons to single-race categories. See *Appendix I, Population Census and Population Estimates, Bridged-Race Population Estimates for Census 2000.*

Beginning with the 2003 data year, multiple-race data were reported by both Pennsylvania and Washington, which used the 2003 revision of the U.S. Standard Certificate of Live Birth, as well as California, Hawaii, Ohio (for births occurring in December only), and Utah, which used the 1989 revision of the U.S. Standard Certificate of Live Birth. These six States, which accounted for 20.7 percent of births in the United States in 2003, reported 2.6 percent of mothers as multiracial, with levels varying from 0.7 percent (Ohio) to 37.5 percent (Hawaii). Data from the vital records of the remaining 44 States and the District of Columbia followed the 1977 OMB standards in which a single race is reported. In order to provide uniformity and comparability of the data during the transition period, before multiple-race data are available for all reporting areas, it is necessary to "bridge" the responses of those who reported more than one race to a single race. See Martin JA, Hamilton BE, et al. Births: Final Data for 2003. National vital statistics reports; forthcoming. Hyattsville, MD: National Center for Health Statistics. 2005.

Although the bridging procedure imputes multiple-race of mothers to one of the four minimum races stipulated in the 1977 race and ethnicity standards, mothers of a specified Asian or Pacific Islander (API) subgroup (Chinese, Japanese, Hawaiian, or Filipino) in combination with another race (American Indian or Alaska Native, Black, or White) or another API subgroup cannot be imputed to a single Asian or Pacific Islander subgroup. In 2003, API mothers were disproportionately represented in the six States reporting multiple-race (44 percent). Data are not shown for the API subgroups or reported alone or in combination with other races or other API subgroups because the bridging technique cannot be applied in this detail. These data are available in the 2003 Natality public-use data file, which can be found at http://www.cdc.gov/nchs/births.htm.

Mortality File—Information about the race and Hispanic ethnicity of the decedent is reported by the funeral director as provided by an informant, often the surviving next of kin, or, in the absence of an informant, on the basis of observation. Death rates by race and Hispanic origin are based on information from death certificates (numerators of the rates) and on population estimates from the Census Bureau (denominators). Race and ethnicity information from the census is by self-report. To the extent that race and Hispanic origin are inconsistent between these two data sources, death rates will be biased. Studies have shown that persons self-reported as American Indian, Asian, or Hispanic on census and survey records may sometimes be reported as white or non-Hispanic on the death certificate, resulting in an underestimation of deaths and death rates for the American Indian, Asian, and Hispanic groups. Bias also results from undercounts of some population groups in the census, particularly young black males, young white males, and elderly persons, resulting in an overestimation of death rates. The net effects of misclassification and undercoverage result in overstated death rates for the white population and black population are estimated to be 1 percent and 5 percent, respectively; understated death rates for other population groups are estimated as follows: American Indians, 21 percent; Asian or Pacific Islanders, 11 percent; and Hispanics, 2 percent. For more information, see Rosenberg HM, Maurer JD, Sorlie PD, et al. Quality of death rates by race and Hispanic origin: A summary of current research, 1999. National Center for Health Statistics. *Vital Health Stat* 2(128). 1999.

Denominators for infant and maternal mortality rates are based on number of live births rather than population estimates. Race information for the denominator is supplied from the birth certificate. Before 1980, race of child for the denominator took into account the races of both parents. Starting in 1980 race information for the denominator was based solely on race of mother. Race information for the numerator is supplied from the death

Samsung Bioepis Exhibit No. 1002
Page 4955

certificate. For the infant mortality rate, race information for the numerator is race of the deceased child; for the maternal mortality rate, it is race of the mother.

Vital event rates for the American Indian or Alaska Native population shown in this book are based on the total U.S. resident population of American Indians and Alaska Natives, as enumerated by the U.S. Census Bureau. In contrast the Indian Health Service calculates vital event rates for this population based on U.S. Census Bureau county data for American Indians and Alaska Natives who reside on or near reservations. Interpretation of trends for the American Indian and Alaska Native population should take into account that population estimates for these groups increased by 45 percent between 1980 and 1990, partly because of better enumeration techniques in the 1990 decennial census and the increased tendency for people to identify themselves as American Indian in 1990.

Interpretation of trends for the Asian population in the United States should take into account that this population more than doubled between 1980 and 1990, primarily because of immigration. Between 1990 and 2000, the increase in the Asian population was 48 percent for persons reporting that they were Asian alone, and 72 percent for persons who reported they were either Asian alone or in combination with another race.

For more information on coding race using vital statistics, see National Center for Health Statistics, Technical Appendix. *Vital Statistics of the United States*, Vol. I, Natality, and Vol. II, Mortality, Part A available on the NCHS home page at www.cdc.gov/nchs/nvss.htm. See related *Hispanic origin; Appendix I, Population Census and Population Estimates.*

Rate—A rate is a measure of some event, disease, or condition in relation to a unit of population, along with some specification of time. See related *Age adjustment; Population.*

▨ *Birth and related rates*

Birth rate is calculated by dividing the number of live births in a population in a year by the midyear resident population. For census years, rates are based on unrounded census counts of the resident population, as of April 1. For the noncensus years 1981–89, rates were based on national estimates of the resident population, as of July 1, rounded to 1,000s. Rounded population estimates for 5-year age groups were calculated by summing unrounded population estimates before rounding to 1,000s. Starting in 1991 rates were based on unrounded national population estimates. Beginning in 1997, the birth rate for the maternal age group 45–49 years includes data for mothers age 50–54 years in the numerator and is based on the population of women age 45–49 years in the denominator. Birth rates are expressed as the number of live births per 1,000 population. The rate may be restricted to births to women of specific age, race, marital status, or geographic location (specific rate), or it may be related to the entire population (crude rate). See related *Cohort fertility.*

Fertility rate is the total number of live births, regardless of age of mother, per 1,000 women of reproductive age, 15–44 years.

▨ *Death and related rates*

Death rate is calculated by dividing the number of deaths in a population in a year by the midyear resident population. For census years, rates are based on unrounded census counts of the resident population, as of April 1. For the noncensus years 1981–89, rates were based on national estimates of the resident population, as of July 1, rounded to 1,000s. Rounded population estimates for 10-year age groups were calculated by summing unrounded population estimates before rounding to 1,000s. Starting in 1991, rates were based on unrounded national population estimates. Rates for the Hispanic and non-Hispanic white populations in each year are based on unrounded State population estimates for States in the Hispanic reporting area. Death rates are expressed as the number of deaths per 100,000 population. The rate may be restricted to deaths in specific age, race, sex, or geographic groups or from specific causes of death (specific rate), or it may be related to the entire population (crude rate).

Fetal death rate is the number of fetal deaths with stated or presumed gestation of 20 weeks or more divided by the sum of live births plus fetal deaths, per 1,000 live births plus fetal deaths. *Late fetal death rate* is the number of fetal deaths with stated or presumed gestation of 28 weeks or more divided by the sum of live births plus late fetal deaths, per 1,000 live births plus late fetal deaths. See related *Gestation.*

Samsung Bioepis Exhibit No. 1002
Page 4956

Infant mortality rate based on period files is calculated by dividing the number of infant deaths during a calendar year by the number of live births reported in the same year. It is expressed as the number of infant deaths per 1,000 live births. *Neonatal mortality rate* is the number of deaths of children under 28 days of age, per 1,000 live births. *Postneonatal mortality rate* is the number of deaths of children that occur between 28 days and 365 days after birth, per 1,000 live births. See related *Infant death.*

Birth cohort infant mortality rates are based on linked birth and infant death files. In contrast to period rates in which the births and infant deaths occur in the same period or calendar year, infant deaths constituting the numerator of a birth cohort rate may have occurred in the same year as, or in the year following, the year of birth. The birth cohort infant mortality rate is expressed as the number of infant deaths per 1,000 live births. See related *Birth cohort.*

Perinatal relates to the period surrounding the birth event. Rates and ratios are based on events reported in a calendar year. *Perinatal mortality rate* is the sum of late fetal deaths plus infant deaths within 7 days of birth divided by the sum of live births plus late fetal deaths, per 1,000 live births plus late fetal deaths.

Perinatal mortality ratio is the sum of late fetal deaths plus infant deaths within 7 days of birth divided by the number of live births, per 1,000 live births.

Maternal mortality rate is defined as the number of maternal deaths per 100,000 live births. The maternal mortality rate is a measure of the likelihood that a pregnant woman will die from maternal causes. The number of live births used in the denominator is a proxy for the population of pregnant women who are at risk of a maternal death. See related *Maternal death.*

Visit rate is a basic measure of service utilization for event-based data. Examples of events include physician office visits with drugs provided or hospital discharges. In the visit rate calculation, the numerator is the number of estimated events, and the denominator is the corresponding U.S. population estimate for those who possibly could have had events during a given period of time. The interpretation is that for every person in the population there were, on average, *x* events. It does not mean that *x* percent of the population had events, because some persons in the population had no events while others had multiple events. The only exception is when an event can occur just once for a person (e.g., if an appendectomy were performed during a hospital stay). The visit rate is best used to compare utilization across various subgroups of interest such as age or race groups or geographic regions (e.g., the rate of hospital discharges in 2002 was 43.4 per 1,000 population for children under 18 years of age and 466.6 per 1, 000 population for adults 75 years and over).

Region—See *Geographic region and division.*

Registered hospitals—See *Hospital.*

Registered nursing education—Registered nursing data are shown by level of educational preparation. Baccalaureate education requires at least 4 years of college or university; associate degree programs are based in community colleges and are usually 2 years in length; and diploma programs are based in hospitals and are usually 3 years in length.

Registration area—The United States has separate registration areas for birth, death, marriage, and divorce statistics. In general, registration areas correspond to States and include two separate registration areas for the District of Columbia and New York City. All States have adopted laws that require registration of births and deaths and reporting of fetal deaths. It is believed that more than 99 percent of births and deaths occurring in this country are registered.

The *death registration area* was established in 1900 with 10 States and the District of Columbia, and the *birth registration area* was established in 1915, also with 10 States and the District of Columbia. Beginning with 1933, all States were included in the birth and death registration areas. The specific States added year by year are shown in "History and Organization of the Vital Statistics System." Reprinted from *Vital Statistics of the United States Vol. I, 1950,* chapter 1. National Center for Health Statistics. 1978. Currently, Puerto Rico, U.S. Virgin Islands, and Guam each constitutes a separate registration area, although their data are not included in statistical tabulations of U.S. resident data. See related *Reporting area.*

Relative standard error—The relative standard error (RSE) is a measure of an estimate's reliability. The RSE of an estimate is obtained by dividing the standard error of the estimate (SE(r)) by the estimate itself (r). This quantity is expressed as a percentage of the estimate and is calculated as follows: RSE = 100 x (SE(r)/r). Estimates with large RSEs

Samsung Bioepis Exhibit No. 1002
Page 4957

are considered unreliable. In *Health, United States* most statistics with large RSEs are preceded by an asterisk or are not presented.

Relative survival rate—The relative survival rate is the ratio of the observed survival rate for the patient group to the expected survival rate for persons in the general population similar to the patient group with respect to age, sex, race, and calendar year of observation. The 5-year relative survival rate is used to estimate the proportion of cancer patients potentially curable. Because over one-half of all cancers occur in persons 65 years of age and over, many of these individuals die of other causes with no evidence of recurrence of their cancer. Thus, because it is obtained by adjusting observed survival for the normal life expectancy of the general population of the same age, the relative survival rate is an estimate of the chance of surviving the effects of cancer.

Reporting area—In the National Vital Statistics System, the reporting area for such basic items on the birth and death certificates as age, race, and sex is based on data from residents of all 50 States in the United States and the District of Columbia (DC). The reporting area for selected items such as Hispanic origin, educational attainment, and marital status is based on data from those States that require the item to be reported, whose data meet a minimum level of completeness (such as 80 or 90 percent), and are considered to be sufficiently comparable to be used for analysis. In 1993–96 the reporting area for Hispanic origin of decedent on the death certificate included 49 States and DC. Starting in 1997 the Hispanic reporting area includes all 50 States and DC. See related *Registration area; Appendix I, National Vital Statistics System.*

Resident—In the Online Survey Certification and Reporting database, all residents in certified facilities are counted on the day of certification inspection. In the National Nursing Home Survey, a resident is a person on the roster of the nursing home as of the night before the survey. Included are all residents for whom beds are maintained even though they may be on overnight leave or in a hospital. See related *Nursing home.*

Resident population—See *Population.*

Residential treatment care—See *Mental health service type.*

Residential treatment centers for emotionally disturbed children—See *Mental health organization.*

Rural—See *Urbanization.*

Self-assessment of health—See *Health status, respondent-assessed.*

Serious psychological distress—The serious psychological distress scale (K6) is a six-item scale developed to measure serious mental illness. The K6 was asked of adults 18 years of age and older. The answers were self-reported and no proxies were allowed. The K6 is designed to identify persons with serious psychological distress using as few questions as possible. The six items included in the K6 are as follows: During the past 30 days, how often did you feel . . .

so sad that nothing could cheer you up?
nervous?
restless or fidgety?
hopeless?
that everything was an effort?
worthless?

Possible answers are all of the time (4 points), most of the time (3 points), some of the time (2 points), a little of the time (1 point), and none of the time (0 points).

To score the K6, the points are added together yielding a possible total of 0 to 24 points. A threshold of 13 or more is used to define serious mental illness. Persons answering "some of the time" to all six questions would not reach the threshold for serious mental illness, because to achieve a score of 13 they would need to answer "most of the time" to at least one item.

For more information, see Kessler RC, Barker PR, Colpe LJ, et al. Screening for serious mental illness in the general population. *Arch Gen Psychiatry* 60:184–189, 2003.

Short-stay hospital—See *Hospital.*

Skilled nursing facility—See *Nursing home.*

Smoker—See *Cigarette smoking.*

Specialty hospital—See *Hospital.*

State health agency—A State health agency is the agency or department within State government headed by the State or territorial health official. Generally, the State health agency

Samsung Bioepis Exhibit No. 1002
Page 4958

is responsible for setting statewide public health priorities, carrying out national and State mandates, responding to public health hazards, and assuring access to health care for underserved State residents.

State Children's Health Insurance Program (SCHIP)—Title XXI of the Social Security Act, known as the State Children's Health Insurance Program (SCHIP), is a program initiated by the Balanced Budget Act of 1997 (BBA). SCHIP provides more Federal funds for States to provide health care coverage to low-income, uninsured children. SCHIP gives States broad flexibility in program design while protecting beneficiaries through Federal standards. Funds from SCHIP may be used to expand Medicaid or to provide medical assistance to children during a presumptive eligibility period for Medicaid. This is one of several options from which States may select to provide health care coverage for more children, as prescribed within the BBA's Title XXI program. See related *Health insurance coverage; Medicaid.*

Substance use—This term refers to the use of selected substances including alcohol, tobacco products, drugs, inhalants, and other substances that can be consumed, inhaled, injected, or otherwise absorbed into the body with possible detrimental effects.

The Monitoring the Future Study (MTF)—The MTF collects information on use of selected substances using self-completed questionnaires to a school-based survey of secondary school students. MTF has tracked 12th-graders' illicit drug use and attitudes towards drugs since 1975. In 1991, 8th and 10th graders were added to the study. The survey includes questions on abuse of substances including (but not limited to) marijuana, inhalants, illegal drugs, alcohol, cigarettes, and other tobacco products. A standard set of three questions is used to assess use of the substances in the past month. "Past month" refers to an individual's use of a substance at least once during the month preceding their response to the survey. See related *Appendix I, Monitoring the Future Study.*

National Survey on Drug Use & Health (NSDUH)—The NSDUH conducts in-person interviews of a sample of individuals 12 years of age and older at their place of residence. For illicit drug use, alcohol use, and tobacco use, information is collected about use in past month. For information on illicit drug use, respondents in the NSDUH are asked about use of marijuana/hashish, cocaine (including crack), inhalants, hallucinogens, heroin, and prescription-type drugs used nonmedically (pain relievers, tranquilizers, stimulants, and sedatives). A series of questions is asked about each substance: "Have you ever, even once, used [e.g., Ecstasy, also know as MDMA/substance]?" "Think specifically about the past 30 days, from [date] up to and including today. During the past 30 days, on how many days did you use [substance]?" Numerous probes and checks are included in the computer-assisted interview system. Nonprescription medications and legitimate uses under a doctor's supervision are not included in the survey. Summary measures such as "any illicit drug use" are produced. See related *Alcohol consumption; Cigarette smoking; Illicit drug use; Appendix I, National Survey on Drug Use & Health.*

Suicidal ideation—Suicidal ideation is having thoughts of suicide or of taking action to end one's own life. Suicidal ideation includes all thoughts of suicide, both when the thoughts include a plan to commit suicide and when they do not include a plan. Suicidal ideation is measured in the Youth Risk Behavior Survey by the question "During the past 12 months, did you ever seriously consider attempting suicide?"

Surgical operation—See *Procedure.*

Surgical specialty—See *Physician specialty.*

Tobacco use—See *Cigarette smoking.*

Uninsured—In the Current Population Survey (CPS), persons are considered uninsured if they do not have coverage through private health insurance, Medicare, Medicaid, State Children's Health Insurance Program, military or Veterans coverage, another government program, a plan of someone outside the household, or other insurance. In addition, if the respondent has missing Medicaid information but has income from certain low-income public programs, then Medicaid coverage is imputed. The questions on health insurance are administered in March and refer to the previous calendar year.

In the National Health Interview Survey (NHIS), the uninsured are persons who do not have coverage under private health insurance, Medicare, Medicaid, public assistance, a State-sponsored health plan, other government-sponsored programs, or a military health plan. Persons with only Indian Health Service coverage are considered uninsured. Estimates of the percentage of persons who are uninsured based on the NHIS (table 134) may differ slightly from those based on

Samsung Bioepis Exhibit No. 1002
Page 4959

the March CPS (table 156) because of differences in survey questions, recall period, and other aspects of survey methodology. In 2003 in the NHIS, 1.1 percent of adults age 65 years and over had no health insurance, but the small sample size precludes the presentation of separate estimates for this population. Therefore the term "uninsured" refers only to the population under age 65. See related *Health insurance coverage; Appendix I, Current Population Survey.*

Urbanization—Urbanization is the degree of urban (city-like) character or nature of a particular geographic area. In this report death rates are presented according to the urbanization level of the decedent's county of residence. Counties and county equivalents were assigned to one of five urbanization levels using Office of Management and Budget's (OMB) standards for metropolitan statistical areas (MSAs) and micropolitan statistical areas and the Rural-Urban Continuum code system to differentiate among metropolitan areas based on population.

There are three major categories of counties. OMB classifies counties as metropolitan or micropolitan. Counties not categorized by OMB are neither metropolitan nor micropolitan.

OMB's classification of metropolitan counties are further differentiated in *Health, United States* by population size using the Rural-Urban Continuum code system (August 2003 Revision) developed by the Economic Research Service, U.S. Department of Agriculture. Metropolitan counties are classified by the population size of their metropolitan area to one of three metropolitan urbanization levels:

(a) *Large*—counties in MSAs with 1 million or more population.
(b) *Medium*— counties MSAs with 250,000 to 1 million population.
(c) *Small*—counties in MSAs with less than 250,000 population.

See *Metropolitan statistical area (MSA)* for definitions of metropolitan and nonmetropolitan counties.

Nonmetropolitan counties are categorized using the OMB's classification of nonmetropolitan micropolitan statistical areas (February 2004 Revision). Nonmetropolitan counties are classified into two categories:

(a) *Micropolitan*—counties defined by OMB as micropolitan based on population criteria.
(b) *Nonmicropolitan*—nonmetropolitan counties that do not meet the population criteria for micropolitan.

See *Micropolitan statistical area* for definitions of micropolitan and nonmicropolitan counties.

Usual source of care—Usual source of care was measured in the National Health Interview Survey (NHIS) in 1993 and 1994 by asking the respondent "Is there a particular person or place that ____ usually goes to when ____ is sick or needs advice about ____ health?" In the 1995 and 1996 NHIS, the respondent was asked "Is there one doctor, person, or place that ____ usually goes to when ____ is sick or needs advice about health?" Starting in 1997 the respondent was asked "Is there a place that ____ usually goes when he/she is sick or you need advice about (his/her) health?" Persons who report the emergency department as their usual source of care are defined as having no usual source of care in this report.

Wages and salaries—See *Employer costs for employee compensation.*

Years of potential life lost (YPLL)—YPLL is a measure of premature mortality. Starting with *Health, United States, 1996–97*, YPLL is presented for persons under 75 years of age because the average life expectancy in the United States is over 75 years. YPLL-75 is calculated using the following eight age groups: under 1 year, 1–14 years, 15–24 years, 25–34 years, 35–44 years, 45–54 years, 55–64 years, and 65–74 years. The number of deaths for each age group is multiplied by years of life lost, calculated as the difference between age 75 years and the midpoint of the age group. For the eight age groups, the midpoints are 0.5, 7.5, 19.5, 29.5, 39.5, 49.5, 59.5, and 69.5. For example, the death of a person 15–24 years of age counts as 55.5 years of life lost. Years of potential life lost is derived by summing years of life lost over all age groups. In *Health, United States, 1995* and earlier editions, YPLL was presented for persons under 65 years of age. For more information, see Centers for Disease Control. *MMWR* 35(2S):suppl. 1986. Available at: www.cdc.gov/mmwr/preview/mmwrhtml/00001773.htm.

Samsung Bioepis Exhibit No. 1002
Page 4960

Appendix III

Click here for spreadsheet version

Additional Data Years Available

For trend tables spanning long periods, only selected data years are shown to highlight major trends. Additional years of data are available for some of the tables in electronic spreadsheets available through the Internet and on CD-ROM.

To access spreadsheet files on the Internet, go to the *Health, United States* Web site at www.cdc.gov/nchs/hus.htm, scroll down to "Spreadsheet Files," and click on 2005 Edition.

Downloadable spreadsheet files for trend tables, many of which include more data years than are shown in the printed report, are available in Excel. Standard errors are included in spreadsheet files for trend tables based on the National Health Interview Survey (NHIS), National Health and Nutrition Examination Survey (NHANES), and National Survey of Family Growth (NSFG).

Spreadsheet files in Excel are also available on a CD-ROM. A limited supply of CD-ROMs are available from the National Center for Health Statistics upon request, while supplies last, or CD-ROMs may be purchased from the Government Printing Office.

Table number	Table topic	Additional data years available
2	Poverty	1986–89, 1991–94, 1996–99
3	Fertility rates and birth rates	1981–84, 1986–89, 1991–94, 1996
4	Live births	1972–74, 1976–79, 1981–84, 1986–89, 1991–94, 1996–99, 2001
5	Twin births	1972–74, 1976–79, 1981–84, 1986–89, 1991–94, 1996, 1998
7	Prenatal care	1981–84, 1986–89, 1991–94, 1996, 1998
9	Teenage childbearing	1981–84, 1986–89, 1991–94, 1996–97
10	Nonmarital childbearing	1981–84, 1986–89, 1991–94, 1996–97
11	Maternal education	1981–84, 1986–89, 1991–94, 1996–97
12	Maternal smoking	1991–94, 1996–97
13	Low birthweight	1981–84, 1986–89, 1991–94, 1996–97
14	Low birthweight	1991–94, 1996–97
15	Low birthweight	1972–74
16	Abortions	1981–84, 1986–89, 1991–94, 1996
19	Infant mortality rates	1996–97
20	Infant mortality rates	1984–89, 1991, 1996–2000
21	Infant mortality rates	1984, 1986–89, 1991, 1996–97, 1996–98, 1999–2001
22	Infant mortality rates	1981–84, 1986–89, 1991–94
27	Life expectancy	1975, 1981–84, 1986–89
29	Age-adjusted death rates for selected causes	1981–89, 1991–94, 1996–99
30	Years of potential life lost	1991–94, 1996–99; Crude 1999–2001
35	Death rates for all causes	1981–89, 1991–94, 1996–97
36	Diseases of heart	1981–89, 1991–99
37	Cerebrovascular diseases	1981–89, 1991–99
38	Malignant neoplasms	1981–89, 1991–99
39	Malignant neoplasms of trachea, bronchus, and lung	1981–89, 1991–99
40	Malignant neoplasm of breast	1981–89, 1991–99
41	Chronic lower respiratory diseases	1981–89, 1991–94, 1996–98
42	Human immunodeficiency virus (HIV) disease	1988–89, 1991–94, 1996–98
43	Maternal mortality	1981–89, 1991–94, 1996–98
44	Motor vehicle–related injuries	1981–89, 1991–99
45	Homicide	1981–89, 1991–99
46	Suicide	1981–89, 1991–99
47	Firearm-related injuries	1981–89, 1991–94, 1996–99
48	Occupational diseases	1981–84, 1986–89, 1991–94, 1996–98
49	Occupational injury deaths	1993–94, 1996

Samsung Bioepis Exhibit No. 1002
Page 4961

51	Notifiable diseases	1985, 1988–89, 1991–99
53	Cancer incidence rates	1991–94
56	Respiratory conditions	1998–2001
57	Severe headache or migraine, low back pain, and neck pain	1998–2001
58	Limitation of activity	1999–2000
59	Vision and hearing limitations	1998–99, 2001
60	Respondent-assessed health status	1998
63	Cigarette smoking	1983, 1987–88, 1991–94, 1997
64	Cigarette smoking	1983, 1987–88, 1991–94, 1997
65	Cigarette smoking	1993–95, 1994–97
67	Use of selected substances	1981–89, 1992–94, 1996–99
68	Alcohol consumption	1998–2001
72	Leisure-time physical activity	1991–2001
75	Health care visits	1998–2001
76	Influenza and pneumococcal vaccination	1991, 1993–94, 1997–2001
77	Vaccinations	1996–97
80	No usual source of health care	1995–96, 1997–98
81	Emergency department visits for children	1998–2001
83	Emergency department visits for adults	1998–99, 2001
84	Dental visits	1998–2001
85	Untreated dental caries	1999–2000
86	Mammography	1993
88	Ambulatory care visits	1997–2000
89	Injury-related visits	1997–98, 1999–2000, 2000–01, 2001–02
90	Ambulatory care visits	1980, 1997–99, 2001–02
91	Prescription drug use	1999–2000
93	Additions to mental health organizations	1992, 1998
96	Discharges	1998–2001
97	Discharges	1991–94, 1996–97, 1999
98	Rates of discharges	1995–99, 2001–02
99	Discharges	1995–99, 2001–02
100	Inpatient procedures	1991–92, 2001–02
101	Hospital admissions	1985, 1991–94, 1996–99
102	Nursing home residents	1997
103	Nursing home residents	1997
106	Physicians	1970, 1980, 1987, 1989–90, 1992–94, 1996–98
107	Primary care doctors of medicine	1994, 1996–99
109	Health professions schools	1996, 1998–99, 2001
110	Total enrollment of minorities in schools	2000–01
111	Enrollment of women in schools	2000–01, 2001–02
112	Hospitals	1985, 1991–94, 1996–99
113	Mental health organizations	1992
114	Community hospital beds	1985, 1988–89, 1995–2001
115	Occupancy rates	1985, 1988–89, 1995–99, 2001–02
116	Nursing homes	1996–99, 2001–02
117	Medicare–certified providers and suppliers	1970, 1998
124	Expenditures for health care	1996, 1998–99, 2001
125	Sources of payment for health care	1996, 1998–99, 2001
128	Employers' costs and health insurance	1992–93, 1995, 1997, 1999
129	Hospital expenses	1975, 1985, 1991–94, 1996–98, 2001
130	Nursing home average monthly charges	All: 1995, 1997; 1977, 1997
131	Mental health expenditures	1992
132	Private health insurance	1994, 1996
133	Medicaid coverage	1994, 1996

Samsung Bioepis Exhibit No. 1002
Page 4962

134	No health insurance coverage	1994, 1996
135	Health care coverage	1984, 1994, 1996–98
136	Health maintenance organization	1997, 1999, 2001
137	Health maintenance organizations	1984, 1986–87, 1989, 1991–94, 1996–97, 2001–02
139	Medicare	1999
141	Medicare	1993–2000
142	Medicaid	1975, 1985–89, 1991–94, 1996
143	Medicaid	1975, 1985–89, 1991–94, 1996
144	Department of Veterans Affairs	1985, 1988–89, 1991–94, 1996–98
145	Personal health care per capita expenditures	1992–93
146	Hospital care per capita expenditures	1992–93
147	Physician services per capita expenditures	1992–93
148	Nursing home care per capita expenditures	1992–93
149	Drugs per capita expenditures	1992–93
153	Medicare	1995–2001
154	Medicaid	1998, 2000
155	Health maintenance organizations	1994, 1996–97, 1999, 2001–02

Samsung Bioepis Exhibit No. 1002
Page 4963

Index to Trend Tables and Chartbook Figures

(Numbers refer to table numbers)

A

Table and Figure

Abortion 16
Access to care (see also Dental visits; Emergency department visits; Health insurance; Hospital utilization; Visits to health professionals) 75, 79, 80, 82
  Health care visits, all persons 75
  No recent health care visit, children 79
  No usual source of care 80, 82
Accidents, see Motor vehicle-related injuries; Unintentional injuries.
Activities of Daily Living (ADL), see Limitation of activity.
Adolescents, see Child and adolescent health.
Adults age 55–64 years, see Special feature: population age 55–64 years.
AIDS, see HIV/AIDS.
Alcohol and drug hospitalizations 98, 99
Alcohol consumption (see also Drinking and driving) 66, 67, 68
Alzheimer's disease 31, 32
Ambulatory surgery centers, Medicare certified 117
American Indian or Alaska Native population
  Access to care 75, 79, 80, 82
  AIDS cases 52
  Alcohol consumption 66, 68
  Asthma attack 56
  Back pain, low 57
  Birth rates 3, 10
  Births, number 4
  Birthweight, low 13, 14, 15
  Cancer incidence rates 53
  Cigarette smoking 12, 65, 66
  Death rates, all causes 28, 29, 35
  Death rates, geographic division and State 28
  Death rates, selected causes 29, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47
  Deaths, leading causes 31
  Dental visits 84
  Education of mother 11, 14, 20
  Emergency department visits 81, 83
  Hay fever 56
  Headache, severe or migraine 57
  Health insurance . . . . . 132, 133, 134, 135, Figure 7
  Health maintenance organization (HMO) 136
  Health status, respondent-assessed 60
  Hearing trouble 59
  Hospital utilization, inpatient 96
  Illicit drug use 66
  Infant mortality . . . . . 19, 20, 23, 24, Figure 28
  Limitation of activity 58
  Mammography 86

A—Con.

Table and Figure

American Indian or Alaska Native population—Con.
  Maternal mortality 43
  Neck pain 57
  Occupational injury deaths 49
  Pap smear 87
  Physical activity 72
  Population, resident . . . . . 1, Figure 3
  Prenatal care 7, 8
  Serious psychological distress 61
  Sinusitis 56
  Smoking status of mother 12
  Students, health occupations 110, 111
  Teenage childbearing 3, 9
  Twin, triplet, and higher order multiple births 4, 5
  Unmarried mothers 10
  Vaccinations 76, 77
  Vision trouble 59
  Years of potential life lost 30
Asian or Pacific Islander population
  Access to care 75, 79, 80 82
  AIDS cases 52
  Alcohol consumption 66, 68
  Asthma attack 56
  Back pain, low 57
  Birth rates 3, 10
  Births, number 4
  Birthweight, low 13, 14, 15
  Cancer incidence rates 53
  Cigarette smoking 12, 65, 66
  Death rates, all causes 28, 29, 35
  Death rates, geographic division and State 28
  Death rates, selected causes 29, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47
  Deaths, leading causes 31
  Dental visits 84
  Education of mother 11, 14, 20
  Emergency department visits 81, 83
  Hay fever 56
  Headache, severe or migraine 57
  Health insurance . . . . . 132, 133, 134, 135, Figure 7
  Health maintenance organization (HMO) 136
  Health status, respondent-assessed 60
  Hearing trouble 59
  Hospital utilization, inpatient 96
  Illicit drug use 66
  Infant mortality . . . . . 19, 20, 23, 24, Figure 28
  Limitation of activity 58
  Mammography . . . . . 86, Figure 21

Samsung Bioepis Exhibit No. 1002
Page 4964

A—Con.

Table and Figure

Asian or Pacific Islander population—Con.
- Maternal mortality . . . 43
- Neck pain . . . 57
- Occupational injury deaths . . . 49
- Pap smear . . . 87, Figure 22
- Physical activity . . . 72
- Population, resident . . . 1, Figure 3
- Poverty status . . . 2, Figures 5, 32
- Prenatal care . . . 7, 8
- Serious psychological distress . . . 61
- Sinusitis . . . 56
- Smoking status of mother . . . 12
- Students, health occupations . . . 110, 111
- Teenage childbearing . . . 3, 9
- Twin, triplet, and higher order multiple births . . . 4, 5
- Unmarried mothers . . . 10
- Vaccinations . . . 76, 77
- Vision trouble . . . 59
- Years of potential life lost . . . 30

Asian subgroups (Chinese; Filipino; Hawaiian; Japanese)
- Birth rates . . . 10
- Births, number . . . 4
- Birthweight, low . . . 13, 14
- Education of mother . . . 11, 14, 20
- Infant mortality . . . 19, 20, Figure 28
- Prenatal care . . . 7
- Smoking status of mother . . . 12
- Teenage childbearing . . . 9
- Twin, triplet, and higher order multiple births . . . 4, 5
- Unmarried mothers . . . 10

Asthma . . . 98, 99

Asthma attack . . . 56, Figure 16

Atherosclerosis . . . 31, 32

B

Back pain, low . . . 57, Figure 17

Birth control, see Contraception.

Births (see also Childless women) . . . 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 98, 99, Figure 10
- Age of mother . . . 3, 10, 12
- Birth rates . . . 3, 10
- Births, number . . . 4, 10
- Birthweight, low . . . 13, 14, 15
- Education of mother . . . 11, 12, 14
- Fertility rates . . . 3
- Geographic division and State . . . 15
- Hospital utilization, delivery . . . 98, 99
- Prenatal care . . . 7, 8
- Smoking status of mother . . . 12, 13, Figure 10
- Teenage childbearing . . . 9

B—Con.

Table and Figure

Births (see also Childless women)—Con.
- Twin, triplet, and higher order multiple births . . . 4, 5
- Unmarried mothers . . . 10

Black or African American population
- Abortion . . . 16
- Access to care . . . 75, 79, 80, 82
- AIDS cases . . . 52
- Alcohol consumption . . . 66, 67, 68
- Asthma attack . . . 56, Figure 16
- Back pain, low . . . 57
- Birth rates . . . 3, 10
- Births, number . . . 4
- Birthweight, low . . . 13, 14, 15
- Breastfeeding . . . 18
- Cancer incidence rates . . . 53
- Cancer survival, 5-year relative . . . 54
- Cholesterol, serum . . . 70
- Cigarette smoking . . . 63, 64, 65, 66, 67, Figure 11
- Cocaine use . . . 67
- Contraception . . . 17
- Death rates, all causes . . . 28, 29, 33, 35
- Death rates, geographic division and State . . . 28
- Death rates, selected causes . . . 29, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47
- Death rates, urbanization . . . 33
- Deaths, leading causes . . . 31
- Dental caries (cavities), untreated . . . 85
- Dental visits . . . 84, 141
- Diabetes . . . 55
- Drugs, prescription, use in past month . . . 91
- Education of mother . . . 11, 14, 20
- Emergency department visits . . . 81, 83, 88
- Employment status . . . Figure 31
- Expenses, health care . . . 124
- Fetal mortality . . . 22
- Hay fever . . . 56
- Headache, severe or migraine . . . 57
- Health insurance . . . 132, 134, 135, Figure 7
- Health maintenance organization (HMO) . . . 136
- Health status, respondent-assessed . . . 60
- Hearing trouble . . . 59
- Hospital utilization, inpatient . . . 96, 141
- Hospital utilization, outpatient department . . . 88, 141
- Hypertension . . . 69
- Illicit drug use . . . 66, 67
- Infant mortality . . . 19, 20, 22, 23, 24, Figure 28
- Inhalants . . . 67
- Life expectancy . . . 27
- Limitation of activity . . . 58, 103
- Mammography . . . 86, Figure 21
- Marijuana use . . . 66, 67

Samsung Bioepis Exhibit No. 1002
Page 4965

B—Con.

Table and Figure

Black or African American population—Con.
- Maternal mortality . . . 43
- Medicaid . . . 125, 133, 135, 142
- Medicare . . . 125, 135, 141
- Neck pain . . . 57
- Nursing home utilization . . . 102, 103
- Occupational injury deaths . . . 49
- Out-of-pocket health care expenditures . . . 124
- Overweight and obesity . . . 73, 74
- Pap smear . . . 87, Figure 22
- Physical activity . . . 72, Figure 13
- Population, resident . . . 1, Figure 3
- Poverty status . . . 2, Figures 5, 32
- Prenatal care . . . 7, 8
- Serious psychological distress . . . 61
- Sinusitis . . . 56
- Smoking status of mother . . . 12
- Students, health occupations . . . 110, 111
- Suicidal ideation, suicide attempts . . . 62
- Teenage childbearing . . . 3, 9
- Twin, triplet, and higher order multiple births . . . 4, 5
- Unmarried mothers . . . 10
- Vaccinations . . . 76, 77
- Vision trouble . . . 59
- Years of potential life lost . . . 30

Breastfeeding . . . 18

C

Calories, see Energy and macronutrient intake.

Cancer (Malignant neoplasms) . . . 29, 30, 31, 32, 38, 39, 40, 53, 54, 94, 95, 98, 99, Figure 29
- Breast . . . 29, 30, 40, 53, 54, 98, 99
- Deaths and death rates . . . 29, 31, 32, 38, 39, 40, Figure 29
- Home health care patients . . . 94
- Hospice patients . . . 95
- Hospital utilization . . . 98, 99
- Incidence rates . . . 53
- Site-specific data . . . 29, 30, 39, 40, 53, 54, 98, 99
- Survival, 5-year relative . . . 54
- Trachea, bronchus, lung . . . 29, 39, 53, 54, 98, 99
- Years of potential life lost . . . 30

Cardiac procedures, see Heart disease, procedures.

Cardiovascular risk factors, see Special feature: population age 55–64 years.

Central and South American population, see Hispanic subgroups.

Cerebrovascular disease (stroke) . . . 29, 30, 31, 32, 37, 98, 99, Figure 29
- Deaths and death rates . . . 29, 31, 32, 37, Figure 29
- Hospital utilization . . . 98, 99
- Years of potential life lost . . . 30

C—Con.

Table and Figure

Cesarean section . . . 100

Chancroid, see Diseases, notifiable.

Child and adolescent health
- Abortion . . . 16
- Access to care . . . 75, 79, 80
- AIDS cases . . . 52
- Alcohol consumption . . . 66, 67
- Asthma attack . . . Figure 16
- Birthweight . . . 13, 14, 15
- Breastfeeding . . . 18
- Cigarette smoking . . . 66, 67, Figures 10, 11
- Cocaine use . . . 67
- Contraception . . . 17
- Death rates, all causes . . . 35
- Death rates, selected causes . . . 41, 42, 44, 45, 46, 47
- Deaths, leading causes . . . 32
- Dental caries (cavities), untreated . . . 85
- Dental visits . . . 84
- Drinking and driving . . . Figure 12
- Drugs, during physician and hospital outpatient department visits . . . 92
- Drugs, prescription, use in past month . . . 91
- Emergency department visits . . . 81, 88, 89, Figure 23
- Expenses, health care . . . 124, 126
- Health insurance . . . 132, 133, 134, Figure 7
- Health status, respondent-assessed . . . 60
- Hospital utilization, inpatient . . . 96, 97, 98, 99, 100
- Hospital utilization, outpatient department . . . 88, Figure 24
- Illicit drug use . . . 66, 67
- Infant mortality . . . 19, 20, 21, 22, 23, 24, 25, Figures 27, 28
- Inhalants . . . 67
- Injury . . . 89, Figure 23
- Limitation of activity . . . 58, Figure 18
- Marijuana use . . . 66, 67
- Medicaid . . . 125, 133, 142
- Out-of-pocket health care expenses . . . 124, 126
- Overweight . . . 74, Figure 15
- Physical activity . . . Figure 13
- Population, resident . . . 1, Figures 2, 3
- Poverty status . . . 2, Figures 4, 5
- Residential treatment centers for emotionally disturbed children . . . 93, 113
- Seatbelt use . . . Figure 12
- Suicidal ideation, suicide attempts . . . 62
- Teenage childbearing . . . 3, 9
- Vaccinations . . . 77, 78

Childless (nulliparous) women . . . 6

Chinese population, see Asian subgroups.

Samsung Bioepis Exhibit No. 1002
Page 4966

C—Con.

	Table and Figure
Chiropractors	104, 108
Chlamydia, see Diseases, notifiable.	
Cholesterol, serum	70, Figures 34, 36
Chronic liver disease and cirrhosis	29, 30, 31, 32
Chronic lower respiratory diseases	29, 30, 31, 32, 41, Figure 29
Deaths and death rates	29, 31, 32, 41, Figure 29
Years of potential life lost	30
Cigarette smoking (see also Births, smoking status of mother)	63, 64, 65, 66, 67, Figures 10, 11
Cirrhosis, see Chronic liver disease and cirrhosis.	
Cocaine use	67
Communicable diseases (see also Diseases, notifiable)	34
Congenital anomalies	31, 32
Consumer Price Index (CPI)	120
Contraception	17
Cost, see Employee costs; Employer costs.	
Cuban population, see Hispanic subgroups.	

D

Deaths, death rates (see also Cancer [malignant neoplasms]; Cerebrovascular disease [stroke]; Chronic lower respiratory diseases deaths; Diabetes mellitus; Firearm-related injuries; Heart disease; HIV/AIDS; Homicide; Infant mortality; Life expectancy; Maternal mortality; Motor vehicle-related injuries; Occupational disease deaths; Occupational injuries; Suicide; Years of potential life lost)	28, 29, 31, 32, 33, 34, 35, Figure 29
All causes	35
Educational attainment	34
Leading causes	31, 32, Figure 29
Selected causes	29
State	28
Urbanization	33
Dental caries (cavities), untreated	85
Dental visits	84, 141
Dentists	104, 108, 109, 110, 111
Diabetes mellitus	29, 30, 31, 32, 55, 94, 98, 99, Figure 36
Deaths and death rates	29, 31, 32
Drugs prescribed during medical visits	92, Figure 36
Home health care patients	94
Hospital utilization	98, 99
Prevalence	55
Years of potential life lost	30
Diagnostic procedures, during hospitalizations	100
Diphtheria, see Diseases, notifiable; Vaccinations.	
Disability	58, 103, 141, 142, 144
Blind and disabled Medicaid expenditures	142
Functional status of nursing home residents	103
Limitation of activity	58, 103
Medicare beneficiaries	141
Veterans with service-connected disabilities	144

D—Con.

	Table and Figure
Diseases, notifiable	51
Drinking and driving	Figure 12
Drug and alcohol hospitalizations	98, 99
Drug use, illicit, see Alcohol consumption; Cigarette smoking; Cocaine use; Illicit drug use; Inhalants; Marijuana use.	
Drugs prescribed during medical visits	92, Figure 36
Drugs, prescription, use in past month	91
DTP (Diphtheria, Tetanus, Pertussis), see Vaccinations.	

E

Education	
Alcohol consumption	67
Asthma attack	56
Back pain, low	57
Births	11, 12, 14
Breastfeeding	18
Cigarette smoking	64, 65, 67
Cocaine use	67
Death rates	34
Hay fever	56
Headache, severe or migraine	57
Hearing trouble	59
Infant mortality	20
Inhalants	67
Mammography	86
Marijuana use	67
Neck pain	57
Pap smear	87
Physical activity	72
Sinusitis	56
Suicidal ideation, suicide attempts	62
Vaccinations	76
Vision trouble	59
Elderly population age 65 years and over	
AIDS cases	52
Alcohol consumption	68
Asthma attack	56
Back pain, low	57, Figure 17
Cholesterol, serum	70
Cigarette smoking	63, 65
Death rates, all causes	35
Death rates, selected causes	36, 37, 38, 39, 40, 41, 42, 44, 45, 46, 47, 48
Deaths, leading causes	32
Dental caries (cavities), untreated	85
Dental visits	84, 141
Drugs prescribed during medical visits	92
Drugs, prescription, use in past month	91

Samsung Bioepis Exhibit No. 1002
Page 4967

E—Con.

Table and Figure

Elderly population age 65 years and over—Con.
- Emergency department visits 75, 83, 88, 89
- Expenses, health care 124, 126
- Hay fever 56
- Headache, severe or migraine 57
- Hearing trouble 59
- Health insurance 135
- Health maintenance organization (HMO) . . . 135, 153, 154
- Health status, respondent-assessed 60
- Home health care patients 94
- Hospice patients 95
- Hospital utilization, inpatient . . . . . 96, 97, 98, 99, 100, 141, 153, Figure 25
- Hospital utilization, outpatient department . . . . . 88, 141, Figure 24
- Hypertension 69
- Injury 89
- Life expectancy at age 65, 75 . . . . . 26, 27, Figure 26
- Limitation of activity . . . . . 58, 103, Figure 20
- Mammography 86
- Medicaid 125, 135, 142
- Medicare 125, 135, 137, 139, 140, 141, 153
- Neck pain 57
- Nursing home expenditures 130, 141, 148
- Nursing home utilization 102, 103, 116, 130, 141
- Nursing homes 116
- Occupational injury deaths 49
- Out-of-pocket health care expenses 124, 126
- Overweight and obesity 73
- Pap smear 87
- Physical activity 72
- Population, resident . . . . . 1, Figures 1, 2
- Poverty status . . . . . Figures 4, 5
- Serious psychological distress 61
- Sinusitis 56
- Vaccinations 76, 77
- Vision trouble 59

Emergency department visits . . . . . 75, 81, 83, 88, 89, Figure 23
Employed health service personnel 104
Employee costs for health insurance 138
Employer costs for health insurance 128
Employment status . . . . . Figure 31
End stage renal disease facilities, Medicare certified 117
Energy and macronutrient intake 71
Ethnicity, see Hispanic or Latino population.
Exercise, see Physical activity.

E—Con.

Table and Figure

Expenditures, national health (see also Consumer Price Index; Hospital care expenditures; Medicaid; Medicare; Mental health expenditures; Nursing home expenditures; Physician services expenditures; Prescription drug expenditures; Veterans' medical care) . . . . . 118, 119, 121, 122, 123, 127, 145, Figures 8, 9
- Amount in billions 119, 122, 123, 127
- Amount per capita 118, 119, 123, 145
- Factors affecting growth 121
- Government 119, 123
- International 118
- Percent of Gross Domestic Product . . . 118, 119, Figure 8
- Personal health care . . . . . 123, 145, Figure 9
- Source of funds . . . . . 119, 123, Figure 9
- Type of expenditure . . . . . 122, 123, Figure 9
- Type of payer 127

Expenses, health care . . . . . 124, 126, Figure 37

F

Fertility rates, see Births.
Fetal mortality 22
Filipino population, see Asian subgroups.
Firearm-related injuries, death rates 47
Food intake, see Energy and macronutrient intake.
Fracture 94, 98, 99, 100

G

Geographic region
- Access to care 75, 79, 80, 82
- Alcohol 68
- Asthma attack 56
- Back pain, low 57
- Death rates 33
- Dental visits 84
- Emergency department visits 81, 83
- Hay fever 56
- Headache, severe or migraine 57
- Health insurance 132, 133, 134, 135, 136
- Health status, respondent-assessed 60
- Hearing trouble 59
- Hospital utilization 96
- Limitation of activity 58
- Neck pain 57
- Physical activity 72
- Serious psychological distress 61
- Sinusitis 56
- Vaccinations 76, 77, 78
- Vision trouble 59

Gonorrhea, see Diseases, notifiable.
Gross Domestic Product (GDP) 118, 119

Samsung Bioepis Exhibit No. 1002
Page 4968

H

Table and Figure

Haemophilus influenzae, invasive, see Diseases, notifiable.
Hawaiian population, see Asian subgroups.
Hay fever 56
Headache, severe or migraine . . . . . 57, Figure 17
Health care expenses, see Expenses, health care.
Health expenditures, national, see Expenditures, national health.
Health insurance (see also Access to care; Emergency department visits; Health maintenance organization [HMO]; Medicaid; Medicare) . . . . . 96, 128, 132, 133, 134, 135, 138, 156, Figures 6, 7, 33
  Employment related 132, 135, 138
  Employer costs 128
  Hospital utilization, inpatient 96
  Race and Hispanic origin 132, 133, 134, 135
  55–64 years of age . . . . . Figure 33
  65 years of age and over 135
  Under 65 years of age . . . . . 132, 133, 134, Figures 6, 7
  Uninsured . . . . . 134, 156, Figures 6, 7, 33
Health maintenance organization (HMO) (see also Health insurance) 136, 137, 153, 154, 155
  Age, sex, race, Hispanic origin 136
  Geographic region and State 155
  Medicaid and State 154
  Medicare and State 153
  Plans and enrollment 137
  Poverty status 136
Health professionals visits, see Visits to health professionals.
Health status, respondent-assessed 60
Hearing trouble 59
Heart disease . . . . . 29, 30, 31, 32, 36, 94, 95, 98, 99, 100, Figures 25, 29, 36
  Cardiovascular risk factors . . . . . Figure 34
  Deaths and death rates . . . . . 29, 31, 32, 36, Figure 29
  Drugs prescribed during medical visits . . . . . 92, Figure 36
  Home health care patients 94
  Hospice patients 95
  Hospital utilization 98, 99
  Ischemic heart disease 29, 30
  Procedures (angiocardiography; cardiac catheterization; coronary artery bypass graft; insertion of stent; incision, excision and occlusion of vessels; pacemaker) . . . . . 100, Figure 25
  Years of potential life lost 30
Hepatitis, see Diseases, notifiable; Vaccinations.
Hib (*Haemophilus influenzae* type b), see Vaccinations.
Hispanic or Latino population
  Abortion 16
  Access to care 75, 79, 80, 82
  AIDS cases 52
  Alcohol consumption 66, 68
  Asthma attack . . . . . 56, Figure 16

H—Con.

Table and Figure

Hispanic or Latino population—Con.
  Back pain, low 57
  Birth rates 3, 10
  Births, number 4
  Birthweight, low 13, 14, 15
  Breastfeeding 18
  Cancer incidence rates 53
  Cigarette smoking . . . . . 65, 66, Figure 11
  Cocaine use 66
  Contraception 17
  Death rates, all causes 28, 29, 35
  Death rates, geographic division and State 28
  Death rates, selected causes 29, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47
  Deaths, leading causes 31
  Dental caries (cavities), untreated 85
  Dental visits 84, 141
  Education of mother 11, 14, 20
  Emergency department visits 75, 81, 83
  Employment status . . . . . Figure 31
  Expenses, health care 124
  Hay fever 56
  Headache, severe or migraine 57
  Health insurance . . . . . 132, 134, 135, Figure 7
  Health maintenance organization (HMO) 135, 136
  Health status, respondent-assessed 60
  Hearing trouble 59
  Hospital utilization, inpatient 96
  Illicit drug use 66
  Infant mortality . . . . . 19, 20, 23, 24, Figure 28
  Limitation of activity 58, 141
  Mammography . . . . . 86, Figure 21
  Marijuana use 66
  Maternal mortality 43
  Medicaid 125, 133, 135, 142
  Medicare 125, 135, 141
  Neck pain 57
  Occupational injury deaths 49
  Out-of-pocket health care expenses 124
  Pap smear . . . . . 87, Figure 22
  Physical activity . . . . . 72, Figure 13
  Population, resident . . . . . 1, Figure 3
  Poverty status . . . . . 2, Figures 5, 32
  Prenatal care 7, 8
  Serious psychological distress 61
  Sinusitis 56
  Smoking status of mother 12
  Students, health occupations 110, 111
  Suicidal ideation, suicide attempts 62
  Teenage childbearing 3, 9

Samsung Bioepis Exhibit No. 1002
Page 4969

H—Con.

Table and Figure

Hispanic or Latino population—Con.
- Twin, triplet, and higher order multiple births 4, 5
- Unmarried mothers 10
- Vaccinations 76, 77
- Vision trouble 59
- Years of potential life lost 30

Hispanic subgroups (Central and South American; Cuban; Mexican; Puerto Rican) (see also Mexican; Puerto Rican)
- Birth rates 10
- Births, number 4
- Birthweight, low 13, 14
- Education of mother 11, 14, 20
- Health insurance . . . . . 132, 133, 134, 135, Figure 7
- Health maintenance organization (HMO) 136
- Infant mortality . . . . . 19, 20, Figure 28
- Prenatal care 7
- Smoking status of mother 12
- Teenage childbearing 9
- Twin, triplet, and higher order multiple births 4, 5
- Unmarried mothers 10

HIV/AIDS 29, 30, 31, 32, 34, 42, 52, 98, 99
- AIDS cases 52
- Deaths and death rates 29, 31, 32, 42
- Educational attainment, death rates 34
- Hospital utilization 98, 99
- Years of potential life lost 30

Home health agencies, Medicare certified 117

Home health care patients 94

Homicide, death rates 29, 30, 31, 32, 45

Hospice patients 95

Hospices, Medicare certified 117

Hospital employees 104

Hospital care expenditures (see also Consumer Price Index; Medicaid; Medicare) 123, 129, 146

Hospital utilization (see also Access to care; Emergency department visits; Medicaid; Medicare; Veterans' medical care) . . . . 88, 96, 97, 98, 99, 100, 101, 141, 153, Figure 24
- Admissions 101
- Average length of stay 96, 97, 99, 101, 153
- Days of care 96, 97, 98
- Diagnoses, selected 98, 99
- Discharges for inpatients 96, 97, 98, 99
- Outpatient department . . . . . 88, 101, 141, Figure 24
- Procedures 100
- Race and Hispanic origin 96, 141

Hospitals (see also Hospital employees; Mental health; Nursing homes) 112, 114, 115
- Beds 112, 115
- Geographic division and State 114, 115
- Occupancy rate 112, 115

Hypertension . . . . . 69, Figure 34

I

Table and Figure

Illicit drug use 66, 67

Immunizations, see Vaccinations.

Income, family, see Poverty status.

Infant mortality (see also Fetal mortality) . . . . . 19, 20, 21, 22, 23, 24, 25, 32, Figures 27, 28
- Age at death . . . . . 19, 22, 24, Figure 27
- Birth cohort data 19, 20, 21
- Birthweight 21
- Cause of death 32
- Education of mother 20
- Geographic division and State 23, 24
- International 25
- Race and Hispanic origin . . . . 19, 20, 22, 23, 24, Figure 28

Influenza and pneumonia 29, 30, 31, 32

Influenza vaccination, see Vaccinations.

Inhalants 67

Injuries, see Emergency department visits; Firearm-related injuries; Homicide; Hospital utilization, diagnoses; Motor vehicle-related injuries; Occupational injuries; Suicide; Unintentional injuries.

Inpatient care, see Hospital utilization; Mental health, additions; Nursing home utilization.

Insurance, see Health insurance.

Instrumental activities of daily living (IADL), see Limitation of activity.

International health (see also Expenditures, international; Infant mortality; Life expectancy) 25, 26, 118

Intervertebral disc disorders 98, 99, 100

Ischemic heart disease, see Heart disease.

J

Japanese population, see Asian subgroups.

L

Leading causes of death, see Deaths, leading causes.

Leisure-time activity, see Physical activity.

Life expectancy . . . . . 26, 27, Figure 26

Limitation of activity . . . . . 58, 103, 141, Figures 18, 19, 20

Liver disease, see Chronic liver disease and cirrhosis.

Low birthweight, see Births; Infant mortality.

Low income, see Poverty status.

Lyme disease, see Diseases, notifiable.

Samsung Bioepis Exhibit No. 1002
Page 4970

M

Table and Figure

Malignant neoplasms, see Cancer.
Mammography . . . . . 86, Figure 21
Managed care, see Health maintenance organization (HMO); Preferred provider organization (PPO).
Marijuana use 66, 67
Maternal health, see Women's health.
Maternal mortality 43
Measles (Rubella), see Diseases, notifiable; Vaccinations.
Medicaid (see also Health insurance) . . . . . 123, 125, 127, 133, 135, 136, 142, 143, 151, 154, Figure 6
- Basis of eligibility 142
- Coverage 133, 135
- Expenditures 123, 127, 151
- Geographic region and State 151, 154
- Health maintenance organization (HMO) 136, 154
- Race and Hispanic origin 133, 142
- Recipients and payments 125, 142, 143, 154
- Type of service 143
- Under 65 years of age . . . . . Figure 6

Medical doctors, see Physicians.
Medicare (see also Health insurance) 117, 123, 125, 135, 139, 140, 141, 150, 153
- Age and sex of beneficiaries 135, 140
- Certified providers and suppliers 117
- Coverage 135
- Enrollment 139, 140, 141, 153
- Expenditures 123, 139, 141, 150
- Geographic region and State 150, 153
- Health maintenance organization (HMO) 135, 153
- Hospital utilization 153
- Payments 125, 140, 150, 153
- Race and Hispanic origin 135, 141
- Type of service 135, 139

Meningococcal disease 32, 51
Men's health
- AIDS cases 52
- Alcohol consumption 66, 67, 68
- Asthma attack 56
- Back pain, low 57
- Cancer incidence rates 53
- Cancer survival, 5-year relative 54
- Cholesterol, serum . . . . . 70, Figure 34
- Cigarette smoking . . . 12, 13, 63, 64, 65, 66, 67, Figure 10
- Cocaine use 67
- Death rates, all causes 29, 35
- Death rates, educational attainment 34
- Death rates, selected causes 29, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47
- Death rates, urbanization 33
- Deaths, leading causes 31
- Dental caries (cavities), untreated 85
- Dental visits 84

M—Con.

Table and Figure

Men's health—Con.
- Diabetes 55
- Drugs prescribed during medical visits . . . . . 92, Figure 36
- Drugs, prescription, use in past month 91
- Emergency department visits 75, 83, 88, 89
- Employment status . . . . . Figure 31
- Energy and macronutrient intake 71
- Hay fever 56
- Headache, severe or migraine 57
- Health status, respondent-assessed 60
- Hearing trouble 59
- Home health care patients 94
- Hospice patients 95
- Hospital utilization, inpatient 96, 97, 98, 99
- Hospital utilization, outpatient department 88
- Hypertension . . . . . 69, Figure 34
- Illicit drug use 66
- Inhalants 67
- Life expectancy 26, 27
- Limitation of activity 58, 103
- Marijuana use 66, 67
- Neck pain 57
- Nursing home utilization 102, 103
- Occupational injury deaths 49
- Overweight and Obesity . . . . . 73, 74, Figure 34
- Physical activity 72
- Poverty status . . . . . Figure 32
- Prescription drug expenses . . . . . Figure 37
- Serious psychological distress 61
- Sinusitis 56
- Vaccinations 76
- Vision trouble 59
- Years of potential life lost 30

Mental health (see also Suicide) 61, 92, 93, 98, 99, 113, 131, 152
- Additions, mental health organizations 93
- Beds and organizations 113
- Drugs prescribed during medical visits 92
- Expenditures 131, 152
- Hospital utilization 98, 99
- Mental illness, serious 98, 99
- Serious psychological distress 61

Metropolitan/nonmetropolitan data
- Access to care 75, 79, 80, 82
- Alcohol 68
- Asthma attack 56
- Back pain, low 57
- Death rates 33
- Dental visits 84
- Emergency department visits 81, 83
- Hay fever 56

Samsung Bioepis Exhibit No. 1002
Page 4971

M—Con.

	Table and Figure
Metropolitan/nonmetropolitan data—Con.	
Headache, severe or migraine	57
Health insurance	132, 133, 134, 135, 136
Health status, respondent-assessed	60
Hearing trouble	59
Hospital utilization	96
Limitation of activity	58
Neck pain	57
Physical activity	72
Serious psychological distress	61
Sinusitis	59
Vaccinations	77, 78
Vision trouble	59
Mexican population (see also Hispanic subgroups)	
Asthma attack	56
Back pain, low	57
Cholesterol, serum	70
Cigarette smoking	65
Dental caries (cavities), untreated	85
Diabetes	55
Drugs, prescription, use in past month	91
Emergency department visits	83
Hay fever	56
Headache, severe or migraine	57
Health insurance	132, 133, 134, 135, 136, Figure 7
Health status, respondent-assessed	60
Hearing trouble	59
Hypertension	69
Limitation of activity	58
Medical students	110, 111
Neck pain	57
No usual source of care	82
Overweight and Obesity	73, 74
Physical activity	72
Poverty status	2
Serious psychological distress	61
Sinusitis	56
Vaccinations	76
Vision trouble	59
MMR (Measles, Mumps, Rubella), see Vaccinations.	
Motor vehicle-related injuries	29, 30, 44, 89, Figure 23
Mumps, see Diseases, notifiable; Vaccinations.	

N

	Table and Figure
National health expenditures, see Expenditures, national health.	
Neonatal mortality, see Infant mortality, age at death.	
Nephritis, nephrotic syndrome, and nephrosis	31, 32
Neck pain	57
Nurses	108, 109, 110, 111
Nursing home employees	104
Nursing home expenditures	123, 130, 141, 148
Nursing home utilization	102, 103, 116, 141, 144
Nursing homes, beds, occupancy	116
Nutritionists/dieticians	108

O

Obesity	73, Figures 15, 34
Occupational disease deaths	48
Occupational injuries	49, 50
Occupational therapists	108, 109
Optometrists	108, 109, 110, 111
Osteoarthritis	94, 98, 99
Osteopaths, see Physicians.	
Out-of-pocket health care expenses	124, 126, 127
Outpatient department, see Hospital utilization, outpatient department.	
Overweight	73, 74, Figure 15

P

Pacemakers	100
Pap smear	87, Figure 22
Parity, see Childless women.	
Perinatal mortality, see Infant mortality, age at death.	
Personal health care expenditures, see Expenditures, national health.	
Pertussis (whooping cough), see Diseases, notifiable; Vaccinations.	
Pharmacists	108, 109, 110, 111
Physical activity	72, Figures 13, 14
Physical therapists	108
Physician services expenditures (see also Consumer Price Index; Medicaid; Medicare)	123, 147
Physician utilization	88, 90, Figures 24, 35
Physicians	90, 104, 105, 106, 107, 108, 109, 110, 111
Active personnel	108
Doctors of osteopathy	108, 109, 110, 111
Employees, in offices of	104
Geographic division and State	105
International medical school graduates	106
Primary care	90, 107
Primary specialty	90, 106, 107
Schools and students	109, 110, 111
Pneumococcal vaccinations, see Vaccinations.	

Samsung Bioepis Exhibit No. 1002
Page 4972

P—Con.

Table and Figure

Pneumonia (see also Influenza and pneumonia) 98, 99
Podiatrists 108, 109, 110, 111
Poliomyelitis (Polio), see Diseases, notifiable; Vaccinations.
Population growth, baby boomers . . . . . Figure 30
Population, resident, by age, race, and Hispanic origin . . . . 1, Figures 1, 2, 3
Postneonatal mortality, see Infant mortality, age at death.
Poverty status . . . . . 2, 56, 57, 58, 59, 60, 61, 72, 75, 76, 77, 79, 80, 81, 82, 83, 84, 85, 86, 87, 96, 132, 133, 134, 135, 136, Figures 4, 5, 14, 32
- Access to care 79, 80, 82
- Asthma attack 56
- Back pain, low 57
- Dental caries (cavities), untreated 85
- Dental visits 84
- Emergency department visits 81, 83
- Hay fever 56
- Headache, severe or migraine 57
- Health care visits 75
- Health insurance . . . . . 132, 133, 134, 135, Figure 7
- Health maintenance organization (HMO) 136
- Health status, respondent-assessed 60
- Hearing trouble 59
- Hospital utilization, inpatient 96
- Limitation of activity 58
- Mammography 86
- Medicaid 133, 135
- Neck pain 57
- Pap smear 87
- Persons and families below poverty level . . . . . 2, Figures 4, 5, 32
- Physical activity . . . . . 72, Figure 14
- Poverty rates by age, race, Hispanic origin . . . Figures 4, 5
- Serious psychological distress 61
- Sinusitis 56
- Vaccinations 76, 77
- Vision trouble 59

Preferred provider organization (PPO) 138
Prenatal care, see Births.
Prescription drug expenses (see also Consumer Price Index; Medicaid; Medicare) . . . . . 123, 124, 149, Figure 37
Prescription drug use, see Drugs, prescription, use in past month.
Primary care physicians, see Physicians.
Private health insurance, see Health insurance.
Procedures 100
Public Health, schools of; students 109
Puerto Rican population (see also Hispanic subgroups)
- Health insurance . . . . . 132, 133, 134, 135, 136, Figure 7
- Medical students 110, 111
- Poverty status 2

R

Table and Figure

Race, see specific race groups.
Respiratory conditions, see Asthma attack; Hay fever; Sinusitis.
Rocky Mountain spotted fever, see Diseases, notifiable.
Rubella (German measles), see Diseases, notifiable; Vaccinations.
Rural data, see Metropolitan/nonmetropolitan data.

S

Salmonellosis, see Diseases, notifiable.
Seatbelt use . . . . . Figure 12
Self-assessment of health, see Health status, respondent-assessed.
Septicemia 31, 32
Serious psychological distress (see also Mental health) . . . 61
Shigellosis, see Diseases, notifiable.
Sinusitis 56
Smoking, see Cigarette smoking.
Socioeconomic status, see Education; Poverty status.
Source of funds or payments (see also Expenditures, national health; Health insurance; Medicaid; Medicare) . . . . . 123, 125, 127, 130, Figures 9, 37
Special feature: population age 55–64 years
- Cardiovascular risk factors . . . . . Figure 34
- Drugs prescribed during medical visits . . . . . Figure 36
- Employment status . . . . . Figure 31
- Health insurance . . . . . Figure 33
- Population growth, baby boomers . . . . . Figure 30
- Poverty status, low income . . . . . Figure 32
- Prescribed medicine expense . . . . . Figure 37
- Visits to health professionals . . . . . Figure 35

Speech therapists 108
State data
- Birthweight, low 15
- Death rates 28
- Expenditures, drug 149
- Expenditures, hospital care 146
- Expenditures, nursing home and home health care . . 148
- Expenditures, personal health care 145
- Expenditures, physician 147
- Expenditures, State mental health agency 152
- Health insurance, uninsured 156
- Health maintenance organization (HMO) . . . 153, 154, 155
- Hospital beds 114
- Hospital occupancy rates 115
- Infant mortality 23, 24
- Medicaid 151, 154
- Medicare 150, 153
- Nursing homes, beds, occupancy, residents 116
- Physicians 105
- Prenatal care 8
- Vaccinations 78

Samsung Bioepis Exhibit No. 1002
Page 4973

S—Con.

Table and Figure

Stent, cardiac, see Heart disease, procedures.
Sterilization, see Contraception.
Stroke, see Cerebrovascular disease.
Sudden infant death syndrome, see Infant mortality, cause of death.
Suicidal ideation, suicide attempts 62
Suicide 29, 30, 31, 32, 46
Surgery, see Hospital utilization.
Syphilis, see Diseases, notifiable.

T

Tetanus, see Diseases, notifiable; Vaccinations.
Tobacco use, see Cigarette smoking.
Tuberculosis, see Diseases, notifiable.
Twin, triplet, and higher order multiple births 4, 5

U

Uninsured, health, see Health insurance, uninsured.
Unintentional injuries . . . . . 29, 30, 31, 32, Figure 29
Urban and rural data, see Metropolitan/nonmetropolitan data.
Usual source of care, see Access to care.

V

Vaccinations 76, 77, 78
Varicella, see Vaccinations.
Veterans' medical care 144
Vision trouble 59
Visits to health professionals . . . . . Figure 35

W

Wages and salaries 128
Women's health
- Abortion 16
- Access to care 75, 82
- AIDS cases 52
- Alcohol consumption 66, 67, 68
- Asthma attack 56
- Back pain, low 57
- Birth rates, fertility rates 3, 10
- Births, number 4, 10
- Breast cancer 40, 53, 54, 98, 99
- Cancer incidence rates 53
- Cancer survival, 5-year relative 54
- Cesarean section 100
- Cholesterol, serum . . . . . 70, Figure 34
- Cigarette smoking . . . 12, 13, 63, 64, 65, 66, 67, Figure 10
- Cocaine use 67
- Contraception 17

W—Con.

Table and Figure

Women's health—Con.
- Death rates, all causes 29, 35
- Death rates, educational attainment 34
- Death rates, selected causes 29, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47
- Death rates, urbanization 33
- Deaths, leading causes 31
- Dental caries (cavities), untreated 85
- Dental visits 84
- Diabetes 55
- Drugs prescribed during medical visits . . . . . 92, Figure 36
- Drugs, prescription, use in past month 91
- Emergency department visits 75, 83, 88, 89
- Employment status . . . . . Figure 31
- Energy and macronutrient intake 71
- Hay fever 56
- Headache, severe or migraine 57
- Health status, respondent-assessed 60
- Hearing trouble 59
- Home health care patients 94
- Hospice patients 95
- Hospital utilization, inpatient 96, 97, 98, 99
- Hospital utilization, outpatient department . . . 88, Figure 24
- Hypertension . . . . . 69, Figure 34
- Illicit drug use 66
- Inhalants 67
- Life expectancy 26, 27
- Limitation of activity 58, 103
- Mammography . . . . . 86, Figure 21
- Marijuana use 66, 67
- Maternal mortality 43
- Neck pain 57
- Nursing home utilization 102, 103
- Occupational injury deaths 49
- Overweight and Obesity . . . . . 73, 74, Figure 34
- Pap smear . . . . . 87, Figure 22
- Physical activity 72
- Poverty status . . . . . 2, Figure 32
- Prenatal care 7, 8
- Prescribed medicine expense . . . . . Figure 37
- Serious psychological distress 61
- Sinusitis 56
- Teenage childbearing 3, 9
- Unmarried mothers 10
- Vaccinations 76
- Vision trouble 59
- Years of potential life lost 30

Y

Years of potential life lost (YPLL) 30

Samsung Bioepis Exhibit No. 1002
Page 4974



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Page 4975

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First Named Inventor/Applicant Name:	Leonard BELL
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	Application Number	15642096
	Filing Date	2017-07-05
	First Named Inventor	Leonard BELL
	Art Unit	1644
	Examiner Name	J. L. Rogers
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STUDY PROTOCOL

BB-IND 11075

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

PROTOCOL NUMBER: C04-001

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

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

PROTOCOL NUMBER: C04-001

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

3-June-04
Date

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

3-June-04
Date

TABLE OF CONTENTS

PROTOCOL SUMMARY 6

ABBREVIATIONS 9

1. INTRODUCTION 11

2. STUDY OBJECTIVES 13

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

3. STUDY PLAN 13

3.1. OVERALL DESIGN 13

4. STUDY TREATMENT 14

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

5. PATIENT ENROLLMENT 20

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

6. STUDY VISITS 24

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

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

7. STUDY ASSESSMENTS 37

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

8. ADVERSE EVENTS 41

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

9. STATISTICAL METHODS 44

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

10. DATA HANDLING AND QUALITY ASSURANCE 49

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

11. ADMINISTRATIVE CONSIDERATIONS 50

Samsung Bioepis Exhibit No. 1002
Page 4991

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

12. INVESTIGATOR'S STATEMENT OF AGREEMENT 54

13. REFERENCES 55

14. APPENDICES 57

APPENDIX A: SUMMARY OF LABORATORY TESTS 58

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

APPENDIX C: FACIT-FATIGUE SUBSCALE VERSION 4 61

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

APPENDIX E: SCHEDULE OF EVENTS – LARGE VERSION 63

[redacted]



4.5. Investigational Product Packaging and Storage

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

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

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

4.6. Test Article Accountability

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

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

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

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

[redacted]

[redacted]

11. ADMINISTRATIVE CONSIDERATIONS

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

11.1. Confidentiality

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

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

[redacted]

12. INVESTIGATOR'S STATEMENT OF AGREEMENT

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

_____

Principal Investigator
(print name)

_____ _____

Principal Investigator
(sign name)

Date

CLINICAL STUDY PROTOCOL
CONFIDENTIAL

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

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

_____ _____
Principal Investigator Date

_____
Principal Investigator (Print)

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

Samsung Bioepis Exhibit No. 1002
Page 4997

TABLE OF CONTENTS

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

Samsung Bioepis Exhibit No. 1002
Page 4998

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

Samsung Bioepis Exhibit No. 1002
Page 4999

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[redacted]

[redacted]

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

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

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Samsung Bioepis Exhibit No. 1002
Page 5000

[redacted]

6.6 Drug Accountability

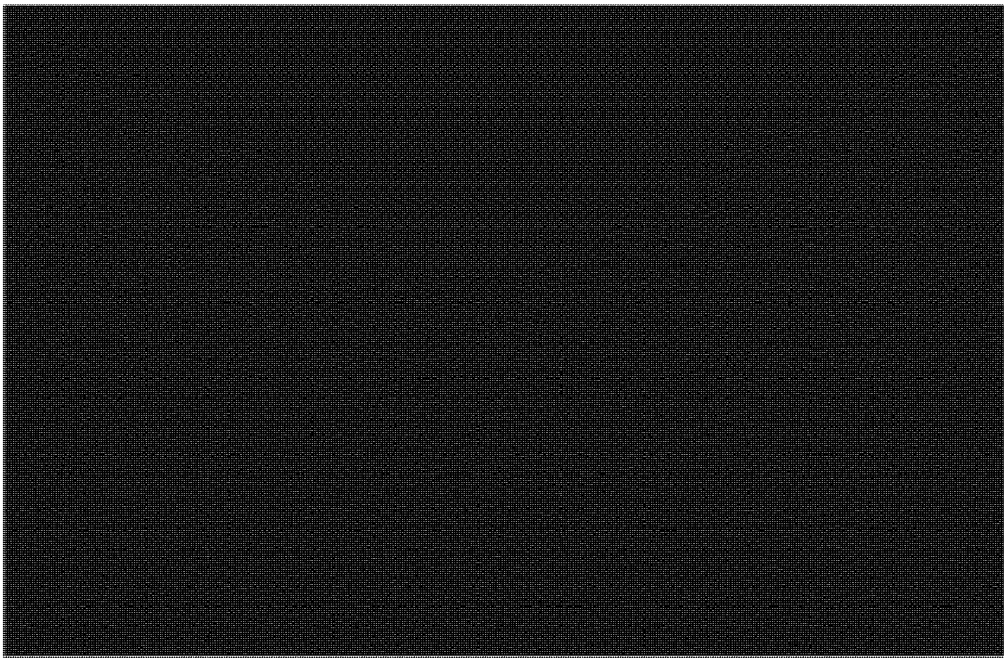
When a drug shipment is received at the site, the pharmacist at each site should verify the contents and sign the Drug Order Form provided with the shipment and maintain the original copy for collection by the Study Monitor/Research Associate. The duplicate copy should be kept in the Pharmacy Binder.

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

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

[redacted]

Samsung Bioepis Exhibit No. 1002
Page 5001



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Page 5002

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

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

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

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

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

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

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

Clinical Data

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

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

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

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

Introducing Soliris OneSource(TM)

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

Important Safety Information

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

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

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

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

Conference Call Information

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

About PNH

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

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

About Alexion

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

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

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

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

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

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

(4) Hillmen P. Lewis SM, Bessler M, Luzzatto L, Dacie JV. Natural history of paroxysmal nocturnal hemoglobinuria. N Engl J Med 1995; 333:1253-1258.

(5) Hillmen P, Young N, et al. The Complement Inhibitor Eculizumab in Paroxsysmal Nocturnal Hemoglobinuria. N Engl J Med 2006; 355:1233-1243.

(6) Young N, Antonioli E, Rotoli B, et al. Safety and efficacy of the terminal complement inhibitor eculizumab in patients with paroxysmal nocturnal hemoglobinuria: SHEPHERD phase III clinical study results. Blood 2006; 108:971.

(7) Hillmen P, Muus P, Duhrsen U, et al. The terminal complement inhibitor eculizumab reduces thrombosis in patients with paroxysmal nocturnal hemoglobinuria. Blood 2006; 108:123.

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

SOURCE Alexion Pharmaceuticals, Inc.

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http://www.alexionpharm.com

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News Provided by COMTEX

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

Dear Sir:

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

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

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

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

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

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

Stephen A. Saxe — January 12, 2018

Stephen A. Saxe, Ph.D., J.D. — Date

Saxe, Stephen

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

Dear Sir or Madam:

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

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

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****
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****

ALEXION pharmaceuticals

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

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

April 28, 2009

Dear Sir or Madam:

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

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

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

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

Samsung Bioepis Exhibit No. 1002
Page 5009

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

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

Disulfide Bonds Connecting the Light and Heavy Chains
Cys136Heavy - Cys214Light

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

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

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

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

Very truly yours,

Stephen A. Saxe

Stephen A. Saxe

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

Heavy Chain

QVQLVQSGAEVKKPGASVKVSCKASGYIFSNYWIQWVRQAPGQGLEWMGEILPGSGSTEYTENFKDRVTMTRDTSTSTVYMELSSLR
SEDTAVYYCARYFFGSSPNWYFDVWGQGTLVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF
PAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVERKCCVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVV
VDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVY
TLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHY
TQKSLSLSLGK

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

Light Chain

DIQMTQSPSSLSASVGDRVTITCGASENIYGALNWYQQKPGKAPKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQ
NVLNTPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST
LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

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

Samsung Bioepis Exhibit No. 1002
Page 5011

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

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

From: Saxe, Stephen Lois Bishop 614-447-3600,
Sent: Monday, April 20, 2009 4:35 PM x 2863
To: 'help@cas.org'
Subject: Registry Number 219685-50-4

Dear Sir or Madam,

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

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

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****

Saxe, Stephen

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

Dear Dr. Saxe,

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

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

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

Dear Sir or Madam:

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

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

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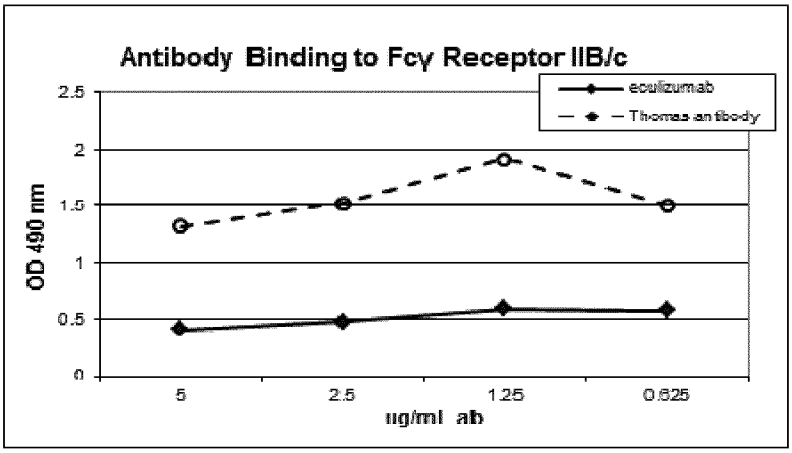
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Experimental Report

Methods

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

Results



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

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

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration
Rockville, MD 20857

MAR 16 2007

Our STN: BL 125166/0

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

Dear Dr. Motola:

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

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

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

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

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

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

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

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

Postmarketing Studies subject to reporting requirements of 21 CFR 601.70.

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

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

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

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

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

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

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

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

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

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

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

      i. Death;

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

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

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

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

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

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

ii. Soliris use patterns including indication for use;

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Sincerely,

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

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

Oral Communication

Paediatric and Red Cell Disorders

1

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

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

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

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

2

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

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

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

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

3

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

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

Samsung Bioepis Exhibit No. 1002
Page 5024

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*

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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

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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

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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

Samsung Bioepis Exhibit No. 1002
Page 5025

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

Oral Communication

Transplantation

7

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

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

8

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

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UK Collaborative Study

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

9

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

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

Samsung Bioepis Exhibit No. 1002
Page 5026

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

10

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

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

11

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

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

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

12

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

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

Samsung Bioepis Exhibit No. 1002
Page 5027

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

Poster Presentation

Haematological malignancies

13

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

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

14

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

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

15

Evidence for a primitive cell origin of acute lymphoblastic leukaemia

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

Samsung Bioepis Exhibit No. 1002
Page 5028

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

16

Characterization of T-ALL progenitor cells

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

17

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

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

18

Protein expression profiling confirms the close association between CLUS and CLL

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

19

The proliferating cells in CLL have a unique immunophenotype

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

Samsung Bioepis Exhibit No. 1002
Page 5029

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

20

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

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

21

Relationship between p53 dysfunction and karyotypic abnormalities in chronic lymphocytic leukaemia

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

22

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

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

Samsung Bioepis Exhibit No. 1002
Page 5030

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

23

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

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

24

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

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

25

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

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

Samsung Bioepis Exhibit No. 1002
Page 5031

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

26

Do we need to monitor MGUS patients indefinitely?

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

27

The natural history of 'early' CLL

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

28

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

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

Samsung Bioepis Exhibit No. 1002
Page 5032

29

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

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

30

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

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

31

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

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

32

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

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

Samsung Bioepis Exhibit No. 1002
Page 5033

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

33

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

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

34

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

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

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

*Fisher's exact test.

35

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

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

Samsung Bioepis Exhibit No. 1002
Page 5034

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

Cyclin D1 by immunohistochemistry

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

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

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

36

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

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

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

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

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

37

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

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

38

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

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

Samsung Bioepis Exhibit No. 1002
Page 5035

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

39

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

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

40

Gene array profiling of the effect of FLT3 mutations in AML

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

41

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

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

Samsung Bioepis Exhibit No. 1002
Page 5036

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

42

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

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

43

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

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

44

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

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

Samsung Bioepis Exhibit No. 1002
Page 5037

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

45

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

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

46

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

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

47

Bcr-Abl down-regulates CCN3 expression

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

Samsung Bioepis Exhibit No. 1002
Page 5038

48

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

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

49

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

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

50

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

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

51

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

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

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Samsung Bioepis Exhibit No. 1002
Page 5039

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

52

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

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

53

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

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

54

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

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

Samsung Bioepis Exhibit No. 1002
Page 5040

55

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

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

56

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

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

57

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

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

58

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

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

Samsung Bioepis Exhibit No. 1002
Page 5041

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

59

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

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

60

Toxic megacolon due to invasive aspergillosis localized to the colon

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

61

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

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

Samsung Bioepis Exhibit No. 1002
Page 5042

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

62

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

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

63

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

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

64

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

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

65

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

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

Samsung Bioepis Exhibit No. 1002
Page 5043

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

66

Sensitivity and As_2O_3 concentration in cell

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

67

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

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

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

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

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

68

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

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

69

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

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

Samsung Bioepis Exhibit No. 1002
Page 5044

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

Poster Presentation

Paediatric and Red Cell Disorders

70

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

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

71

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

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Unrelated donor stem cell transplantation (SCT) in patients with severe MPS 1H (Hurler's syndrome) has been reported to have poor outcome. Registry data shows a long term, engrafted survival of 35%. This data have influenced parent's adversely about unrelated donor SCT when no HLA-matched sibling was available. We report the results of unrelated donor SCT in MPS 1H from three UK centres: Manchester, Great Ormond Street and Birmingham. Methods: between January 2000 and December 2003 seven patients with MPS 1H underwent SCT from an unrelated donor. Median age at time of transplantation was 17 months (range 14–22 months). Three patients received conditioning with Fludarabine 30 mg/m^2/d × 5, Busulphan 5 mg/kg/d × 4, Cyclophosphamide 50 mg/kg/d × 4 and rabbit Anti-Thymocyte Globulin (ATG) 2.5 mg/kg/d × 3. Two patients received Fludarabine 30 mg/m^2/d × 5, Busulphan 5 mg/kg/d × 4, Cyclophosphamide 50 mg/kg/d × 4 and Alemtuzumab 0.2 mg/kg/d × 5. One patient received Melphalan 125 mg/m^2 × 1, Cyclophosphamide 50 mg/kg/d × 4 and ATG 2.5 mg/kg/d × 3. One patient received Fludarabine 30 mg/m^2/d × 5, Busulphan 5 mg/kg/d × 4, Cyclophosphamide 50 mg/kg/d × 4. All patients were given Cyclosporin A (CsA) as graft-versus-host disease (GvHD) prophylaxis.

Results: All conditioning regimens were well tolerated. Six of seven patients (86%) survived with a median follow up of 25 months (range 7–45 months). All long-term survivors show full donor engraftment. One of seven patients died 44 d post-SCT with grade 4 GvHD of skin, liver, gastrointestinal tract and lungs. Conclusions: We show improved engraftment and long-term survival in our patients with MPS 1H undergoing unrelated donor SCT when compared with historical data. We believe this is due to more intensive conditioning regimens used. Improving alternate donor transplant results in MPS 1H should be communicated to families to add decision making prior to SCT.

72

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

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

Samsung Bioepis Exhibit No. 1002
Page 5045

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

73

Assessment of liver disease in transfusion dependent thalassaemia major

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

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

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

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

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

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

74

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

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

75

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

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

Samsung Bioepis Exhibit No. 1002
Page 5046

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

76

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

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

77

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

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

78

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

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

Samsung Bioepis Exhibit No. 1002
Page 5047

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

79

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

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

80

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

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

81

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

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

Samsung Bioepis Exhibit No. 1002
Page 5048

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

Table. Patient characteristics, surgery risk and postoperative complications

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

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

82

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

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

83

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

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

84

HFE (haemochromatosis) mutations and myocardial infarction

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

Samsung Bioepis Exhibit No. 1002
Page 5049

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

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

85

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

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

86

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

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

87

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

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

Samsung Bioepis Exhibit No. 1002
Page 5050

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

88

Windy weather and acute pain in sickle cell disease

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

89

Vascular endothelial growth factor and proliferative retinopathy in sickle cell disease

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

Poster Presentation

Transplantation

90

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

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

Samsung Bioepis Exhibit No. 1002
Page 5051

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

91

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

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

92

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

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

93

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

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

Samsung Bioepis Exhibit No. 1002
Page 5052

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

94

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

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

95

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

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

96

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

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

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Samsung Bioepis Exhibit No. 1002
Page 5053

97

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

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

98

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

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

99

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

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

Samsung Bioepis Exhibit No. 1002
Page 5054

100

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

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

101

In vitro generation of T cells cytotoxic to allogeneic AML blasts

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

102

Nucleated cell doses collected from unrelated donor bone marrow harvests

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

Samsung Bioepis Exhibit No. 1002
Page 5055

Oral Communication

General Haematology and Transfusion

103

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

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

104

Automated analysis of reticulated platelets and its potential clinical value

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

105

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

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

106

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

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

Samsung Bioepis Exhibit No. 1002
Page 5056

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

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

107

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

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

108

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

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

Samsung Bioepis Exhibit No. 1002
Page 5057

Oral Communication

Haemostasis & Thrombosis and Anticoagulation

109

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

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

110

Gene defects in patients with factor XI deficiency

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

111

Heparin cofactor II, new insights into heparin binding

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

112

Thrombophilia in pregnancy: a systematic review

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

Samsung Bioepis Exhibit No. 1002
Page 5058

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

113

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

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

114

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

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

Poster Presentation

Cellular & Molecular Biology

115

Ras promotes monocyte lineage selection

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

Samsung Bioepis Exhibit No. 1002
Page 5059

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

116

Profiling proteasome activity in multiple myeloma and lymphoma

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

117

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

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

118

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

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

Samsung Bioepis Exhibit No. 1002
Page 5060

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

119

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

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

120

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

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

121

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

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

Samsung Bioepis Exhibit No. 1002
Page 5061

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

122

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

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

123

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

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

124

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

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

Samsung Bioepis Exhibit No. 1002
Page 5062

125

Analysis of anagrelide-induced apoptosis in megakaryocytic cell lines

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

126

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

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The isolation of murine mesenchymal stem cells (muMSC) is complicated by the lack of specific markers and by haemopoietic contamination of the murine CFU-F. We describe the isolation of muMSC from femurs following mechanical depletion of bone marrow. Following enzymatic digestion of bone fragments with Collagenase type II the adherent population is expanded in MesenCult murine media (StemCell Technologies) and subsequently depleted of $CD45^+$ and $CD31^+$ cells. The initial cell count following enzymatic digestion of bone was half that of whole bone marrow cells (11×10^6 vs. 23×10^6 cells/femur). However, after 14 d the adherent population from bone was fivefold higher than that from bone marrow (4×10^6 vs. 0.8×10^6 adherent cells). The $CD45^-/CD31^-$ population in bone was an average of 21.5% vs. 7.6% for bone marrow resulting in a total output of 3.5×10^6 cells/femur compared with 0.05×10^6 from bone marrow. The muMSC derived in this manner can be expanded for over 60 population doublings and following transfer to stimulant media are able to differentiate into both adipocytes and osteocytes. Following staining with LacZ the muMSC derived from the Rosa26 murine strain can be demonstrated to express β-galactosidase. Exposure to HoxB4-GFP producers resulted in a transduction efficiency of 27% and expression was maintained for over 30 population doublings and was not seen in either in untransduced or mock-transduced mock-transduced muMSC. HoxB4-GFP-muMSC exhibited no difference in expansion kinetics or differentiation potential compared with non-transduced muMSC. In conclusion, muMSC are more abundant in bone compared with bone marrow but maintain the properties described for human bone marrow derived MSC. The muMSC isolated in this manner are suitable for preclinical studies in a murine model.

127

Proteolytic activities of the proteasome in chronic myeloid leukaemia

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The proteasome is a multicatalyic enzyme complex that plays a key role in cell cycle regulation. It has three distinct peptidase activities: chymotrypsin-like (CT-L), trypsin-like (T-L) and postglutamyl peptide hydrolase (PGPH). The aims of this study were to measure these individual proteolytic activities in a murine model for chronic myeloid leukaemia (CML), human CML cell lines and primary human cells. Proteasome was extracted in a DTT/ATP lysis buffer. Individual proteolytic activities were measured by rate of turnover of specific fluorogenic substrates (Succ-Leu-Leu-Val-Tyr-AMC, Z-Ala-Arg-Arg-AMC and Z-Leu-Leu-Glu-AMC for CT-L, T-L and PGPH respectively) and expressed as arbitrary fluorescence units (AFU)/50 μg protein/min. Addition of the novel proteasome inhibitor Bz-Leu-Leu-Leu was used to confirm reaction specificity. Proteasome activities were measured in an FDCP-Mix murine haematopoietic stem cell line transfected with a temperature sensitive Bcr-Abl gene. All three activities were higher in the Bcr-Abl positive cells compared with Bcr-Abl negative controls (CT-L 316 ± 26 vs. 163 ± 22, T-L 645 ± 97 vs. 393 ± 152 and PGPH 1169 ± 97 vs. 837 ± 29 AFU/50 μg protein/min) with PGPH highest and CT-L lowest ($n = 3$). Addition of Bz-Leu-Leu-Leu caused >95% inhibition of all three activities. Three human CML cell lines were studied: K-562, LAMA-84 and MEG-01. In all lines, T-L activity was highest and CT-L lowest, e.g. for LAMA-84 cells: CT-L 223 ± 6, T-L 1296 ± 134 and PGPH 683 ± 97 AFU/50 μg protein/min ($n = 3$). Addition of Bz-Leu-Leu-Leu caused >95% reduction in T-L and PGPH. Proteasome activity in one newly diagnosed CML patient was similar to Bcr-Abl positive cell lines while levels in three normal subjects were significantly lower and comparable with Bcr-Abl negative cells. First generation proteasome inhibitors such as Bortezomib target the CT-L peptidase of the proteasome. This study indicates future potential for targeting the T-L and PGPH proteases.

128

Are we getting as much information as possible from bone marrow biopsies?

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Immunocytochemical definition of 'macroscopic' microscopic disease visible in H&E stained sections of bone-marrow biopsies is now a routine process. However, haematologists are frequently looking for minimal evidence of malignant disease or other disease processes to account for cytopenias or other phenomena. In this paper, we provide data from four instructive biopsies in which very small, but clinically significant, populations of cells were shown although H&E/Giemsa

Samsung Bioepis Exhibit No. 1002
Page 5063

stained sections did not suggest their presence and currently would not routinely have been investigated further. The populations exposed were: (i) a tiny λ light chain-restricted population of IgD positive small lymphocytes in a patient with λ light chain amyloidosis; (ii) a relatively large polyclonal T lymphocytosis in a patient with pure red cell aplasia; (iii) a large population of CD20 positive lymphocytes of which one-third were CD5 positive in a patient with unexplained neutropenia; (iv) a tiny, but significantly increased, population of IgD positive plasma cells in the iliac crest biopsy from a patient with 'plasmacytoma' of cervical vertebrae. We conclude that aggressive investigation of bone marrow biopsies is warranted to determine the presence of 'clones' either possibly responsible for symptomatology (and possibly influencing their treatment) or for determining the presence of minimal neoplastic disease. Only by building a body of knowledge from such investigations will it be possible to determine fully and accurately the exact significance of these populations in individual patients in the future.

129

Detection in serum of tumour-derived antigen receptor DNA in chronic lymphoproliferative disorder patients

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Detection of minimal residual disease in haematological malignancies is best achieved by PCR-based molecular or flow cytometric techniques. Molecular studies have usually involved isolation of mononuclear cells from blood or bone marrow aspirate samples, followed by extraction of DNA and subsequent molecular analysis. This study was designed to investigate previous reports suggesting that tumour DNA may be detectable in the plasma or serum of patients with active disease. Tumour-derived antigen receptor DNA in serum, and DNA extracted from peripheral blood mononuclear cells, were analysed in 56 patients with B ($n = 54$) or T ($n = 2$) cell chronic lymphoproliferative disorders. DNA was amplified using PCR and analysed using polyacrylamide gel electrophoresis and a fragment analyser with Genescan software. In all 56 patients, clonal antigen receptor gene rearrangements were detected in DNA extracted from peripheral blood mononuclear cells. In 49 of the 56 (87%) serum samples, an antigen receptor gene clone was demonstrated. Twenty-nine of the 49 positive serum clones were identified using polyacrylamide gel electrophoresis, while the remaining 20 were detected using Genescan. Degradation of clonal tumour-derived DNA *in vitro* at room temperature reduced the sensitivity of the assay. Fresh serum gave optimal results. Biochemical and immunophenotypic markers for tumour load and active disease were not found to be statistically significantly different in patients in whom clonal tumour-derived DNA was detected in serum, either by polyacrylamide gel electrophoresis or Genescanning, and those in whom no clonal DNA was detected in serum. The use of Genescan in this study has significantly enhanced sensitivity, and hence accuracy, compared to previous studies. The use of PCR to detect tumour-derived DNA in serum is time conserving and could have benefits in diagnostic screening and for detection of minimal residual disease or prediction of clinical relapse.

130

Effect of calcium on bone marrow-derived stromal cell growth and differentiation

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Bone marrow-derived stromal cells (BMdSCs) were isolated from the mononuclear cell fraction by plastic adherence and were grown and passaged in α-modified minimal essential medium (α-MEM) *in vitro*. When subconfluent, these cells were induced to differentiate down the osteogenic lineage in α-MEM, which has a relatively high calcium concentration of 200 mg $CaCl_2$/l. Induction involved the widely used differentiation protocol: dexamethasone (0.1 μmol/l), ascorbic acid-2-phosphate (50 μmol/l) and sodium β-glycerophosphate (10 mmol/l). We found that within several days of induction in α-MEM (variable between cultures), scattered mineral deposits began to form over the cell layer. These deposits gradually became so dense that in <21 d of induction (sometimes as little as 7 d) the cells had begun to break up, releasing the cytoplasmic contents. This made it difficult to analyse bone lineage gene expression quantitatively for >21 d as the extractable levels of RNA from differentiating BMdSC decreased as the mineralization in culture increased. We have therefore examined the effects of calcium concentration on stromal cell growth and differentiation with a view to limiting the rate of mineralization by reducing the medium calcium concentration. An alternative strategy is also being investigated by varying the concentration of sodium β-glycerophosphate. The addition of calcium chloride (1.56–400 mg/l) to stromal cell growth in α-MEM resulted in increased growth to 400 mg/l. On average, a twofold reduction in calcium concentration resulted in <20% reduction in stromal density. It was therefore possible to reduce the calcium concentration without seriously compromising stromal cell growth. Reducing the concentration of calcium chloride in α-MEM slowed the rate of mineralization in cultures allowing for RNA extraction for >21 d.

131

Acetyl carnitine stimulates *in vitro* myeloid progenitor growth

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It has been reported that erythroid colony formation is stimulated in cultures of fetal mouse liver cells by carnitine derivatives (Matsumura *et al* Am. J. Nephrol. 1998; 18: 355). This prompted us to check the effect of carnitine on our erythroid and myeloid clonal assays. Carnitine is involved in transporting long chain fatty acids across membranes for oxidative catabolism and for incorporation of lipid material into membranes. Carnitine also reduces the *in vitro* toxicity of glutamine-derived ammonia; and has been investigated as a possible treatment for anaemia in patients undergoing haemodialysis. As carnitine is only synthesized in liver, kidney and brain cells it is likely to be deficient in cultured haemopoietic cells, especially under serum-free conditions. Our serum-free assay of burst forming units erythroid (BFU-E) from normal peripheral blood was unaffected by acetyl carnitine levels between 0.3 and 10.0 μmol/l, in combination with optimal (2 U/ml) or suboptimal (0.5 U/ml) concentrations of erythropoietin (carnitine = 0 μmol/l, mean = 171 BFU-E/ml blood, 95% confidence range = 102–287; carnitine = 2.5 μmol/l, mean = 192, 138–267; $n = 15$). The growth of normal peripheral blood myeloid progenitors from the same patients (cultured with 10% FCS) was significantly stimulated by acetyl carnitine at all concentrations between 0.3 and 10.0 μmol/l ($P < 0.001$ at 5.0 μmol/l; $n = 9$). Colony forming unit granulocyte macrophage (CFU-GM) numbers were more than doubled with 2.5 μmol/l acetyl carnitine (mean = 30.2 CFU-GM/ml blood; 95% confidence range = 17.3–52.6; $n = 9$) compared with no addition (mean = 12.7; 4.9–32.9; $P < 0.01$, $n = 9$). The reason for the unexpected difference in the response to acetyl carnitine of myeloid and erythroid progenitors has yet to be resolved. The specific mechanism of myeloid stimulation is being further investigated. In the meantime, we have a useful increase in the myeloid clonal assay sensitivity.

Samsung Bioepis Exhibit No. 1002
Page 5064

132

Improving the accuracy of detection of t(11;14)(q13;q32) and t(14;18)(q32;q21) chromosomal translocations by PCR

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The polymerase chain reaction is commonly used to identify chromosomal translocations for diagnostic purposes. A translocation is generally considered to be present upon amplification of a product of a similar size to the positive control. However, non-specific products may also be generated using PCR, in particular when using nested reactions to amplify from low templates. If these non-specific products are of a similar size to the positive control then it may be difficult to distinguish them from genuine translocation products, using agarose gel detection. It may also be difficult to determine if the product is genuine if it is significantly different in size from the positive control. We report the use internal control primers and sequencing to improve the accuracy of detection of t(14;18)(q32;q21) and t(11;14)(q13;q32) translocations. Samples were analysed from patients with suspected non-Hodgkin's lymphoma over a 5-year period (1997–2003). Internal control primers were introduced into published t(11;14)(q13;q32) and t(14;18)(q32;q21) translocation PCR reactions. Sequencing was employed to distinguish between translocation and non-specific PCR products. The number of non-specific products was decreased but not eliminated using internal control primers. The sizes of t(11;14)(q13;q32) and t(14;18)(q32;q21) PCR products amplified from patient samples were often very different in size from the positive controls, with products varying by up to 700 base pairs (bp). Confirmatory sequencing was required in 65% of cases, of which 70% were found to contain genuine translocation products. Genuine t(11;14) translocation products ranged in size from 234 to 934 bp and 143 to 560 bp for the MTCA and MTCB breakpoints respectively. Similarly two t(14;18) MCR products sequenced were found to be genuine despite the products being only 219 and 237 bp, in contrast to the positive control which spanned 498 bp. This data highlights the importance of using internal control primers and sequencing to accurately identify genuine translocation products.

Poster Presentation

Haemostasis & Thrombosis and Anticoagulation

133

Acute endothelial cell activation in the pathogenesis of severe and cerebral malaria

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Plasmodium falciparum malaria is a major cause of death in African children. Clinical features of severe and cerebral malaria are well described, but the pathogenesis remains unknown. Sequestration of malaria-infected erythrocytes in the peripheral circulation has been implicated, and *in vitro* studies have shown parasitized erythrocytes adhere to endothelial cells (EC). To investigate EC activation in severe malaria, we studied 87 Ghanaian children with severe ($n = 73$) or cerebral ($n = 14$) malaria, compared with 24 febrile but malaria-negative OPD controls. Plasma VWF:Ag and FVIII:C levels were both increased in severe malaria (medians 3.41 and 2.39 IU/ml), compared with malaria-negative controls (medians 1.21 and 1.5 IU/ml). However, the FVIII:VWF ratio was significantly reduced in malaria (0.7), a finding consistent with EC activation. VWF-propeptide levels provide a more specific measure of acute EC perturbation, and were markedly elevated in cases of severe malaria compared with controls (medians 25.3 and 10.4 nmol/l; $P < 0.001$). The highest VWF-propeptide levels were found in children with cerebral malaria (28.1 nmol/l) and in those children who died. Lactic acidosis (>5 mmol/l) is a reliable marker of malaria severity, and in children ($n = 20$) with lactic acidosis VWF-propeptide levels were significantly higher (medians 31.7 and 21.3 nmol/l; $P < 0.001$). In cerebral malaria ($n = 14$), follow-up samples demonstrated a rapid fall in propeptide (to 18.3 nmol/l at 24 h, and 10.9 mmol at 72 h) concordant with clinical recovery. In contrast, VWF:Ag and FVIII:C levels had increased further by 24 h, but declined by 72 h. Although VWF and FVIII are acute phase reactants, CRP values were normal in 73% of cases, and demonstrated no correlation with VWF-propeptide levels. In conclusion, acute EC activation is a hallmark of severe malaria and correlates with disease severity. Further studies are required to investigate the role of HMW-VWF multimers, VWF-propeptide, P-selectin, or other Weibel-Palade body components, in the pathogenesis of cerebral malaria.

134

Prospective evaluation of a new algorithm for diagnosis of pulmonary embolism

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There is a lack of consensus regarding the optimal diagnostic strategy for pulmonary embolism (PE). We assessed the utility of a new diagnostic algorithm for PE at King's. All subjects with suspected PE were initially assessed using clinical pretest probability (PTP), plasma D-dimer (DD) and CXR. Subjects with low PTP and negative DD (<200 mg/l) were discharged without further investigation. In remaining subjects CXR result determined further imaging. Subjects with normal CXR underwent ventilation-perfusion (VQ) scan, requiring subsequent spiral CT (CTPA) only if results were non-diagnostic. Subjects with abnormal CXR underwent CTPA, requiring further investigation with lower limb venous duplex or pulmonary angiography only if results were equivocal. Over a 12-month period, we prospectively assessed 437 patients (62% female) with suspected PE (mean age 56.8 years, SD 19.5). PTP scores were as follows: 15.3% high, 81.2% moderate and 3.5% low. DD level (range 14–9807 mg/l, median 599 mg/l) was >200 mg/l in 90.8% of subjects and CXR was abnormal in 57.2% subjects. VQ scan was performed in 42.1% (60.9% low probability, 29.3% intermediate probability and 9.8% high probability scans) and CTPA in 59.7% of subjects (72.4% normal, 3.8% equivocal, 23.4% positive and 0.4% failed) with 10.1% having both types of scan. Of subjects selected for CTPA, 75.5% had an abnormal presentation CXR; of those having VQ scan 84.2% had a normal presentation CXR; 18.9% of subjects were diagnosed as having PE (66.3% by CTPA and 22.9% by VQ, 9.6% both). In PE subjects, CXR was abnormal in 57.8%. DD levels of <200 mg/l had a 100% negative predictive value (NPV) for PE (range 201–8265 mg/l, median 1227 mg/l). Low PTP had a 92.3% NPV for PE (1.2% low PTP, 75.9% moderate PTP and 22.9% high PTP). Use of such an

Samsung Bioepis Exhibit No. 1002
Page 5065

algorithm helps streamline PE diagnosis, improves diagnostic accuracy and allows rational and efficient use of imaging resources.

135

Potentiation of fibrinolysis by inhibition of thrombin activatable fibrinolysis inhibitor in whole human blood

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Exogenous tissue plasminogen activator (t-PA) fails to achieve reperfusion of the infarct related artery in *c.* 30% patients presenting with acute myocardial infarction. Thrombin-activatable fibrinolysis inhibitor (TAFI) is a carboxypeptidase present in human plasma that inhibits t-PA mediated fibrinolysis in animal models of coronary artery occlusion. Investigation of the role of TAFI in man has been limited by the lack of a suitable bioassay. Thromboelastography (TEG) is a potential method for assessing clot dynamics *in vitro* through changes in the viscoelastic properties of whole blood over time. Using TEG of human whole blood from healthy volunteers ($n = 5$ for each study), we investigated the effects of potato carboxypeptidase inhibitor (PCI), a specific inhibitor of TAFI, and tranexamic acid (TA), a competitive inhibitor of plasminogen activation, on t-PA mediated clot lysis. Incremental doses of recombinant t-PA (0–560 ng/ml) were applied with saline placebo, PCI (27 μg/ml) or tranexamic acid (420 ng/ml) during tissue factor initiated TEG and the percentage of clot lysed at 60 min was recorded. In the presence of placebo, t-PA produced a significant dose-dependent increase in clot lysis in all studies ($P < 0.001$). PCI or TA alone did not alter clot lysis. The presence of PCI or TA, caused left and rightward shifts respectively in the dose response curve for t-PA mediated lysis ($P < 0.001$). The mean concentrations of t-PA producing 50% clot lysis in the presence of PCI, t-PA alone or TA were 160 (130–190) ng/ml, 220 (186–268) ng/ml and 372 (125–1111) ng/ml respectively. Thromboelastography is a suitable bioassay in which to assess inhibitors and adjuncts of t-PA mediated lysis in human whole blood. Tissue plasminogen activator-mediated clot lysis is potentiated by PCI, a specific inhibitor of TAFI, and inhibited by TA suggesting functional activity of TAFI in human whole blood. Inhibition of TAFI may be a novel adjuvant to thrombolytic therapy in man.

136

Low incidence of high titre FVIII inhibitors in previously untreated children with severe haemophilia A treated only with intermediate FVIII concentrate (BPL 8Y). Experience from five comprehensive care centres in the UK

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Development of high titre FVIII inhibitors in severe haemophilia A (SHA) remains the most significant complication of replacement therapy. It remains controversial whether the type of FVIII concentrate used influences the development of FVIII inhibitors in SHA. Previous reports have shown a low incidence of inhibitors in children who had only received an intermediate purity FVIII concentrate (BPL 8Y). The aim of the current study is to obtain data on BPL 8Y use in relation to the incidence of high titre inhibitors (>5 BU/ml) and underlying FVIII gene mutations. Seventy-four children with SHA, from five centres, were treated solely with BPL 8Y as the first FVIII concentrate. The median age at first exposure to BPL 8Y was 12 months (range 0.5–70 months) and median duration of treatment was 78 months (range 15–149 months). In this cohort, only two high titre inhibitors developed (an incidence of 2.7%). This compares with an incidence of high titre FVIII inhibitors of between 11.3% and 18% seen in three studies of recombinant FVIII concentrate use in previously untreated children. Although molecular analysis remains incomplete, 26 of 54 (48%) individuals tested possess the intron 22 inversion. In a further 20 individuals, an underlying mutation in the FVIII gene has been detected. A review of the Hamsters FVIII database revealed there was information on inhibitor development for the mutations in 12 individuals, of which the mutations in six individuals had been associated with FVIII inhibitor development. ITI has failed to provide long-term tolerization in the two individuals with FVIII inhibitors. The underlying FVIII gene mutation has been identified in one of the inhibitor patients: this nonsense mutation (C1336T) is associated with inhibitor development. In conclusion, the use of BPL 8Y was associated with a low incidence of high titre FVIII inhibitors.

137

Circulating endothelial cells predict outcomes in patients with acute coronary syndrome: relationship to plasma indices of endothelial damage/dysfunction and interleukin-6

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It is well recognized that markers of inflammation and coagulation as well as endothelial activation are altered in patients with acute coronary syndrome (ACS). The aim of this study was to compare the short-term prognostic value of interleukin-6 (IL-6, an inflammatory marker), von Willebrand factor levels (VWF, an index of endothelial damage/dysfunction) and circulating endothelial cells (CECs, indicating endothelial damage) in patients presenting with ACS. A total of 156 patients (107 males, 64 ± 12 years) consecutively admitted to the coronary care unit with ACS were included. Blood was drawn within 24 h of admission (T1) and 48 h thereafter (T2) for plasma levels of VWF and IL-6 (ELISA). CECs were quantified using epifluorescence microscope after immunomagnetic separation. Baseline values were compared with 35 matched patients with stable coronary heart disease (CHD) and 35 healthy controls. Levels of VWF, IL-6 and CECs were significantly higher in ACS patients compared with stable CHD and controls (all $P < 0.01$). CECs, VWF and IL-6 were highly correlated with each other (all $P < 0.001$). Forty-eight patients had a major adverse clinical event (MACE; 10 deaths, 33 refractory angina, five readmitted with ACS) at 30 d. CECs and IL-6 levels at T1 and T2, VWF levels at T2 and changes of CECs, VWF and IL-6 levels over 48 h of admission were strongly and significantly correlated with 30-d MACE (all $P < 0.002$). CECs at T2 were the strongest independent predictor for 30-d MACE, followed by the changes of IL-6 and VWF levels over 48 h (all $P < 0.05$). CECs level at T2 was the only single independent predictor of death at 30 d ($P = 0.007$). Raised plasma levels of VWF, IL-6 and numbers of CECs during the first 48 h of ACS were strongly associated with MACE at 30 d. CECs levels at 48 h were the only independent predictor of both death and MACE at 30 d, indicating the crucial role of endothelial/vascular damage in the pathogenesis of ACS.

Samsung Bioepis Exhibit No. 1002
Page 5066

138

Effects of hypobaric hypoxia on platelet activation and reactivity

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Links between air travel and deep vein thrombosis (DVT) have been widely reported but it is unclear if specific factors in the aeroplane cabin increase the risk over that of seated immobility at ground level. The role of platelets in the pathogenesis of DVT is also uncertain, although aspirin has been advocated as prophylaxis. We investigated the effects of hypobaric hypoxia, similar to that which might be experienced during a commercial long-haul flight, on platelet activation and reactivity. Thirty-one healthy volunteers sat for 8 h in a hypobaric chamber and were exposed to normobaric normoxia (ground level) or hypobaric hypoxia (equivalent to an altitude of 8000 feet). Subjects were exposed to both conditions, in a double-blind randomized fashion, 1–2 weeks apart. Blood was taken before and within 10 min after exposure. Whole blood flow cytometry was performed to measure platelet response *ex vivo* [percentage of platelets binding FITC-fibrinogen (Dakocytomation)] following activation with ADP (1×10^{-6} mol/l; Sigma) and TRAP (1×10^{-6} mol/l; in-house) and platelet activation *in vivo* [percentage of leukocyte-platelet aggregates (LPA), separating leukocyte populations by forward and side scatter characteristics and the presence of platelets by anti-CD42b antibodies (BD Pharmingen)]. There was no significant change in fibrinogen binding to platelets following either normobaric or hypobaric exposures, in response to ADP (69.3 ± 12.3% vs. 67.3 ± 12.5%, $P = 0.11$, and 69.6 ± 10.6% vs. 70.0 ± 12.1%, $P = 0.93$ respectively) or TRAP (4.36 ± 1.6% vs. 5.33 ± 3.1%, $P = 0.15$, and 4.55 ± 1.7% vs. 4.71 ± 2.07%, $P = 0.41$ respectively). The percentage of LPA increased after both normobaric (monocytes: 9.15 ± 1.3% vs. 10.16 ± 1.4%, $P < 0.0001$; lymphocytes: 8.51 ± 1.3% vs. 9.21 ± 1.4%, $P < 0.0001$; granulocytes: 8.44 ± 1.3% vs. 9.20 ± 1.4%, $P < 0.0001$) and hypobaric (monocytes: 10.1 ± 3.3% vs. 10.43 ± 2.5%, $P = 0.708$; lymphocytes: 8.88 ± 1.6% vs. 9.71 ± 2.0%, $P = 0.004$; granulocytes: 8.59 ± 1.7% vs. 9.49 ± 2.1%, $P = 0.0002$) exposures but there was no significant difference in the change between the two conditions. In conclusion, mild hypobaric hypoxia has no effect on platelet activation or reactivity.

139

Thrombin generation and correlation with bleeding tendency in inherited FXI deficiency

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Factor XI (FXI) deficiency is characterized by a variable bleeding tendency but routine coagulation assays do not readily identify individuals at risk of excessive haemorrhage. We have developed and evaluated a thrombin generation assay in a group of 23 patients with FXI deficiency with well-defined bleeding phenotypes and in a subgroup of patients treated with recombinant factor VIIa (rFVIIa). Defibrinated plasma was incubated with phospholipid and coagulation initiated with a low concentration of recombinant tissue factor (10 pg/ml) and $CaCl_2$. Subsamples of the reaction mix were withdrawn into a stop solution at 30–60 s intervals for 15–30 min and thrombin generation determined using the chromogenic substrate (S2238). Study participants consisted of 12 patients with severe deficiency of FXI and 11 with partial deficiency. Significantly less thrombin generating potential was observed in severely deficient plasmas compared with partially deficient plasmas, with a median *AUC* of 1574.2 U/ml vs. 3199.5 U/ml (ranges 1260.4–3119.4 U/ml and 1402.5–5354.1 U/ml; $P = 0.0062$). The median peak thrombin was also less in severely deficient plasmas at 1.95 U/ml vs. 6.5 U/ml [ranges 1.4–4.3 U/ml and 3.7–12.8 U/ml; $P < 0.0001$]. There was a non-significant trend for higher *AUC* and peak thrombin in non-bleeders *versus* bleeders (median *AUC* 1790.9 U/ml vs. 1357.6 U/ml and 3480.6 U/ml vs. 2322.1 U/ml in severely and partially deficient plasmas). Six patients received rFVIIa to cover elective surgical procedures. A marked increase in the *AUC* and peak thrombin (median *AUC* 2147.55 U/ml vs. 3804.5 U/ml, median peak thrombin 4.9 U/ml vs. 11.5 U/ml) was seen after the first dose of rFVIIa. These preliminary results suggest that the thrombin generation test can differentiate severe and partial FXI deficiency and may be effective in identifying patients with a bleeding tendency. Treatment with rFVIIa normalizes thrombin generation. A continuous thrombin generation assay is currently under development to investigate the relationship between bleeding tendency and thrombin generation in a larger patient group.

140

Successful use of magnetic resonance direct thrombus imaging (MRDTI) and inferior vena cava (IVC) filter insertion in pregnancy and the puerperium

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Magnetic resonance direct thrombus imaging (MRDTI) is a new imaging technique. It allows assessment of deep vein thrombosis (DVT) and pulmonary emboli (PE) as a single investigation. Intravenous contrast is unnecessary and there is no radiation exposure. This makes its use appealing, particularly in pregnancy. inferior vena cava (IVC) filters have a limited but important role in the treatment of venous thromboembolism (VTE). Filter usage in pregnancy and the puerperium is not widely reported. We present a series of three women who developed VTE in either the third trimester or puerperium. Standard management with therapeutic heparin failed. Two patients presented with DVT. Despite therapeutic anticoagulation they developed PE. The third patient had bleeding that necessitated minimization of anticoagulation. MRDTI accurately evaluated both DVT and PE. All three patients had IVC filters inserted. In the two patients with recurrent VTE despite anticoagulation MRDTI delineated thrombus in both the pulmonary vessels and the proximal extent of clot in the pelvic veins. In pregnancy visualization of pelvic thrombus with ultrasound is difficult owing to the presence of a gravid uterus. One patient received a permanent filter, the other a temporary one. The third patient presented with chest symptoms. Ventilation/perfusion scanning gave an intermediate probability of PE. Definitive diagnosis was made by MRDTI. She developed a rectus sheath haematoma. A temporary filter was inserted and the baby was delivered safely by caesarean section. The filter was later removed without complication. We believe MRDTI is the gold standard imaging modality for VTE in pregnancy. These cases illustrate a role for IVC filters in pregnancy and the puerperium. Long-term follow-up data in this setting is currently lacking. Given the rarity of these events a national database would be of value.

Samsung Bioepis Exhibit No. 1002
Page 5067

141

Circulating platelet microparticles in patients with antiphospholipid antibodies

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Platelet microparticles (PMPs) are released upon platelet activation that are prothrombotic. The aim of this study was to assess PMPs in the pathogenesis of thrombosis in patients with antiphospholipid antibodies (aPL). Ethical committee approval was obtained. PMP levels were measured using both a functional assay of PMP procoagulant activity, and flow cytometry assay. There were 20 healthy controls (median age 27 years, range 20–62 years), and 17 patients with aPL (median age 42 years, range 29–74 years). All patients with aPL had a positive lupus anticoagulant or anticardiolipin antibody on at least two occasions 8 weeks apart. Fifteen had previous thrombotic events and were using oral anticoagulation. Blood was collected by flawless venepuncture into trisodium citrate. PMPs were detected by flow cytometry (Becton Dickinson) using CD42b-PE (PharMingen) as a platelet marker and annexin V-FITC (PharMingen). Platelet-derived procoagulant activity of the following plasma fractions; PRP (platelet rich plasma), PPP (platelet poor plasma), 0.1-μm filtered PFP (particle free plasma) and 0.2-μm filtered PFP was assayed by Russells Viper Venom Test (RVVT) on the ACL300R (IL, UK Ltd.). Results were compared with RVVT times of doubling dilutions of brain phospholipid (Diagen) in filtered pooled plasma. Statistical analysis was performed using the Mann–Whitney test. All patients receiving anticoagulant therapy had significantly prolonged RVVT times in comparison to normal controls, making this method unsuitable for determining the procoagulant activity of PMP. The PMP levels in patients with aPL were significantly higher (median 1.72, range 0.59–3.72) than normal controls (1.03, 0.19–2.29) ($P = 0.02$). Thus PMPs may play a role in the pathogenesis of thrombosis in antiphospholipid syndrome. Unfortunately, the procoagulant activity of PMP using RVVT is not reliable in patients receiving oral anticoagulation.

142

Defective fibrinolysis in children with severe haemophilia A

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In addition to the coagulation defect in children with haemophilia A, there is increasing evidence that they have abnormal regulation of fibrinolysis compared with non-haemophiliacs, which is thought to result from reduced generation of activated TAFI because of an insufficient thrombin burst at the initiation of coagulation. We present clot lysis data from 19 children (aged 3 months to 15 years) with severe haemophilia A (FVIII <1%), 14 untreated and another five treated 9–48 h previously. Platelet poor plasma, tPA (1.43 nmol/l), Hepes buffer and either BSA or recombinant FVIII (final concentration *c.* 1 IU/ml) were combined and then a thrombin/calcium chloride mixture added before pipetting in triplicate onto a microtitre plate and reading absorbance at 405 nm at 10–15-min intervals until clot lysis was complete. The time to 50% clot lysis was calculated graphically. Children with baseline FVIII levels had shorter lysis times (mean ± SD, 59.9 ± 8.9 min) compared with 10 normal adults (85.4 ± 23.9 min); this was corrected with *in vitro* FVIII concentrate (88.0 ± 15.3 min, $P < 0.000$ post-FVIII). We found no significant correction of the lysis times with *in vitro* addition of rVIIa (Novo Nordisk). Two children given FVIII <12 h previously had lysis times greater than those of normal plasma (mean = 119.5 min vs. 85.4 min for normal plasma) and lysis was only prolonged marginally by further *in vitro* FVIII. Three children who received FVIII 48 h previously had lysis times similar to those with baseline FVIII levels. Severe haemophilia A is associated with shortened clot lysis times which are prolonged by addition of FVIII, an effect which is maintained *in vivo* for up to 12 h, but with this assay more than a trace amount of FVIII is needed to have an impact on the clot lysis time.

143

A second cohort of patients with coronary heart disease and reduced VWF cleaving protease activity

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In a previous study (Br. J. Haematol. 2003; 121(Suppl. 1): 21–22), we found a mild reduction of von Willebrand factor cleaving protease (VWFcp), using a collagen binding assay, in four of 26 acute coronary syndrome patients. We now present a separate cohort of 33 patients (age 44–83 years) with ischaemic heart disease (stable angina), attending cardiology outpatients or having elective angiography. Twelve patients had previous MI, eight PTCA and nine CABG surgery. VWFcp was measured by an electrophoretic method based on the measurement of residual VWF multimers after incubation of patient plasma with pure VWF. Of 33 patients, nine had reduced VWFcp (median ratio 0.26, IQR 0.48 and normal range 0.80–1.25). Three of these showed a gross reduction (ratios: 0.02, 0.06 and 0.07), similar to that commonly seen in acute TTP. Mixing tests suggested the presence of an inhibitor in three of the nine patients with low VWFcp. There was no association between low VWFcp and clinical history or current medication. All patients were receiving 75 mg daily aspirin, but a large proportion showed apparent aspirin resistance as measured by the PFA-100. Six of the nine patients with reduced VWFcp had PFA-100 closure times <200 s using CEPI cartridges, although 13 of 24 patients with normal VWFcp ratios also had closure times <200 s. There was a trend towards shorter closure times and lower VWFcp with higher VWF:Ag values ($rs = -0.38$, $P = 0.03$; $rs = -0.4$, $P = 0.02$ respectively). The reduction in VWFcp confirms our previous findings and is in agreement with Yoo *et al* (2003), where 14 patients with stable coronary heart disease were found (using a method based on residual VWF:RCo activity) to have a mild reduction in VWFcp, as well as increased VWF:Ag. These results suggest that a large study of VWF function and metabolism in coronary syndromes is merited, to investigate the potential contribution of VWFcp in cardiovascular disease.

144

The combined use of D-dimers and diagnostic imaging in the exclusion of deep vein thrombosis in a nurse led outpatient service

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The use of D-dimers in the exclusion of venous thromboembolism is of maximal clinical benefit when incorporated into an algorithm in combination with diagnostic imaging. This approach has been adopted in our DVT clinic over the past 2 years to screen 3182 patients using the diagnostic scoring categories of Low, Intermediate and High probability as described by Wells *et al* (1997; 191: 3182) (6%) were categorized as low probability of which 100 (52%) were excluded from further testing with a negative (<500 μg/l FEU)

Samsung Bioepis Exhibit No. 1002
Page 5068

D-dimer (Biomerieux, UK). Those that were D-dimer positive (48%) went on to have ultrasonography with all but one being negative. Those in the high probability group (1186/3182, 37%) went straight to have ultrasonography with 333 being positive. Those that were negative (853) had a repeat scan in 1 week. This identified 25 more positive cases. In the intermediate group, 1805 (57%) were identified. Ultrasonagraphy and D-dimers were performed in all cases; 1662 (92%) of the patients were ultrasound negative with approximately 48% of those D-dimer negative. Repeat scanning was withdrawn in this category with <1% positive after 1 week. Of those that were D-dimer positive 23 of 870 (3%) were subsequently found to be positive on repeat so follow-up in this category is currently maintained. In the intermediate group, 138 (8%) were ultrasound positive of which 13 (9%) had a negative D-dimer. These figures give a negative predictive value of >98% for D-dimers in the intermediate probability group, similar to the recent medical devices agency (MDA) recommendations. In conclusion, D-dimers in conjunction with a diagnostic algorithm have saved potentially 1231 of 4147 (30%) ultrasound investigations without adverse clinical outcome. Currently, the goal is to further evolve the categories similar to that of Wells *et al* (2003) limiting still further the numbers of ultrasounds that are performed.

145

Elevated factor VIII:C levels in patients with pulmonary hypertension. A marker of endothelial dysfunction?

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Patients with pulmonary hypertension are known to have endothelial dysfunction and an increased risk of thrombosis. We prospectively performed tests for thrombophilia over a 6-month period in patients referred with suspected pulmonary hypertension, up to 8 weeks prior to cardiac catheterization. Fifty-three patients (37F, 16M) were subsequently confirmed to have pulmonary hypertension; 11 patients had chronic thromboembolic pulmonary hypertension (CTEPH) and 42 had pulmonary arterial hypertension (PAH). Of the patients with PAH, 18 had primary pulmonary hypertension (PPH), 17 in association with connective tissue disease (CTD) and seven associated with other diseases. Standard thrombophilia tests were performed and the abnormalities identified were antithrombin deficiency in 5.6%, protein C deficiency 5.6%, protein S deficiency 13.2%, factor V Leiden 1.8%, prothrombin 20210A 3.8%, lupus anticoagulant 6.9%, IgG anticardiolipin antibodies 15.1%, IgM anticardiolipin antibodies 13.2% and anti-β_2 glycoprotein 1 in 1.8%. Factor VIII, a recently recognized prothrombotic risk factor, was measured using a two-stage assay. We also measured the FVIII:C in 103 healthy individuals. Patients with CTEPH and PAH had mean FVIII:C levels of 2.77 and 1.84 IU/ml, respectively, which were significantly higher than the mean in normal subjects which was 1.18 IU/ml ($P < 0.001$). Patients with CTEPH had significantly higher FVIII:C than the PPH (2.04 IU/ml) and CTD (1.74 IU/ml) groups ($P < 0.001$). There was no significant difference between the PAH subgroups themselves. Our normal range for FVIII:C is 0.52–1.84 and the percentage of patients with a result above the upper limit of normal were CTEPH 91%, PAH 45%, PPH 61% and CTD 41%. We conclude that elevated FVIII:C is the most frequent haemostatic abnormality in patients with pulmonary hypertension and although highest in CTEPH, it is elevated in all the other subgroups of PAH. Further work is required to determine if the high FVIII levels are causal in the pathogenesis of pulmonary hypertension or the result of endothelial dysfunction/damage.

146

Neutral endopeptidase inhibition potentiates bradykinin mediated vasodilatation and tissue plasminogen activator release in patients with heart failure on chronic angiotensin converting enzyme inhibitor therapy

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Angiotensin converting enzyme (ACE) and neutral endopeptidase (NEP) degrade kinins. ACE inhibition potentiates bradykinin mediated vasodilatation and endothelial release of the pro-lytic factor, tissue plasminogen activator (t-PA) in patients with chronic heart failure (CHF). We investigated whether additional NEP inhibition with thiorphan further augments the vascular actions of bradykinin in CHF patients on chronic ACE inhibitor therapy. Ten patients with CHF on chronic ACE inhibitor therapy received an intrabrachial infusion of thiorphan (30 nmol/min) or saline placebo for 3 h in a randomized, double blind, cross over trial. Thiorphan or placebo was co-infused with Lys-des-Arg9-bradykinin (B1 kinin receptor agonist; 1–10 nmol/min), bradykinin (B2 kinin receptor agonist; 30–300 pmol/min), atrial natriuretic peptide (10–100 pmol/min) and sodium nitroprusside (2–8 mcg/min) in random order. Bilateral forearm blood flow (FBF) was recorded using strain gauge plethysmography and plasma t-PA antigen determined using ELISA. There were no changes in heart rate, blood pressure or non-infused FBF or t-PA release during thiorphan or placebo infusion. Bradykinin, atrial natriuretic peptide and sodium nitroprusside caused dose dependent increases in infused FBF in all studies ($P < 0.001$). There were no significant changes in FBF during Lys-des-Arg9-bradykinin infusion. Bradykinin but not Lys-des-Arg9-bradykinin or atrial natriuretic peptide caused a dose dependent increase in t-PA antigen and estimated t-PA antigen release in all studies ($P < 0.001$). Compared with placebo, thiorphan augmented the increase in infused FBF, t-PA antigen and estimated t-PA antigen release ($P < 0.001$, $P = 0.076$, $P < 0.005$ respectively) to bradykinin but not atrial natriuretic peptide or sodium nitroprusside. In the presence of chronic systemic ACE inhibition, local NEP inhibition potentiates bradykinin mediated vasodilatation and endothelial t-PA release. Despite the results of recent trials of vasopeptidase inhibition, these findings support the hypothesis that combined ACE and NEP inhibition may afford greater cardiovascular protection than ACE inhibition alone.

147

Rebound elevation of factor VIII:C levels following successful treatment for acquired haemophilia: a risk factor for venous thrombosis?

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Acquired haemophilia A is a rare, life-threatening immune-mediated haemorrhagic disorder, due to autoantibodies against factor VIII (FVIII). After eradication of the autoantibody with immuno-modulatory treatment, FVIII:C returns to normal levels. Rebound elevation of FVIII:C following treatment has not previously been reported. We reviewed 13 cases of acquired haemophilia treated at our centre over 14 years (seven females, six males; age range 30–88 years). Of these, FVIII:C returned to normal (50–200 IU/dl) in seven patients. In four patients, FVIII:C rebounded to very high levels. Patient characteristics were: three females, one male; age range 35–87 years. All had pre-existing conditions known to be associated with acquired haemophilia: two had rheumatoid arthritis, one systemic lupus erythematosus and one chronic lymphocytic leukaemia. Immuno-modulatory therapy with oral prednisolone was given to all four, and additional agents including cyclophosphamide, vincristine and intravenous

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Samsung Bioepis Exhibit No. 1002
Page 5069

immunoglobulin were used in three cases. FVIII:C levels rebounded to between 307 and 616 IU/dl after 2–12 months of treatment. One patient, a male aged 56 years with rheumatoid arthritis and end stage chronic renal failure, developed a pulmonary embolus when his FVIII:C was 463 IU/dl. Elevated FVIII:C (FVIII:C>150 IU/dl) has been shown to be a risk factor for venous thrombosis. Very high levels may confer a substantially greater risk of thrombosis in a dose dependent fashion. Our review suggests that FVIII:C can rebound to very high levels after recovery from acquired haemophilia in some cases. This phenomenon may be associated with pre-existing autoimmune or iymphoproliferative disorders and could increase thrombotic risk.

148

Audit of the management of thrombotic thrombocytopenic purpura in adult patients in Scotland

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This audit aimed to review the management of adult patients with TTP in Scotland against guidelines produced by the British Committee for Standards in Haematology (2002). Data were collected by questionnaire and clinical note review as necessary. In Scotland in 2001, 23 patients were treated for a presumed diagnosis of TTP; 3 were subsequently given alternative diagnoses. The overall incidence was 4.1/million with a mean age of presentation of 50.3 years. Six patients died (this included all three with alternative diagnoses), two entered a partial remission and 14 had a complete remission with a mean follow-up of 6 months. There were three relapses. A normal coagulation screen in addition to the defined criteria of thrombocytopenia, red cell fragmentation and a raised LDH was found to be diagnostically important. All three cases of misdiagnosis had evidence of DIC at initial presentation. All patients were treated with plasma exchange, over 50% within 24 h of diagnosis. The median number of exchanges was 10, although there was large variability in exchange regimens, suggesting the need for comparative trials. Treatment complications occurred in 16 patients, which is significantly higher than anticipated by the guidelines. Infusions of fresh frozen plasma were useful as initial treatment pending transfer to a plasmapheresis centre and as maintenance therapy in two patients. The centralization of treatment in specialized centres throughout Scotland did not delay treatment. This audit has demonstrated the true incidence of TTP requiring treatment in Scotland. The sharing of knowledge has led to a review of the way in which TTP is managed and has enabled the introduction of standard apheresis protocols. The work is ongoing and the establishment of a Scottish TTP database will promote further evidence-based improvements in patient care.

149

Thrombophilia in the black population: results of the Camberwell Thrombophilia Study

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In the Camberwell Thrombophilia Study conventional thrombophilia testing was unrewarding in black subjects with venous thromboembolism (VTE) revealing an abnormality in only 9% black cases as opposed to 30% white cases. We previously identified elevated FVIII:C levels as a major risk factor for VTE in blacks. We now studied levels of fibrinogen, D-dimer and thrombin-antithrombin complexes (TAT) in 61 black patients with DVT (27 males, 34 females, mean age 56.2 years, SD 14.6) and 64 healthy black volunteers (nine males, 55 females, mean age 55 years, SD 14). Cases had completed standard anticoagulant treatment for objectively confirmed DVT at least 3 months previously. Controls had no known history of VTE, malignancy or intercurrent illness. Mean fibrinogen was significantly higher ($P = 0.048$) in cases (mean 3.19 g/l, SD 0.72, range 1.8–4.9 g/l) than controls (mean 2.94 g/l, SD 0.67, range 1.7–4.4 g/l). Fibrinogen level was independent of gender ($P = 0.10$) and age ($P = 0.11$). Odds ratio (OR) for DVT was significantly higher for subjects with fibrinogen in excess of 3 g/l (OR 2.41, 95% CI 1.11–5.30, $P = 0.025$) using levels less than 3 g/l as comparator. Median D-dimer was significantly higher ($P = 0.007$) in cases (median 179 ng/ml, interquartile range 86–432 ng/ml) than controls (median 101 ng/ml, interquartile range 54–203 ng/ml). OR for DVT of D-dimer levels >347 ng/ml (the 90th centile value for controls) was 4.3 (95% CI 1.48–13.5, $P = 0.005$). D-dimer levels were independently associated with FVIII:C ($P = 0.034$) in cases but not in controls. We could not demonstrate any association between fibrinogen and either D-dimer or FVIII:C in either cases or controls. D-dimer levels were associated with elevated TAT levels ($P = 0.03$) in cases but there was no association between TAT and either fibrinogen or FVIII:C level. In blacks elevated levels of coagulation proteins may constitute significant risk factors for thrombosis and should be considered when evaluating such patients for thrombophilia.

150

A UK survey of prophylaxis in paediatric patients with haemophilia

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A questionnaire was sent to all haemophilia centres in the UK to determine the use of prophylaxis in patients with severe or moderate haemophilia A or B aged <16 years on 30 September 2002. The ISTH definitions of severity of haemophilia and type of prophylaxis were given. Forty-one centres responded which included 17 of 21 Comprehensive Care Centres treating children. Eleven responders treated no children. A total of 753 children with severe or moderate haemophilia were identified; 82% had haemophilia A, of whom 78% were severe and 18% had haemophilia B, of whom 56% were severe. Seventy-three percentage of patients with haemophilia A were on prophylaxis, both primary and secondary, compared with 52% of patients with haemophilia B. The median frequency of dosing for both haemophilia A and B was ×3/week with a range of twice per week to daily. The median dose range of factor replacement was 25–40 units/kg for factor VIII and 25–50 units/kg for factor IX. Twenty-three centres monitored prophylaxis using trough levels, with fewer using half-life studies or recovery instead or in addition; three centres used clinical criteria alone; 27 centres (90%) used clinical outcome measures to judge efficacy whereas only 20% used the X-ray or orthopaedic scores. Prophylaxis was given via peripheral veins in 86% of children, although 28% had used a central venous access device at some point, usually for poor venous access. Carers administered factor in 71% and the patients themselves in 24%; 31% were not on prophylaxis, the most frequent reason being a mild phenotype. Compliance was the commonest reason for discontinuing prophylaxis. In the UK, 69% of those with moderate or severe haemophilia are on prophylaxis. In 95% of children, the carer or child gives the prophylaxis. Continued support is important to prevent non-compliance with such demanding treatment.

Samsung Bioepis Exhibit No. 1002
Page 5070

151

u-PA expression in normal myeloid differentiation and leukaemia

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The urokinase-type plasminogen activator (uPA) plays an important role in extra- and intravascular proteolysis. We have shown that u-PA is present in myeloid leukaemic cells and so can induce primary hyperfibrinolysis that may cause severe bleeding, particularly in APL patients. We have also shown that normal peripheral blood stem cells do not express functional u-PA but acquire this ability during the process of myeloid differentiation, suggesting that u-PA production by myeloid leukaemia cells may be a reflection of their stage of maturation and not aberrant gene expression. However, not all APL express functional u-PA. To study this further, we analysed cells from six APL patients and the HL-60 cell line (human-derived APL cell line). HL-60 cells were stimulated with 1.2% DMSO to induce granulocytic differentiation and examined at serial intervals. uPA antigen and activity were detected using ELISA or APAAP, SDS–PAGE/zymography and clot lysis assay and uPA mRNA using RT-PCR. PAI -1 antigen and activity were detected using ELISA and SDS–PAGE/reverse zymography. Results from the HL-60 cell line showed that uPA antigen and activity was detected in Hl-60 blasts and promyelocytes prior to and in the progeny following differentiation induction with 1.2% DMSO. The pattern of uPA mRNA expression paralleled that of uPA activity. PAI-1 antigen/activity was undetected. Mononuclear cells and plasma were obtained from six APL patients at presentation. u-PA antigen was detected in six of six and u-PA activity in four of six patients. Plasma PAI-1 was found to be within normal limits in all patients. These results suggest that the expression of u-PA antigen in APL is not dependent on the leukaemic process while the expression of u-PA function is more complex and may reflect upregulation of u-PA, high cell load or absence of functional inhibitor.

152

Protein Z concentrations in normal pregnancy

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Protein Z (PZ) is a vitamin K dependent glycoprotein that functions as a natural anticoagulant. We have previously shown that PZ concentrations are significantly reduced in subjects with antiphospholipid syndrome and may contribute to the thrombotic phenotype observed in this condition. Data suggest PZ deficiency may also play a role in fetal loss. However, data relating to PZ concentrations during normal pregnancy are lacking. In a prospective study, we assayed PZ concentrations in all three trimesters and 3 months following delivery in 40 healthy women. Mean maternal age was 28.4 (SD 5) years and BMI 28.6 (SD 6.4) kg/m^2. Mean PZ increased from 1.73 mcg/ml (SD 0.7) in the first trimester (T1) to 1.96 mcg/ml (SD 0.7, $P < 0.001$) in T2 and 1.9 mcg/ml (SD 0.8) in T3 ($P = 0.7$ vs. T2). Levels fell at 3 months following delivery to 1.3 mcg/ml (SD 0.5, $P < 0.001$ vs. T3; $P < 0.001$ vs. T1 and T2). There was no significant difference in non-pregnant PZ concentrations between lean (BMI ≤28 kg/m^2) and obese (BMI >28 kg/m^2) individuals. Mean PZ concentration was significantly higher in T2 and T3 in obese compared with lean women. However, mean PZ also increased significantly even in lean women during pregnancy. We conclude that PZ concentrations increase early in pregnancy. This may be a physiological response to control the hypercoagulability of pregnancy. Failure of this mechanism may predispose to a pro-thrombotic state explaining the association with poor pregnancy outcome.

153

An analysis of the β_2-glycoprotein I Cys306Gly polymorphism in patients with thrombotic and obstetric disorders

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The Cys306Gly polymorphism of β_2-glycoprotein I (B2GPI) results in the disruption of a disulphide bond between Cys281 and Cys306. These results in a change in the conformation of the fifth domain of B2GPI, a region crucial for the interaction of the molecule with negatively charged phospholipids. We have analysed the frequency of this polymorphism in 284 normal healthy controls and 290 consecutive patients referred to our clinic for investigation of a thrombotic episode or obstetric morbidity. Morbidity among the patients was distributed as follows: thrombosis (arterial and/or venous) 165; late pregnancy morbidity 55; recurrent/repeated miscarriage 86; miscellaneous 11; thrombocytopenia three (some patients had more than one clinical condition). Ninety-seven patients were persistently positive for antiphospholipid antibodies (APA) (anticardiolipin antibodies and/or lupus anticoagulant and/or anti-B2GPI antibodies). No significant differences were found between the APA positive and negative patient groups with regard to the frequency of the allele coding for the Cys306Gly polymorphism. This finding remained after analysis of patient subgroups by clinical presentation. In addition, no significant differences were observed between the normal control group and patients with thrombosis or late pregnancy morbidity. However, the frequency of the allele was increased in APA negative patients with miscarriage (7.35%) compared with female only controls (2.79%, $P = 0.01$, chi-squared test). This difference however failed to reach statistical significance when only Caucasian patients were considered (frequency in miscarriage APA negative group 6.9%, frequency in control group 2.9%; $P = 0.06$, chi-squared test). This effect was not seen in either case with APA positive patients. Our results suggest that the Cys306Gly polymorphism has little influence on the antigenicity of B2GPI with regard to the production of APA or does it affect the incidence of thrombosis. However, the polymorphism may increase the risk of miscarriage via a mechanism independent of the production of APA.

154

An analysis of the antiphospholipid antibody status of 111 women with late pregnancy morbidity

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Late pregnancy complications associated with the antiphospholipid syndrome include pre-eclampsia (PET) and placental insufficiency. We have analysed the clinical presentations and thrombophilia screen results of 111 such consecutive patients referred to our haematology department. Patients were considered positive for antiphospholipid antibodies (APA) only if they were positive on two separate occasions at least 6 weeks apart [tests were performed for anticardiolipin antibodies (aCL) of IgG and IgM class, anti-β_2-glycoprotein I (b2GPI) antibodies of IgG class and for lupus anticoagulant activity]. Clinical presentations were as follows: PET 59; intrauterine growth restriction (IUGR) 40; stillbirth 33; placental abruption 15 (some patients had more than one presentation). The patient's thrombophilia screens showed eight heterozygotes for the factor V Leiden mutation; one homozygote and 39 heterozygotes for the thermolabile variant of the MTHFR gene; two heterozygotes for the G20210A prothrombin gene mutation and two patients deficient for protein S. Sixteen of the

Samsung Bioepis Exhibit No. 1002
Page 5071

patients were found to be persistently APA positive: 11 had persistent IgG aCL (median titre 15 GPLU, range 5–85 GPLU); four persistent IgM aCL; two persistent lupus anticoagulant (LA) activity; four persistent IgG anti-b2GPI antibodies (median 26%, range 13–35%). Both of the LA positive patients were strongly positive for IgG aCL (>50 GPLU). Six of the APA positive patients were heterozygous for the thermolabile variant of the MTHFR gene and one was homozygous; no other additional thrombophilia makers were observed in the APA positive group. Eight of the APA positive patients had PET and one of these had suffered a cerebral infarction previously. Seven of the APA positive patients had IUGR (some overlap with PET cases). The prevalence of APA in women with late obstetric complications was 14.4% and the proportion of APA positive patients with PET in this cohort was 13.5%. The optimal haematological management of these patients requires a larger evidence base.

155

High pulse pressure but not mean arterial blood pressure is associated with abnormal thrombogenesis and endothelial damage/dysfunction in patients with coronary heart disease with and without hypertension

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Both pulse pressure (PP) and mean arterial blood pressure (MAP) have been implicated as independent predictors of cardiovascular (CV) risk. PP measures the pulsatile component, whereas MAP measures the steady component of BP. We hypothesized that high 24-h ambulatory PP in patients with coronary heart disease (CHD) is associated with an increased risk of haemostasis, which may be related to abnormal haemorheology (raised haematocrit, plasma viscosity and fibrinogen levels), endothelial damage/dysfunction (raised plasma von Willebrand factor, VWF), thrombogenesis (raised D-dimer, Dd) and platelet activation (raised soluble P-selectin, sPsel). We studied 72 patients with stable CHD who were in sinus rhythm and with preserved left ventricular systolic function (mean ejection fraction 61 ± 10%). All completed a 24-h ambulatory BP monitoring. Patients were divided into two groups according to high (above median) or low (below median) values of the ambulatory PP (51 mmHg). The two groups were not different in clinical and demographic characteristics. Twenty-four hours PP was positively correlated with age ($r = 0.48$), VWF ($r = 0.41$), Dd ($r = 0.25$), fibrinogen ($r = 0.3$) and sPsel ($r = 0.22$) levels (all $P < 0.05$; see table), but not with plasma viscosity or haematocrit. None of haemostatic indices correlated with 24-h MAP. On multivariate ANCOVA analysis, after controlling for age and MAP, VWF, fibrinogen, Dd and sPsel levels remained significantly elevated in patients with high 24-h PP (all $P < 0.05$; see table). High 24-h PP load in patients with CHD was associated with a prothrombotic state (with higher levels of fibrinogen, Dd and sPsel) and evidence of endothelial damage/dysfunction (VWF), independently of age. These may contribute to higher CV risk associated with increased PP.

	High-PP ($n = 37$)	Low-PP ($n = 35$)	P-value
Age (year)	63 ± 11	55 ± 9	<0.001
VWF (IU/dl)	212 ± 58	172 ± 49	0.002
D-dimer (mg/l)	0.35 (0.23–0.59)	0.24 (0.21–0.35)	0.004
Fibrinogen (g/l)	3.4 ± 0.8	3.0 ± 0.8	0.025
Soluble P-selectin (ng/ml)	87 (77–98)	78 (63–91)	0.034

Mean ± SD or median (IQR).

156

Impaired left ventricular diastolic function is associated with abnormal haemorheology, thrombogenesis and endothelial damage/dysfunction in patients with coronary heart disease

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Heart failure (HF) is associated with an increased risk of thromboembolic events. About 35% of patients with HF have normal left ventricular (LV) systolic function, with diastolic dysfunction as the underlying cause. We hypothesized that LV diastolic dysfunction is associated with an increased risk of haemostasis, which may be related to abnormal haemorheology [raised plasma fibrinogen (Fg) and plasma viscosity (PV)], endothelial damage/dysfunction (raised von Willebrand factor, VWF) and thrombogenesis (raised D-dimer, Dd). Seventy-two patients with CHD (58 males, 59 ± 11 years) who were in sinus rhythm and with preserved LV systolic function (mean ejection fraction 61 ± 10%) and 35 matched healthy controls (23 males, 57 ± 6 years) were studied. Transmitral pulsed Doppler indices [early/atrial (E/A) velocity ratio, E-wave deceleration time (EDT), isovolumic relaxation time (IVRT)] and pulsed Doppler Tissue imaging indices [peak systolic (S'), early and late diastolic velocities (E', A')] obtained at the septal and lateral mitral annuli were measured and related to plasma haemostatic markers levels. Patients with CHD have significant prolonged EDT (median 240 ms vs. 204 ms) and IVRT (149 ms vs. 125 ms), shortened S' (10 cm/s vs. 12 cm/s), E' (12 cm/s vs. 14 cm/s) and A' (12 cm/s vs. 13 cm/s) velocities with higher E/E' ratio (6.2 vs. 5.4) compared with controls (Mann–Whitney test, all $P < 0.05$). PV (median 1.7 vs. 1.6 mPa/s), Fg (3.3 g/l vs. 2.5 g/l), Dd (0.28 ng/ml vs. 0.18 ng/ml) and VWF (182 IU/dl vs. 120 IU/dl) levels were significantly higher in CHD patients than in controls (all $P < 0.01$). PV and Fg levels were correlated with S', E' and E/E' ratio (Spearman's $r = 0.3$, $P < 0.01$). VWF levels were correlated with E/A ratio ($r = 0.2$), EDT ($r = 0.3$), IVRT ($r = 0.3$), S' ($r = 0.4$) and E' ($r = 0.2$) (all $P < 0.05$). Dd was correlated with EDT ($r = 0.3$). On multivariate analysis, VWF was independently related to S' ($P = 0.03$) and IVRT ($P = 0.02$), whilst PV was independently related to E/E' ratio ($P = 0.03$). Diastolic LV dysfunction in patients with CHD is significantly associated with abnormal haemorheology, thrombogenesis and endothelial damage/dysfunction.

157

Platelet activation, coagulation and angiogenesis in breast and prostate cancer

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In health, haemostasis and angiogenesis are tightly regulated processes, but may become deregulated in cancer. Recent evidence suggests that platelet activation may link these processes as platelets can release angiogenic factors, such as vascular endothelial growth factor (VEGF). Recent data also implicate inflammation in regulating both coagulation and angiogenesis, possibly by activating platelets directly and increasing (e.g. fibrinogen). We hypothesized relationships between these plasma markers of the processes in two common forms of cancer. Plasma levels of VEGF (reflecting angiogenesis), soluble P selectin (sP-sel), marking platelet activation, tissue factor (TF), fibrinogen and D-dimer (coagulation markers), and serum levels of IL-6 (inflammation) were measured by ELISA in 30 patients with biopsy-proven breast cancer, 30 patients with biopsy-proven prostate cancer, and 30 age- and sex-matched controls per group. Prostate specific antigen was also measured in the men. Release of VEGF from IL-6 stimulated platelets was assessed by ELISA. Results were that plasma levels of IL-6 ($P < 0.02$), VEGF, sP-sel, fibrinogen and fibrin D-dimer (all $P < 0.01$)

Samsung Bioepis Exhibit No. 1002
Page 5072

were significantly raised in breast cancer, whereas plasma fibrinogen ($P < 0.05$), VEGF, sP-sel and fibrin D-dimer (all $P < 0.01$) were significantly raised in prostate cancer. Strongly significant correlations were found between IL-6 and VEGF in breast and prostate cancer, and between PSA and sP-sel in prostate cancer (all $P < 0.01$). There was an IL-6 dose-dependent release of VEGF from platelets. We conclude that strong relationships between IL6 and VEGF, but not with coagulation or platelet markers, and release of VEGF from IL-6 stimulated platelets, suggest a role for inflammation and platelets in angiogenesis.

158

Vaccino-associated immune thrombocytopenic purpura in childhood

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We observed 96 children aged 1–15 years with ITP, associated with vaccination, in the period of possible postvaccinal complications. ITP developed: in 25 children after rubella vaccination on the 15th day (in the average); in 17 children after DTP-vaccination on the 14th day; in 16 children after influenza vaccination on the 12th day; in 12 children after measles vaccination on the 10th day; in 10 children after hepatitis B vaccination on the 17th day; in nine children after poliomyelitis vaccination on the 11th day; in five children after mumps vaccination and in two children after BCG revaccination on the 9th day. The ITP in all patients was characterized with skin bleeds. Besides at 60% of the patients, the various bleedings (epistaxis, mouth bleedings, haematuria and menorrhagia) were marked. At 88% of children there was revealed the deep thrombocytopenia (10 × 10 milliards/l). The hardest ITP was marked after hepatitis B and influenza vaccination. At these patients in 90% and 75% of cases, accordingly, there were marked various bleedings. At the majority of the patients (73%) were revealed high level of antiplatelet antibodies. There was investigated a condition of general immunity in all patients, that marked on changes, characterized for infectious and autoimmune mechanisms. There also was researched the specific immunity in the majority of patients, that revealed higher level of antibodies to vaccin-associated infections, which required the exact connection of ITP and vaccination. Fifty-five patients received corticosteroid therapy and 35 received symptomatic therapy. Six children received IVIG. Among children received corticosteroid therapy complete recovery has come at 84% of the patients. Among children received symptomatic therapy the recovery has come at 45% of children. The efficiency of therapy IVIG was 66.7%.

159

Multi-centre survey of the management of antenatal venous thromboembolism in the UK and Ireland 2003–2004

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Antenatal venous thromboembolism (VTE) occurs in <1 in 1000 pregnancies (Simpson *et al* BJOG 2001; 108: 56–60); therefore, many hospitals manage fewer than five such patients per year. Most haematologists and obstetricians follow UK guidelines published by the RCOG and the BSH, some of which are extrapolations from level 1 evidence in non-pregnant patients. The dearth of randomized studies on VTE in pregnancy is partly due to the lack of any multi-centre organization to conduct such research. This multi-centre MREC approved study was set up after requests from many haematologists to share information on current diagnosis and treatment of antenatal VTE. An informal survey by the BSH Obstetric Haematology group confirmed that management varied widely, with different heparins, dosage regimes and monitoring protocols being used (Copplestone *et al* Br. J. Haematol. 2004; 124: 124–125). The primary objective of the study is to record current management of over 200 antenatal VTE patients in the UK and Ireland, from March 2003, using information sheets and data forms designed by participants and obtained from the website http://www.ukivtepsurvey.co.uk/. Forty-eight haematologists have signed up to date and the study is still open. Data are being submitted to a secure database for analysis in 2004 and subsequent publication. A secondary objective is to find out whether an on-line data collection survey of this type will be successful. If it is, this method of multi-centre audit of the management of rare conditions will be a cost-effective tool for future multi-centre work. Having established a national group interested in this clinical area, overcome problems in setting up the study and demonstrated that such a survey can be conducted mainly by e-mail, we will be in a strong position to support, participate in and recruit for randomized research.

160

A novel R1205C candidate type 1 von Willebrand disease mutation in the same codon as type 2 'Vincenza' in two affected siblings of a Scottish family

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Type 1 von Willebrand disease is characterized by reduced, but qualitatively normal von Willebrand Factor (VWF) and the reported gene mutations are scattered throughout the gene. We have investigated two affected members of a Scottish family with reduced plasma levels of FVIII:C, VWF:Ag and VWF:Ricof, and having normal VWF:Ag multimers. Although these two siblings had minimal bleeding symptoms, their father, paternal grandfather and an aunt had repeated bleeding episodes. Initially, DNA from these two patients was used in targeted direct DNA sequencing of exon 27 and 28 of VWF gene, revealing an heterozygous 3613C>T transition, predicting the substitution of R1205C in exon 27. This mutation was confirmed using *Nae*I restriction enzyme, and found to be absent in 50 normal individuals. The Arginine 1205 codon is the site of other reported changes leading to R1205L and R1205H 'Vincenza' phenotypes, which results in presence of ultra high molecular weight multimers (UHMWM) in plasma. We have previously reported five affected patients from two unrelated families with R1205H 'Vincenza' mutation who similar to present patients did not show UHMWM [Lester *et al* J. Thromb. Haemost. 2003; 1(Suppl. 1): July (ISSN 1740 3340). Abstract no.: P0096]. The findings from this study also refute other reports suggesting that any mutation involving destruction or creations of Cysteine will result in an abnormal multimer smearing effect. It is likely that the Arginine 1205 codon is target of many mutations which occur frequently: this amino acid has a significant position in VWF gene and different base changes are likely to lead to a spectrum of bleeding phenotypes in these patients.

Samsung Bioepis Exhibit No. 1002
Page 5073

161

Changes in plasma haemostatic markers with hospital-based versus home-based cardiac rehabilitation in patients with coronary heart disease: the Birmingham Rehabilitation Uptake Maximization Study (BRUM)

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Raised levels of plasma viscosity (PV), fibrinogen, D-dimer (Dd, an index of thrombogenesis) and von Willebrand factor (VWF, an index of endothelial damage/dysfunction) are known independent predictors of cardiovascular (CV) risk in patients with coronary heart disease (CHD). The effects of home-based (nurse facilitated, based on the UK Heart Manual) *versus* hospital-based cardiac rehabilitation (CR) programmes on these haemostatic indices in patients with CHD are unknown. Seventy-two patients (58 males, 59 ± 11 years) with CHD (following myocardial infarction or coronary revascularization) were randomized to either a hospital- ($n = 35$) or home-based ($n = 37$) CR programme. Plasma levels of VWF, Dd, fibrinogen and PV were measured at baseline and after 3 months of CR. Baseline values were compared with 35 matched healthy controls (23 males, 57 ± 6 years). Patients with CHD had significantly elevated PV [(mean ± SD) 1.7 ± 0.2 mPa/s vs. 1.6 ± 0.8 mPa/s], fibrinogen (3.3 ± 0.8 g/l vs. 2.6 ± 0.6 g/l), VWF (189 ± 58 IU/dl vs. 127 ± 32 IU/dl) and D-dimer [median (IQR) 0.28 (0.2–0.5) ng/ml vs. 0.18 (0.13–0.3) ng/ml] when compared with healthy controls (all $P < 0.001$). The baseline levels of haemostatic indices were not significantly different between the two CR programmes. After 3 months of CR, PV, fibrinogen, VWF and Dd levels were significantly reduced [to 1.6 ± 0.1 mPa/s, 2.9 ± 0.7 g/l, 172 ± 50 IU/dl and 0.2 (0.12–0.3) ng/ml respectively] in the whole patient cohort when compared with baseline (all $P < 0.001$). The improvements in these haemostatic markers levels were not significantly different between patients of the home- and hospital-based programmes. In conclusions, CR improves haemostatic profiles in patients with CHD, with no significant differences between home- and hospital-based CR programmes. These effects may contribute to the beneficial effects of CR on CV risks.

162

Incremental shuttle walking is associated with activation of haemostatic and haemorheologic markers in patients with coronary heart disease

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Activation of platelet reactivity and coagulation cascade after short-term physical exertion has been shown in patients with CHD. However, most previous studies have used either bicycle ergometer or treadmill exercise protocol, which is not widely available to most patients. Most CHD patients choose walking as a mean of exercise and therefore the aim of this study was to assess the effect of an incremental shuttle walk test (ISWT) on plasma haemostatic markers. Plasma viscosity (PV), fibrinogen (Fg), D-dimer (Dd), plasma soluble P-selectin (sPsel) and von Willebrand factor (VWF) levels were measured before and immediately after ISWT in 52 CHD patients (47 males, 58 ± 10 years) who had completed a 12 weeks course of cardiac rehabilitation and in 19 matched healthy controls (15 males, 59 ± 7 years). The ISWT is a standardized incremental field-walking test that provokes maximal performance and has been shown to correlate strongly with maximum oxygen uptake (see table). Baseline Fg, VWF and sPsel levels were significantly higher in CHD patients than in controls (all $P < 0.05$). Fg, vWf, Dd and PV but not sPsel levels were significantly raised immediately after ISWT. Increment of Fg was correlated with total walking distance and peak heart rate ($P = 0.02$). A single bout of shuttle walking in patients with CHD appears to elevate fibrinogen, VWF, Dd and PV compared with healthy individuals with intact endothelium and normal haemostasis.

Table. Pre- and post-ISWT in CHD patients *versus* controls.

	CHD		Control	
	Pre	Post	Pre	Post
VWF(IU/dl)	163 ± 44	179 ± 49*	122 ± 17	126 ± 17
Dd(ng/ml)	0.23 ± 0.1	0.26 ± 0.2*	0.22 ± 0.1	0.23 ± 0.1
Fg(g/l)	2.9 ± 0.7	3.2 ± 0.7*	2.5 ± 0.7	2.7 ± 0.7*
PV(mPa/s)	1.6 ± 0.1	1.7 ± 0.1*	1.6 ± 0.1	1.6 ± 0.1

Values are given as mean ± SD.
*$P = 0.001$.

163

Anti-hypertension treatment reduces platelet derived VEGF and angiopoietin-1 in hypertension

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Platelets carry angiogenic growth factors vascular endothelial growth factor (VEGF) and angiopoietin 1 (Ang-1). Although both growth factors are important in the pathogenesis and metastasis of malignancy, their role in the pathogenesis of complications and the response to treatment in hypertension is not known. To test the hypotheses (i) that there are differences in VEGF and Ang-1 in the plasma and within platelets from patients with hypertension, and (ii) that levels change with successful treatment. We recruited 42 previously untreated patients with hypertension (25 males; mean age 53 years) and 30 age- and sex-matched controls. Plasma VEGF, Ang-1 and soluble P-selectin (sPsel, an index of platelet activation), and total platelet [platelet VEGF (pVEGF) and platelet Ang-1 (pAng-1)] were measured by ELISA. The patients were treated for 6 months with amlodipine-base therapy, achieving a blood pressure below 140/80 mmHg. Patients with hypertension had significantly higher levels of plasma sPsel ($P = 0.01$), VEGF ($P < 0.001$) and Ang-1 ($P = 0.01$), as well as pVEGF ($P < 0.001$) and pAng-1 ($P = 0.02$). The levels of plasma and platelet angiogenic growth factors were significantly reduced after antihypertensive treatment (VEGF, $P = 0.01$; pVEGF $P < 0.001$; Ang-1, $P < 0.001$; pAng-1 $P = 0.04$). There were no correlations with blood pressure or the levels of sPsel. Levels of plasma and intra-platelet VEGF and Ang-1 are increased in hypertension and are decreased with treatment. Platelet levels of VEGF and Ang-1 are probably not related to platelet activation and may involve other mechanisms, such as the general vascular and haemodynamic changes that are seen in hypertension.

164

Soluble CD40 ligand, P-selectin, interleukin-6 and tissue factor in diabetes with and without overt cardiovascular disease

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CD40L-CD40 interaction has been linked to vascular inflammation and the hypercoagulability. High levels of soluble CD40 ligand (sCD40L) have been found in diabetes (DM). We hypothesized that sCD40L is related to indices of platelet activation, inflammation and hypercoagulability in patients with DM with and without clinically

Samsung Bioepis Exhibit No. 1002
Page 5074

overt cardiovascular disease (OCVD). We measured plasma sCD40L, soluble P-selectin (sP-sel, a marker of platelet activation), interleukin-6 (IL-6, a pro-inflammatory cytokine) and tissue factor (TF, an initiator of coagulation) by ELISA in 97 patients with DM (41 with and 56 without OCVD) and 39 age- and sex-comparable controls. [All data as mean (SD) or median (IQR) and in the following order: controls, DM without OCVD, DM with OCVD. Differences between patient groups by ANOVA] HbA1c [5.4 (0.3), 7.5 (0.5), 7.8 (1.2), $P < 0.001$] and systolic blood pressure [128 (17), 138 (15), 137 (21), $P = 0.048$] were higher in patients compared to controls, but not between patient groups. Plasma sCD40L [30 (10–175), 520 (183–1475), 700 (228–1425) pg/ml], sP-sel [85 (65–95), 95 (80–120), 100 (85–115) ng/ml], IL-6 [1.0 (1.0–2.0), 1.0 (1.0–5.7), 2.6 (1.0–17.5) pg/ml] and TF [300 (11–925), 560 (250–1400), 975 (400–2574) pg/ml] were higher in patients with DM compared with controls (all $P < 0.01$). IL-6 and TF were highest in patients with OCVD ($P < 0.01$). Plasma sCD40L correlated significantly with IL-6 and TF (all $r > 0.5$, $P < 0.001$) but not with sP-sel and HbA1c in both patient groups. On multivariate regression analysis, patient group was the only significant predictor of plasma sCD40L ($P = 0.04$). Our data suggest a link between the CD40-CD40L system and the inflammatory and hypercoagulable state in DM. Higher levels of plasma IL-6 and TF in patients with OCVD may explain the higher cardiovascular risk in these patients.

165

The effect of intensive multi-factorial intervention on soluble CD40 ligand, interleukin-6 and tissue factor in diabetes with and without overt cardiovascular disease

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CD40L–CD40 interaction has been linked to vascular inflammation, a hypercoagulable state and may be involved in the pathogenesis of atherothrombotic complications. We sought to determine if: (i) intensified multi-factorial intervention could reduce plasma sCD40L, indices of inflammation and the hypercoagulable state in diabetes, and (ii) patients with overt CVD (OCVD) would derive similar benefit when compared with those without. Sixty-eight patients with diabetes (36 patients with and 32 without OCVD) participated in multi-factorial intervention, targeting glycaemia, hyperlipidaemia and blood pressure over 1 year. We measured plasma sCD40L, interleukin-6 (IL-6, a pro-inflammatory cytokine) and tissue factor (TF, an initiator of coagulation) by ELISA at baseline and 1 year. [The significance of changes in clinical and biochemical indices were evaluated with paired t-test or Wilcoxon's signed rank test] HbA1c (by mean of 0.9% and 0.8%) and total cholesterol (by mean of 1.1 and 0.8 mmol/l) were reduced in patients without and with OCVD respectively (all $P < 0.01$). Blood pressure was reduced to 135/77 and 135/74 mmHg at the end of the study, but this was not statistically significant. These changes were associated with reductions in sCD40L in both patient groups (by mean of 195 and 280 pg/ml, $P < 0.001$). TF fell only in patients without OCVD (by mean of 180 pg/ml, $P < 0.001$). There was no significant change in IL-6 in both patient groups. Changes in sCD40L and TF did not correlate with changes in metabolic parameters. Contemporary multi-factorial intervention is associated with reduction in plasma sCD40L but can only be partially correct the underlying vascular inflammation and hypercoagulability in diabetes, particularly in patients with OCVD. This highlights the limitations of contemporary management strategies and suggests the need for earlier, possibly more aggressive intervention in diabetes.

166

Plasma vascular endothelial growth factor, angiopoietin-1, -2 and soluble Tie-2 levels in diabetes: relationship with glycaemia, microalbuminuria, endothelial damage/dysfunction and the effect of multi-factorial intervention

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Vascular endothelial growth factor (VEGF) and angiopoietin/Tie-2 are angiogenic growth factors implicated in the pathogenesis of diabetic vascular disease. Plasma levels of these growth factors in patients with diabetes and their relationship with the presence of overt cardiovascular disease (OCVD), measures of glycaemia, albuminuria and endothelial perturbations are unclear, as are the effects of intensified diabetes and cardiovascular risk management. We measured plasma von Willebrand factor (VWF, a marker of endothelial damage/dysfunction), VEGF, angiopoietins-1&2 (Ang-1, Ang-2) and soluble Tie-2 (all ELISA) in 94 patients (38 with OCVD) with diabetes and 34 normal controls. Thirty-three patients with and 31 without OCVD were subjected to a year's intensified cardiovascular risk management. Plasma VWF ($P = 0.009$), VEGF ($P = 0.001$) and Ang-2 ($P = 0.001$), but not Ang-1 ($P = 0.432$) or soluble Tie-2 ($P = 0.052$), were higher in both groups of diabetics, both with and without OCVD, compared with controls. Ang-2 correlated with urine albumin: creatinine ratio ($r = 0.307$, $P = 0.021$) and VWF ($r = 0.398$, $P = 0.005$) in patients without OCVD but not in patients with OCVD. VEGF correlated with HbA1c in patients with ($r = 0.367$, $P = 0.012$) and without ($r = 0.338$, $P = 0.024$) OCVD. Correlation between Ang-2 and HbA1c was significant only in patients without OCVD ($r = 0.356$, $P = 0.007$). There were significant reductions in HBA1c and total cholesterol after 1-year treatment of 33 patients with and 31 without OCVD associated with reductions in plasma VWF, and VEGF in both groups, but Ang-2 fell only in the patients without OCVD (all $P < 0.001$). Our data indicate that plasma Ang-2, like VEGF and VWF, is elevated in patients with diabetes regardless of overt vascular disease and may be associated with glycaemia, microalbuminuria and endothelial damage/dysfunction. Intensive multi-factorial intervention results in improvements in plasma VEGF and (in patients without overt CVD) Ang-2 levels. Further studies to assess the prognostic value of these pathophysiological changes are warranted.

167

Comparison of multiplex PCR with thirdwave invader DNA analysis for the detection of the factor V Leiden and prothrombin 3'UTR gene mutations

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Screening for factor V Leiden and prothrombin 3' UTR mutations is part of the assessment of many patients with a suspected prothrombotic disorder. Analysis is commonly performed by PCR followed by restriction enzyme analysis. This approach is labour intensive, time consuming and involves multiple steps. A novel approach to the detection of the factor V Leiden and prothrombin 3' UTR mutations, developed by thirdwave technologies (Madison, WI, USA) involves using two partly overlapping DNA probes one of which contains the allele specific sequence of interest. Hybridization to a nucleic acid target results in the formation of a structure recognized by a proprietary Cleavase enzyme. In a secondary reaction, the cleaved product releases a detectable fluorescent signal. We have retrospectively evaluated the thirdwave invader DNA technique

Samsung Bioepis Exhibit No. 1002
Page 5075

in 151 patients proven by PCR and restriction analysis to be normal, heterozygous or homozygous for factor V Leiden and/or the prothrombin 3' UTR mutations. Our current multiplex PCR method uses mutant primers that create a *Hin*dIII restriction site if the mutation is present. Results using the 3'UTR prothrombin gene invader assay identified 10 of 151 patients as heterozygous, four of 151 had equivocal results, nine of 151 had no result and the remainder were normal. Results using the factor V Leiden invader assay identified 17 of 151 as heterozygous, two of 151 as homozygous, 11 of 151 had no result, one of 151 had an equivocal result and the remainder were normal. All patients were correctly identified by the invader assay. Failure to generate a result or an equivocal result was due solely to the quality/quantity of DNA used. Overall, the thirdwave invader DNA assay is a simple, accurate rapid and reproducible procedure for correctly identifying Factor V Leiden and the 3' UTR prothrombin gene mutations.

168

Risk factor profile and provisional outcomes findings for confirmed venous thromboembolism: the VEenous thromboembolism RegIsTrY (VERITY)

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VERITY is a UK, prospective, observational registry of patients with suspected venous thromboembolism (VTE). Data on demographics, medical history, symptoms, diagnosis, treatment and outcomes are recorded. Uniquely, data from VTE-negative cases are available for comparison. All confirmed cases are treated with low-molecular-weight heparin, enoxaparin (LMWH). VERITY is expanding rapidly (15 December 2003: 11 276 entries; 34 centres active; 11 new centres confirmed). The 2003 analysis will be reported (February 2004) in five key areas: (i) DVT and PE diagnosis/outcomes, (ii) provision of thromboprophylaxis, (iii) cancer, (iv) pregnancy and (v) practice variations. In a preliminary analysis undertaken for this submission (5 December 2003), there were 10 268 patient entries, 2167 confirmed cases of DVT and 176 confirmed PE ± DVT (22.8% of referrals). In VTE-positive cases ($n = 2343$), previous medical or surgical admissions were a common risk factor (10.9% and 14.3% respectively). In VTE cases with a recent history (<4 weeks) of surgery ($n = 310$), 136 (44%) had undergone orthopaedic surgery. In VTE cases with recent hospital admission, about 33% received thromboprophylaxis (aspirin, heparin or LMWH). In VTE cases with cancer (ongoing or within previous 6 months) ($n = 273/2343$, 11.7%), the most common cancers described were colorectal ($n = 30$), prostate ($n = 22$), pancreatic ($n = 21$) and leukaemia ($n = 16$). To date, 186 cases of suspected VTE in pregnancy have been recorded; additional data are being collated to characterize thromboprophylaxis patterns in pregnancy. Initial registry findings highlight the current inadequate provision for assessment of post-treatment outcomes (recurrence and complications) both in primary and secondary care. Focused efforts, using novel follow-up initiatives, are being undertaken to define outcomes for different populations of VTE-positive patients. Available outcome data will be analysed and presented. Previous interim data (August 2003) showed 333 patients with follow-up. Provisional findings on bleeding complications were: early (<5 d) in 0.6%; late (>5 d) in 1.5%.

169

A novel variant of acquired von Willebrand syndrome associated with systemic lupus erythematosus successfully managed with recombinant activated factor VIIa and rituximab

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Our patient was diagnosed with systemic lupus erythemastosus (SLE) aged 9 years. Spontaneous petechiae and symptomatic oropharyngeal mucosal bleeding were noted shortly after initial diagnosis. All laboratory investigations were normal with the exception of impaired *in vitro* aggregation of platelets in response to Ristocetin (RIPA). A mixture of our patient's platelet poor plasma and normal control platelet rich plasma showed a markedly reduced RIPA consistent with the presence of an inhibitor despite the patient having a normal platelet count, von Willebrand factor (VWF):Ag, VWF:RCo and factor VIII and normal VWF:Ag multimers. The patient required frequent inpatient admissions for bleeding but slowly improved with standard immunosuppressive therapy and monthly infusions of IV immunoglobulins (IVIg). 6 years after diagnosis the patient remained on oral prednisolone, methotrexate and cyclophosphamide but developed a life threatening mucocutaneous haemorrhage in her neck requiring intubation and intensive care. Laboratory investigations were identical to those at diagnosis. Bleeding failed to respond to infusions of platelets and intermediate purity plasma derived factor VIII, but transient haemostasis was observed after treatment with 90 μg/kg recombinant activated factor VIIa. Methylprednisolone, cyclophosphamide, IVIg and plasma exchange were administered during the first week with a subsequent reduction in haemorrhagic manifestations. Treatment was consolidated with rituximab and cyclophosphamide. 6 months later, there has been no recurrence of serious haemorrhage and the patient's plasma does not inhibit normal control RIPA. In summary, we report a clinically severe novel variant of acquired von Willebrand syndrome associated with SLE but with no depletion of platelets or clotting factors despite apparent inhibitor activity against VWF platelet interaction, which responds clinically to immunosuppressive therapy.

170

Acquired haemophilia associated with clopidogrel; report of two cases

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Acquired haemophilia is a rare bleeding disorder caused by autoantibodies against factor VIII. Bleeding is often severe and may be life threatening. In 50% of patients, no underlying disorder is found, however, common associations are with autoimmune disease, malignancy, dermatological disorders, pregnancy and drugs. We report two female patients age 70 and 67 years, who recently presented with a history of excessive bruising and bleeding 2–3 months after commencing clopidogrel for peripheral vascular disease. One patient had had a documented normal aPTT at the time of starting clopidogrel whilst the other patient had not been tested. Investigation showed that both patients had a low factor VIII (3.9 and 1.0 IU/dl) with a detectable anti-factor VIII inhibitor (2.2 and 17.6 Bethesda units) respectively. There was no evidence of any other underlying cause. Both patients responded to discontinuation of clopidogrel and commencing high dose steroids. The factor VIII levels rose to 119 and 136 IU/dl and the inhibitors became undetectable (<0.4 Bethesda units) within 8 weeks of treatment. A possible link between acquired haemophilia and clopidogrel has not previously reported. Clopidogrel is a relatively new anti-platelet drug that is becoming increasingly common in patients with cardiovascular disease. Physicians who pre-

Samsung Bioepis Exhibit No. 1002
Page 5076

scribe clopidogrel should be aware of this possible association, particularly if patients develop bruising after starting this drug. Increased bruising should not be ascribed to the anti-platelet action of clopidogrel unless a coagulation screen has been found to be normal. Further investigation for an anti-factor VIII inhibitor should be undertaken if indicated.

171

Analysis of predisposing factors and six-month outcomes of DVT management in 668 patients treated with LMWH (tinzaparin) as outpatients

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In Swindon, eligable patients with a suspected or proven DVT are treated as outpatients using a once daily low molecular weight heparin (LMWH). Prospective audit using an electronic database has been ongoing since 1998. As a result of this data analysis, we can identify patients at greater risk of recurrent thromboembolic disease and bleeding complications. As of August 2003, there was a total cohort of 717 patients: average age was 61 years, with 53% female patients. The major predisposing factors were recorded as immobility (32.5%), previous VTE (21.3%) and surgery (14.8%). Six-month follow-up data were available for 668 patients: 20 (3.0%) patients had confirmed thromboembolic complications, of which 18 (2.7%) occurred subsequent to the initial LMWH treatment phase (late). Bleeding complications occurred in 12 (1.8%), with four (0.6%) being classified as major (three gastrointestinal bleeds and one retroperitoneal haematoma). Of the 20 confirmed thromboembolic complications, two (10%) had no predisposing factor at initial presentation. Other risk factors were malignancy (six events), immobility (six events), surgery/trauma (four events), oral contraception/HRT use (three events), previous VTE (two events), family history of VTE (two events) and a long haul flight (one event). Six patients had more than one risk factor; two of the patients had two events and both of these patients had a malignancy. Incidence of thromboembolic and bleeding events at 6 months were low (comparable with clinical trial data and other UK audit data) in our centre. The most significant risk factors for recurrent thromboembolic complications were malignancy and immobility. The database has allowed analysis of the data over different time periods enabling us to show that the service has been extended to more patients but with a reduction in complication rates. The service is now beginning to manage selected patients with a confirmed PE on an ambulatory basis.

172

Heparin induced thrombocytopenia (HIT): a 5-year review at University Hospital Birmingham, UK

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We performed a retrospective analysis of patients suspected of having heparin-induced thrombocytopenia during the 5-year period of January 1998 and January 2003.Of 15 evaluable patients who underwent HIT screening, eight (44%) patients (five males and three females) were found to have heparin/PF4 antibodies by ELISA assay. The median age was 69 years (range 26–73 years). Unfractionated heparin (UFH) had been given in three patients, low molecular weight (LMWH) heparin in three patients and in two patients both UFH and LMWH was used. Five patients received therapeutic doses of heparin (UFH-3, LMWH-1, both-1) and three patients received prophylactic doses (LMWH-2, both-1). Median date of onset of thrombocytopenia from the start of heparin was 5.5 d (range 2–20 d). The median platelet count nadir was 47×10^9/l (range 24–86). The patient who developed thrombocytopenia on day 2 had past history of heparin exposure over a year previously. Only one of eight patients (12.5%) developed a thrombotic event (a below knee DVT). Heparin was stopped in all except one patient who was not considered to have HIT clinically. Danaparoid was substituted in five patients and prostacycline in one patient. Two patients received no ongoing anticoagulation. The mean time of platelet recovery to $>100 \times 10^9$/l was 6 d. Two patients had persistent thrombocytopenia suggesting that there was likely to be another cause. Four patients subsequently died. No death was attributable to HIT. It is well established that the platelet count needs to be monitored in patients who are on UFH or LMWH beyond 5 d. Results of sensitive diagnostic tests such as the ELISA assay should be interpreted in the clinical context so as to accurately assess the probability of HIT. Of interest is the low incidence of clinically overt thrombosis in this group of patients with HIT.

173

Acquired FVIII inhibitor associated with nephrotic syndrome in a child

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Acquired factor VIII (FVIII) inhibitors usually occur in adults and may be triggered by pregnancy or malignancy. They usually present with rapid onset bruising and bleeding and if untreated are associated with a high mortality rate. We present a child from West Africa who developed steroid-resistant nephrotic syndrome at the age of 19 months, which was not initially associated with any thrombotic or haemorrhagic symptoms. When he was first seen in our centre he had been pretreated with cyclophosphamide and prednisolone. He was noted at this time to have an APTT of 121.2 s (27–38 s) with a 50:50 mix of 50.2 s and FVIII activity of <1 IU/dl. Although the Exner was positive on the first occasion the DRVVT and autoantibody screen were negative and anticardiolipin antibodies were within the normal range. He had a FVIII inhibitor of 143 Bethesda units (BU). He was found to be negative for the inversion 22 FVIII mutation and although further detailed genetic studies were carried out, no evidence of congenital haemophilia was ever found. There was no personal or family history of haemophilia and he had had an uncomplicated circumcision at 3 weeks of age. Over the subsequent 3 years this boy developed a target joint in one knee, but he was otherwise remarkably free from serious bleeding despite a maximum inhibitor titre of 1558 BU. Attempts to wean his immune suppression resulted in a rebound of his nephrotic syndrome and ultimately this necessitated repeated hospital admissions in Ghana, culminating in his death in the fourth year of his illness. We present a child with nephrotic syndrome complicated by a very high titre acquired FVIII inhibitor, which did not respond to prolonged immunosuppression, in whom there were remarkably few bleeding episodes.

174

The effect of citrate and citrate theophylline adenosine dipyridamole (CTAD) anticoagulants on the determination of plasminogen activator inhibitor-1 (PAI-1) antigen

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PAI-1 deficiency is an extremely rare disorder characterized by a bleeding diathesis due to hyperfibrinolysis as a result of low activity and antigen. Forty-five samples, comprising 20 adult controls and 25 test samples from a children's haemostasis clinic were collected, as part of screening investigations for easy bruising, bleeding and/or non-accidental injury. All the samples were collected in duplicate in both citrate and CTAD anticoagulant bottles between 10 and 12 a.m. and by the same experienced haemophilia nurse to standardize any diurnal variation and phlebotomy technique. The aim was to determine

Samsung Bioepis Exhibit No. 1002
Page 5077

whether the samples with CTAD as anticoagulant were better suited to determine PAI-1 antigen than using 3.8% citrate as an anticoagulant. Earlier studies have shown that plasma determination of PAI-1 antigen must be performed in blood collected on an antiplatelet/anticoagulant mixture. CTAD inhibits the release of PAI-1 from platelets unlike citrate. Using the PAI-1 Tintelize antigen assay (Biopool, Sweden), the results showed a significantly higher antigen value with the citrate bottles than with the CTAD anticoagulant ($P < 0.001$). This is of major clinical significance when using the PAI-1 antigen assay as a screening test to eliminate PAI-1 deficiency in patients with a bleeding diathesis in the absence of any haemostatic dysfunction or when used as part of screening tests for exclusion of non-accidental injury in medico-legal cases. These are preliminary findings of a larger study of the evaluation of suitable assay for the determination of PAI-1 activity and antigen from blood plasma samples for routine laboratory use.

175

Increased circulating endothelial cells as an index of endothelial damage in congestive heart failure

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Background: Circulating endothelial cells (CECs) in the peripheral blood probably represent the most direct evidence of endothelial cell damage. CECs are increased in haematological diseases, myocardial infarction, unstable angina and critical limb ischaemia. As congestive heart failure (CHF) is associated with endothelial abnormalities, we hypothesized that CECs are raised in CHF, and that they would correlate with the more established plasma indices of endothelial perturbation, that is, von Willebrand factor (vWf) and soluble E-selectin (sE-sel).

Methods: We studied 30 patients with acute CHF, 30 patients with chronic stable CHF [all in sinus rhythm with ejection fraction (40%) and 20 controls]. Patients with acute CHF were venesected within 24 h of hospital admission. CECs were quantified using epifluorescence microscopy after immunomagnetic separation. Citrated plasma was analysed for sE-sel and vWf by ELISA.

Results: Levels of CECs, vWf and E-sel were significantly higher (all $P < 0.01$) in patients with CHF compared to controls, with no significant differences between acute and chronic CHF. CECs correlated with plasma vWf ($P < 0.0001$) and E-selectin ($P = 0.022$) but not ejection fraction or NYHA class. In stepwise multiple regression analysis, CHF was the only independent predictor of raised CECs ($P < 0.0001$).

Conclusion: CECs may be used as a novel measure of endothelial damage in CHF, which may have implications for the thrombotic risk associated with CHF and prognosis in this condition.

176

Abnormal vascular, platelet and coagulation markers in essential thrombocythaemia are not reversed by treatments that reduce the platelet count

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Essential thrombocythaemia (ET) is characterized by increased platelet counts, and abnormalities in plasma markers of platelet activation (e.g. P-selectin), vascular dysfunction (von Willebrand factor, E-sepectin) and coagulopathy (fibrinogen, D-dimers). Treatment centres on lowering platelet counts. We hypothesized that successful treatment of thrombocytosis would result in a reduction in selected specific markers of these processes, and therefore compared such levels in 32 ET patients on treatment, 13 patients not on treatment, and in 15 healthy age- and sex-matched controls. As expected, ET patients on treatment had a higher platelet count than the controls, and those patients untreated had higher numbers than those on treatment (all $P < 0.01$). However, fibrinogen (Clauss), soluble P-selectin, platelet P-selectin, von Willebrand factor and soluble VCAM (all ELISA) were all higher in both patient groups compared to controls (all $P < 0.05$), but there was no difference between patient groups. Levels of D-dimers were higher in those on treatment compared to the controls ($P < 0.05$). Overall, there were no differences in haemoglobin, white blood cell count, soluble E-selectin or soluble ICAM amongst the three groups. We conclude that successful treatment of thrombocytosis does not bring about normalization of additional markers of the pathophysiology of platelets, the endothelium or coagulation.

177

The effect of multi-factorial intervention on markers of endothelial damage/dysfunction and thrombogenesis in diabetes mellitus

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Background: Endothelial abnormalities and a hypercoagulable state may contribute to increased cardiovascular risk in diabetes mellitus, particularly in patients with overt cardiovascular disease (OCVD). We sought to determine if a 'package of care' of intensified multi-factorial cardiovascular risk intervention could improve parameters of endothelial abnormality and hypercoagulability in diabetes, and if diabetic patients with OCVD would derive similar benefit as compared to those without.

Methods: We measured plasma von Willebrand factor (vWf, an index of endothelial damage/dysfunction), soluble E-selectin (sE-sel, marking endothelial activation) and tissue factor (TF, an initiator of coagulation) by ELISA in 94 patients with diabetes mellitus (38 with CVD and 56 without OCVD and 38 comparable controls). Thirty-three patients with and 31 without OCVD then participated in multi-factorial cardiovascular risk intervention over 1 year.

Results: Plasma levels of vWf, sE-sel and TF were significantly higher in diabetic patients compared to controls, with TF highest in patients with OCVD ($P < 0.01$ by ANOVA). Intensive multi-factorial intervention was associated with significant reductions in HbA1c, total and LDL cholesterol (all $P < 0.001$) associated with reductions in vWf in both patient groups (both $P < 0.01$). TF was reduced only in patients without OCVD ($P = 0.03$). There were no significant changes in sE-sel levels in either group.

Conclusion: Patients with diabetes mellitus demonstrate endothelial perturbation, which can be partially influenced by a 'package of care' of intensified multi-factorial cardiovascular risk intervention. These data suggest the need for earlier and more aggressive risk factor intervention.

178

What is the role of thrombophilia screening in eye disease?

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Requests for thrombophilia screens and the apparent indications keep on increasing with significant workload pressures on haematology laboratories. There has been much debate on the merits of these screens with those in favour and those against. Now ophthalmic surgeons have been made aware of the potential link between retinal vein occlusions (RVOs) and thrombophilic defects especially the factor V Leiden mutation (FVL). During the past 12 months we have seen 167 patients in our thrombophilia clinic. This group comprised 90 females and 77 males with an age range of 13–80 (mean 52). The majority of referrals

Samsung Bioepis Exhibit No. 1002
Page 5078

were patients after completion of anticoagulation therapy for venous thromboembolic events (VTEs). There were also 12 ophthalmic referrals comprising nine females and three males. Reason for referrals were RVOs in seven, unexplained visual field loss in three and unexplained retinal infarcts in two. Comprehensive screens were performed and the findings were presence of anticardiolipin antibodies ± lupus inhibitor in four, no abnormality in four, raised homocysteine in one, type 1 protein C deficiency in one, type 2 antithrombin deficiency in one and heterozygous FVL in one. Several patients had other risk factors such as hypercholesterolaemia making it difficult to know the significance of the results. The majority received aspirin whether the screens were abnormal or not with good effect. The only exception was the patient with type 1 protein C deficiency who started long-term warfarin because of his increased risks of VTEs as a consequence of being a frequent long haul flyer. We as a group feel the jury is out as to whether thrombophilia testing is useful in these patients, bearing in mind the complexity and costs of the tests, and also will an abnormal result change management. Without doubt larger studies are needed to clarify the situation.

179

The effect of hormone replacement therapy on the expression of monocyte tissue factor

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Hormone replacement therapy (HRT) is associated with an increase in both venous and arterial thrombotic events during the first year of treatment. This could conceivably be mediated via an increase in monocyte tissue factor (TF) expression, which has been shown to be elevated in patients with previous idiopathic venous thromboembolism. A double-blind randomized control trial was undertaken to investigate TF expression during the first 3 months of treatment with oral HRT. Healthy postmenopausal women ($n = 32$, mean age 59) were randomized to either 1 mg oestradiol and 0.5 mg norethisterone ($n = 8$), 1 mg oestradiol and 5 mg dydrogesterone ($n = 14$) or placebo ($n = 10$). Monocyte TF was measured by flow cytometry on fresh blood incubated with lipopolysaccharide 40 μg/ml at 37°C, 5% CO_2 for 3 h. The sample was labelled with FITC-conjugated CD14 for monocyte identification and a PE-conjugated anti-TF antibody. The number of TF binding sites was calculated by comparing the fluorescence of the monocyte population with standard beads. There was no significant difference between TF expression at baseline and 3 months for the dydrogesterone group (mean ± SD, 855 ± 355 molecules/cell versus 1209 ± 656 molecules/cell), the norethisterone group (751 ± 426 molecules/cell versus 839 ± 347 molecules/cell), and the placebo group (787 ± 291 molecules/cell versus 1110 ± 489 molecules/cell). These results provide no evidence that the early increase in thrombotic events poststarting HRT is mediated via an increase monocyte tissue factor expression.

180

Self-management of anticoagulation: a randomized trial (SMART)

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Introduction: The increased demand for oral anticoagulation has led to a need for alternative models of care. Self-management (PSM), analogous to diabetic self-care has been proposed as one such alternative. This paper reports the first large-scale UK trial of PSM compared with routine care.

Method: All adult patients receiving warfarin from 50 general practices were invited to participate. Following consent patients were randomized to either control or intervention. Intervention patients were trained in 2 × 3 h sessions to measure INR and interpret their dose of warfarin. Patients completing training were followed up for 12 months.
Results: A total of 617 patients were randomized (337 intervention/280 control, from 2470 invited). Using intention to treat analysis, there was no significant difference in percent time in range (PSM 70% versus control 68%). There were no significant differences in adverse events. Cost-effectiveness data will be available for presentation.

181

A study of the effect of introduction of clinical guidelines on the management of major haemorrhage in patients on warfarin

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The management of major haemorrhage in patients on warfarin is highly variable and often suboptimal. In an attempt to develop standardized, evidence-based care we developed a guideline based on BCSH recommendations. Practice, compared with the guideline was assessed for 14 months prior to and 12 months after the production of the guideline. In 82 patients (34 pre and 48 postguideline) over a 26-month period, indication for use of PCC, INR at time of request, PCC dose, use of the appropriate dose and route of administration of vitamin K, follow-up monitoring of coagulation status, and clinical thrombotic events were recorded. Eighty-two episodes of PCC use were recorded compared with 108 expected. Comparing the respective periods pre and postguideline the following were observed: of requests which were granted 100% and 92% were for appropriate indications, the dose of PCC was correct in 52% and 67% of cases, patients who received the wrong dose were more likely to be under-dosed, 73% and 80% respectively. Concomitant use of vitamin K improved from 65% to 79% and the dose was correct in 35% and 47% of occasions respectively. Monitoring of coagulation post-PCC was performed in only 65% and 53% of occasions respectively. One thrombotic event was recorded in the 82 treatment episodes. Introduction of a guideline has only marginally improved practice and verbal re-iteration by a specialist in haematology at the time of all PCC issues is now being evaluated.

182

Patient self-testing of INR is a safe and acceptable alternative to laboratory-based testing

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Approximately 750 000 UK patients currently take warfarin and, as this is expected to increase this by approximately 10% annually, alternatives to hospital-based anticoagulation clinics are being explored. Following a satisfactory laboratory evaluation (MDA 01026) of the Roche Diagnostics CoaguChek S (CCS), we studied the accuracy and patient acceptability of self-testing for INR using the CCS. Patients were randomized to either self-testing or control groups. Both groups continued to have their anticoagulation monitored every 4 weeks in the hospital laboratory. Dosage adjustments were performed by the anticoagulant clinic staff using the laboratory INR. Eighty-four patients were randomized to the study; 44 to self-testing and 40 to the control group. Following training, and subject to satisfactory assessment, the self-testing group performed weekly INR measurements using the CCS over a 6-month period. Fifteen patients (18%) failed to complete the study; five were unable to obtain an adequate sample, three had deteriorating health, two moved from the area, one discontinued

Samsung Bioepis Exhibit No. 1002
Page 5079

warfarin and four demonstrated compliance problems. Of 31 self-testing patients who completed a questionnaire, 27 found the CCS easy to use, 27 were confident in the result they obtained and 24 preferred self-testing to the hospital anticoagulation clinic. When comparative INR data were analysed ($n = 234$), excellent agreement was obtained between the methods ($r = 0.95$), median INR CCS = 2.90 (IQR 1.15) laboratory = 2.96 (IQR 1.17) with a mean difference of 0.05. Overall 85% of results were within 0.5 INR units of each other. On four occasions differences of >1 unit INR were obtained, but in each case the patient's anticoagulation was poorly controlled (INR >4.5 by both methods) and the differences in INR would not have significantly altered patient management. We conclude that self-testing is a safe alternative to attending the hospital clinic and is acceptable to patients.

183

The role of medication adherence in warfarin anticoagulation control and its cognitive determinants

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Non-adherence is frequently considered as a factor to explain fluctuating INRs in warfarin-anticoagulated patients. Others argue that patients on warfarin are more adherent than those on diuretics. We measured level of adherence to medication taking and regimen in warfarin-anticoagulated patients and explored determinants of this behaviour based on social cognition theories. A survey of a simple random sample of attendees of a single hospital-based anticoagulation clinic was conducted. Of the 162 respondents, typical of anticoagulated population on all background characteristics, 146 (92%) scored 29 or the maximum score of 30 on the Medication Adherence Report Scale. Calculation of adherence from warfarin plasma levels identified eight (5%) patients with too low plasma warfarin levels. A total of 160 (99%) reported taking warfarin at the same time each day, mostly in the evening (120, 76%). A total of 293 (59%) of 499 INR measurements were within the therapeutic range. On the 'effects of warfarin treatment' 7-question battery, 108 (67%) to 129 (80%) did not perceive warfarin a risk (four questions). Fifty-nine (37%) to 92 (57%) perceived warfarin to have a great deal of benefits, while 63 (39%) to 83 (52%) did not know if warfarin had benefits (three questions). Scores on beliefs about Medication Questionnaire General ranged from 16 to 39 with 141 (88%) of scores above the theoretical mean, suggesting acceptance of allopathic medication. Intrinsic Desire for Information scale scores were normally distributed, suggesting a range of desires for information within the sample. We have identified high adherence to medication taking and regimen in our sample although 41% of INRs were out of range; non-adherence was not the cause of INR fluctuation. Warfarin was not perceived as risk but there was uncertainty about benefits. Most patients considered allopathic medication as good. These general beliefs about medication were more important determinants of high adherence than specific beliefs. Influencing these opinions by providing information would have to be carefully considered and tailored as not all required information.

184

The effect of initiation of oral anticoagulation on protein Z and coagulation activation

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Protein Z (PZ) is a vitamin K dependent glycoprotein with a similar structure to coagulation factors VII, IX, X and to protein C. During the initiation of warfarin therapy patients may be at an increased thrombotic risk. This study aims to determine whether the rate at which PZ falls contributes to this prothrombotic tendency and to compare the rates at which the levels of other vitamin K dependent factors fall. We correlated the changing levels of vitamin K dependent proteins with alterations in the level of the coagulation activation marker, prothrombin fragment F1 + 2. We recruited seventeen patients (M = 10 and F = 7; mean age = 68.6) with atrial fibrillation (AF) electively starting warfarin (5 mg induction regimen). None had prior history of thrombosis or bleeding. Patients with antiphospholipids were excluded. Samples were taken at baseline (day 1) and at days 2, 3, 4, 5, 6, 9, 16 and 30 for analysis of factors II, VII, IX, X, protein C, protein S, F1 + 2 and PZ antigen (ELISA assay). Fourteen of 17 had INRs >2 by day 9. PZ falls slowly but significantly during warfarinization, in a time frame similar to factor II and X (see table). The 5 mg anticoagulant regime did not cause a precipitous fall in protein C nor was there evidence of transient hypercoaguability as measured by the F1 + 2 level. To summarize, PZ drops relatively slowly during 5 mg warfarin induction regimen this slow fall is unlikely to contribute to transient hypercoaguability. This study suggests that a 5-mg daily warfarin outpatient-loading regime, is a safe and gentle approach to anticoagulation.

	Half-life decline (d)	Percentage of baseline at Nadir	Days to Nadir
Factor II activity	3.1	26	9
Factor VII activity	1.3	15	5
Factor IX activity	2.1	27	9
Factor X activity	2.5	19	9
Protein Z antigen	3.0	11	16

185

Bleeding complications of oral anticoagulant therapy: a prospective audit

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Bleeding is the most serious complication of the use of oral anticoagulation in the prevention and treatment of thromboembolism. We designed a tool to prospectively audit the frequency and severity of over anticoagulation and bleeding in two anticoagulant services; a district hospital serving a population of 330 000 patients (3400 anticoagulant patients) and a University hospital with a population of 300 000 (4000 anticoagulant patients), both providing Anticoagulant practitioner-led inpatient and outpatient anticoagulant dosing. Data was collected over 10 months including INR at time of event, whether complications necessitated hospital attendance and/or admission, investigations carried out and treatment given. There were 347 events; 133 were asymptomatic high INRs (>8). The majority of these were treated with oral vitamin K as outpatients. There were 214 bleeding episodes, 54 major and 160 minor. The most common sites of bleeding were epistaxis (70/214), GI bleeding (45/214), haematoma (36/214) and haematuria (24/214). A total of 106 patients were admitted to hospital, constituting 0.61% of the total emergency medical admissions over this period. Patients spent a total of 887 d in hospital. Fourteen patients died, four directly as a result of the bleeding episode. INR stability in the period prior to the event was measured by ±1 standard deviation from the mean. Fifty-two of 347 patients were new at the time of the event (receiving anticoagulant therapy for less than 4 weeks), 164/347 were unstable, 121/347 were stable and 10/347 were GP monitored patients with no INR data available. At the time of event 68% of patients had an INR above target range (major bleed 72%; minor 66%). The most common factors contributing to the event were

Samsung Bioepis Exhibit No. 1002
Page 5080

concurrent illness (78/347), prescription of potentiating medication (65/347), increase in prescribed warfarin dose (21/347) or patients inadvertently exceeding their prescribed dose (15/347).

186

Health care costs of treating bleeding and other complications associated with warfarin treatment of atrial fibrillation and venous thromboembolism in the UK

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Oral anticoagulation with warfarin is an effective therapy for the treatment and prevention of thromboembolic events in patients with atrial fibrillation (AF) and venous thromboembolism (VTE). However, bleeding is a common complication of warfarin therapy. This study assesses the cost of treating this complication from the perspective of the UK National Health Service (NHS). A prospective clinical audit of adverse events associated with warfarin treatment was carried out over a 10-month period at two hospital-based anticoagulation clinics monitoring 7400 patients. Resource use associated with the treatment of bleeding and asymptomatic high INR values (>8) was obtained from hospital records or from the patient where no hospital admission occurred. Events occurring in inpatients were excluded due to the difficulty in assessing which costs were due to the original admission. Event costs were calculated by multiplying the resource units by the corresponding published national 2002 reference costs. A total of 325 bleeding events and asymptomatic high INRs requiring treatment were recorded with a total cost of £367 850. Of these 124 were in patients treated for AF and 107 in VTE patients. The remaining events occurred in patients treated for other conditions and are not considered in this analysis. The average costs associated with treatment for any event were £1121 (95% CI: £675–£1567) in AF patients and £1032 (95% CI: £641–£1423) in VTE patients. The average costs associated with major bleeds ($n = 32$) (£4584, 95% CI: £3256–£5912) were significantly higher than the costs associated with minor bleeds ($n = 111$) (£715, 95% CI: £432–£998) or asymptomatic high INR values (£251, 95% CI: £118–£384) ($P < 0.001$). The average costs in those admitted to hospital (£3298, 95% CI: £2555–£4041) were greater than in those not admitted (£106, 95% CI: £83–129). Treatment of bleeding and asymptomatic high INR values associated with warfarin therapy results in a significant consumption of NHS resources.

187

Management of anticoagulated patients before and after elective urological surgery

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Literature suggests that the risk of bleeding associated with perioperative intravenous heparin therapy is higher than the risk of thromboembolism associated with subtherapeutic anticoagulation perioperatively. This audit is an initial step to establish an evidence-based guideline on perioperative management of anticoagulated patients undergoing elective urological surgery. Seventy-five questionnaires were sent to urologists and consultant physicians (haematologists and cardiologists) in the East of Scotland. This is to find out the established practice and the expert opinion of the consultant physicians. They were asked various questions on the perioperative management of anticoagulation. A review of literature was also performed to assess the current recommendation. Sixty-three per cent of urologists and 51% of physicians replied; 59% of urologists would stop warfarin 3–4 d preoperatively and 64% would admit the patients the day before surgery; 67% of urologists would operate with international normalized ratio (INR) less than 1.5 and majority would not use Vitamin K in elective surgery. Some urologists would convert warfarin into intravenous heparin in all warfarinized patients. All physicians think the conversion is unnecessary if the indication of warfarin therapy is atrial fibrillation or venous thromboembolism, although some physicians agree that it is required in prosthetic heart valves cases. Postoperatively, most urologists would re-start intravenous heparin within 4–6 h and re-start warfarin the day after surgery; 62% of urologists agree that patients can be discharged earlier if subcutaneous instead of intravenous heparin is used because this can be managed in the community. Literature review shows that the available guidelines are subjective. No optimal strategy has been identified, as the established practice is inconsistent. There is a need to establish an objective guideline in each unit or specialty to reduce the risk of bleeding or thromboembolism in these patients.

188

Monitoring low molecular weight heparin in pregnancy: the timing of the peak measurement

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The role of monitoring low molecular weight heparin (LMWH) by an anti-Xa assay remains disputed. Much confusion arises because different centres undertake this using different protocols. The timing of the peak measurement has been recommended at 2, 3 and 4 h in pregnant women. This study aimed to identify the true time of a peak LMWH level in pregnant women. Fourteen pregnant women took part in the study. Samples were taken at time 0 immediately prior to LMWH and then at 1, 2, 3, 4 and 6 h after LMWH. LMWH concentration was measured using a one stage clotting anti-Xa assay. Thirty-seven separate studies were done. Six women were studied only once. Eight women had studies two to six times during their pregnancy. Data showed that the mean time of peak LMWH is 4.1 h (95% CI 3.6–4.5). The median is 3.7 h (95% CI 3.4–4.4). The interquartile range is 3.2–4.8 h. Where a pregnant woman has no detectable LMWH at time 0 we change their dosing to a twice-daily regime. Further analysis of the data showed that there is no significant difference in the time that women reach peak LMWH level between women having once daily and those having twice daily heparin ($P = 0.68$) although those on once daily regimes have a wider scatter of results. This study shows that although pregnant women have increased rates of clearance of LMWH the time for LMWH to reach a peak is close to 4 h, which is very similar to results obtained in non-pregnant individuals. Even where women need twice daily dosing there is no change in the time of the peak LMWH level.

Poster Presentation

General Haematology and Transfusion

189

Results of a digital web-based exercise for UK NEQAS(H), blood cell and bone marrow morphology

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We present results from a fourth national web based morphology exercise. Participants of UK NEQAS(H), General Haematology Scheme

Samsung Bioepis Exhibit No. 1002
Page 5081

were invited to www.manlab.co.uk or via NEQAS(H) site to review Case 0301BF1, a myelodysplastic syndrome with complex morphology. Digital images were presented in three formats. Part 1 had 12 single static images with expert morphological comment and participant's consensus comments from the original national survey. Part 2 provided four images with interactive tutorial requiring participants to identify specific cells. Part 3, was comprised of nine 'Stitched' images that allowed movement across the image and a zoom facility. Of 426 participants 128 (30%) returned results to UK NEQAS(H). Part 1: Over 90% of responders gave positive opinion regarding image quality, access to the site, presentation of expert opinion and agreed that the single images represented the morphological features they had originally reported. Part 2: Over 95% responded positively to the educational element and commented that the tutorial element was a useful tool. Part 3: Of 128 responders 34 were unable to download the software necessary to view the 'Stitched' image due to Trust policy. Of the remaining 94 responders, 85 found image quality adequate and representative. A high proportion (71%) thought the Stitched image offered advantages over single static images, including ease of navigation, overview of morphology and magnification control. This positive feedback supports our intention to place images of all six annual blood film surveys on the WWW. We aim to expand the tutorial aspect by using specific surveys to highlight interesting morphology. Whilst software problems are to be addressed the development of 'Stitched' images into a virtual slide is ongoing. The potential educational aspect of digital morphology is creating keen interest; future development will provide a mechanism for continuing professional development as part of external quality assessment.

190

The hypergammaglobinaemia of Gauchers disease is not reversed by enzyme replacement therapy

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Accumulation of glucosylceramide in Gaucher disease leads to characteristic storage cells and multiorgan pathology. Enzyme replacement therapy (ERT) with recombinant glucocerebrosidase has been successful in reversing symptoms relating to bulk storage, e.g. hepatosplenomegaly and bone marrow infiltration but its effects on immune system pathology are unknown. We have measured the immunoglobulin levels in 57 patients with Gaucher disease receiving ERT for up to 10 years. Twenty-eight of 57 patients (43.8%) were found to have levels of immunoglobulins significantly higher than those found in healthy adults. There were no sex or age related differences between those patients with elevated immunoglobulins (15 male; median age 44 years, 13 female; median age 48 years) and those with normal levels (13 male median age 41 years, 14 female; median age 44.5 years). Nineteen patients had elevation of a single immunoglobulin (eight IgM, seven IgG and five IgA), seven had elevation of two immunoglobulin classes (four IgA + IgG, three IgA + IgM) and one had elevation of all three immunoglobulin classes. Six patients had monoclonal gammopathy. Lymphocyte subset analysis of the peripheral blood of eight patients failed to show evidence of B-cell clonality. In patients with IgM polyclonal gammopathy levels of immunoglobulin normalized with ERT. This was not the case with IgG and IgA gammopathies which were unaffected by ERT even after 10 years of treatment. No patient with normal immunoglobulin levels prior to treatment developed a gammopathy after initiation of ERT. Our research suggests that in contrast to organomegaly, which is due to bulk storage of glucosyl ceramide the persistence of polyclonal gammopathy relates to constant immune stimulation which may occur at low levels of storage product not reversed by ERT. This may in turn contribute to the pathogenesis of haematological malignancy, which is notably elevated in Gaucher disease.

191

Abnormal distribution of cell surface receptors in glycosphingolipid storage disorders: an *in vitro* model of Gaucher's and Fabry disease

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Gaucher and Fabry Disease are lysosomal storage disorders resulting from deficiency of specific hydrolytic enzymes. Storage of un-degraded substrate results in characteristic pathologies but despite recent advances in the understanding of their natural history the pathophysiology underlying these conditions remains poorly defined. We have developed an *in vitro* assay of lysosomal storage in order to investigate the role of cellular interactions in the pathology of Gaucher and Fabry disease. Monocytes were selected from peripheral blood mononuclear cells of patients with Gaucher or Fabry disease and cultured in serum medium. Cells were cultured alone or with effete red cells to stimulate glycosphingolipid storage. The validity of the model was confirmed by measurement of chitotriosidase, an enzyme highly expressed in Gaucher disease. Over 7-d morphological differentiation into macrophages was accompanied by a 15-fold elevation in chitotriosidase in the supernatant of Gaucher cells compared to threefold elevation in controls. Cells from Gaucher and Fabry patients exhibited extensive cytoplasmic vacuolation. This was visible in only a subpopulation of cells from female carriers of Fabry disease (an X-linked condition) and in no cells from controls. The vacuolation was associated with an abnormal distribution of the adhesion and phagocytic receptors, the type-three complement receptor (CR3) and the mannose-6-phosphate receptor (MPR). In contrast to controls where the receptors were detected on the cell surface, the receptors of storage cells were located in a peri-nuclear compartment, defined as lysosomal by the expression of the LAMP-1 glycoprotein. Reduced cell surface expression of CR3 was further confirmed by our finding of reduced adhesion of Gaucher-derived monocytes to artificial substrata. Failure of recycling of cell surface receptors out of the lysosomal compartment is likely to be a consequence of disordered membrane trafficking due to substrate storage. This has implications for cellular interactions and also for the efficient delivery of recombinant enzyme, which is targeted to the prelysosomal compartment via the MPR.

192

Use of the galactomannan ELISA in the detection of invasive aspergillosis: a retrospective review and potential pitfalls in application

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Invasive aspergillosis (IA) remains a significant source of morbidity and mortality amongst haemato-oncology patients. The development of IA compromises treatment options and has a high economic burden. Much effort has been directed to the early detection and treatment of IA. However, the diagnosis and management of IA remains a challenge. Detection of galactomannan antigen in serum and body fluids has been intensively studied in recent years. An ELISA based test has been used in Europe for several years and recently the FDA approved it for use with a cut-off of 0.5 optical density (OD). Between October 2000 and September 2003, we analysed approximately 2500 serial samples from 290 haematology patients for the presence of galactomannan antigen using the Platelia *Aspergillus* EIA (ASPEIA) test kit. There were positive results in 46 patients (>0.5 OD). Results were charted and each individual highest test was correlated with clinical and radiological findings. The patients were assessed for diagnosis of IA based on the EORTC criteria. Of the 46 patients, five had probable and six had possible IA. Of the 244 patients with negative ASPEIA results, 15 had probable and 15 had possible IA. The sensitivity, specificity, positive predictive value (PPV)

Samsung Bioepis Exhibit No. 1002
Page 5082

and negative predictive values (NPV) were 26.8%, 85.9%, 23.9% and 87.7% respectively. The majority of our patients received oral itraconazole prophylaxis, which has been reported to reduce the sensitivity of the test. Half of the patients had also received piperacillin and tazobactam, which is known to cause false positive results. Negative ASPEIA test results (<0.5 OD) when evaluated together with radiological findings had good specificity and NPV. However the PPV is poor. Our findings suggest that ASPEIA tests should be interpreted with care, probably in conjunction with other diagnostic modalities. Newer techniques, such as fungal PCR may have an important role to play in future detection and monitoring of IA.

193

Only a minority of age eligible patients with myeloma undergo treatment with autologous transplantation. A population based study of the contributory factors. A combined survey on behalf of The Northern Ireland Regional Haematology Group (NIRHG) and The Newcastle Regional Haematology Group (NRHG)

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Although autologous transplantation is recognized as a standard of care for younger patients with myeloma, clinical trials do not represent fully the spectrum of patients who present with myeloma in this age group. The aim of this survey was to identify the number of patients eligible by age, who had failed to receive a transplant, the reason for this, what proportion of these patients might in different circumstances have received transplantation and to identify if there was unmet need for transplantation. The survey was carried out between January 2000 and December 2001. Of 418 patients with myeloma, 131 were aged <65 years (31.3%) and 43% of these were transplanted leaving a total of 75 patients in the survey. The main reasons for non-transplantation were co-morbid medical conditions 30%, early death 15%, patient decision 11%, refractory disease 12% and failed mobilization 11%. Only a small number of patients over 65 had transplants. Comparison of transplant rates for NIRHG and NRHG were lower than the UK national average obtained from the EBMT Annual Activity Survey suggesting the reduced transplantation rate may be a further marker of the poor health status of these populations. This study highlights the need for early diagnosis and effective non-toxic therapies for induction of patients with myeloma.

194

The impact of the National Screening Program on the affected births and β-thalassaemia mutations in transfusion-dependent thalassaemic patients in Taiwan

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Taiwan is a country where the prevalence rate of β-thalassaemia carrier is at least 1.1%. To prevent thalassaemia major, the Taiwan government has initiated a National Screening Program since 1993. We are now examining the differences before and after the initiation of National Screening Program. The data of the total number of patients with thalassaemia major are obtained from the register of Taiwan Thalassemia Association. The data about the affected birth rate and the affected births per year are obtained from Bureau of Health Promotion, Taiwan. Ninety-one patients with transfusion-dependent thalassaemia treated in National Taiwan University Hospital and Tao-Yuan General Hospital were included for analysis. Of which, DNA analysis of 86 patients were recorded. There are now 361 patients with transfusion-dependent thalassaemia in Taiwan. The affected births of thalassaemia major was 15 in 1991, 10 in 1992, and 17 in 1993 respectively. And the affected births have fallen to three per year since 2000. The affected birth rate per 100 000 births was 5.59 in 1994 and 1.21 in 2002. Fourteen patients treated in our hospitals were born after the initiation of National Screening Program. DNA analysis of them revealed a new mutation [IVS-1-5 (G-C)], which came from a foreign bride form Indonesia. Otherwise, most of the mutations were common β-thalassaemia mutations in Taiwan. Despite the great success in the National Screening Program for thalassaemia, there are still few doctors failed to detect the parents at risk. Hence, postgraduate education programmes for primary care physicians, health education for general population, update management guidelines and decision support should be offered by the national register system in the future.

195

Variable recovery of the 3rd international ferritin standard 94/572 shown by UKNEQAS haematinics survey of ferritin immunoassays

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A serum pool of low ferritin was spiked with 63 and 189 μg/l of the 3rd international serum ferritin standard 94/572 (supplied by National Institute for Biological Standards and controls) and distributed to laboratories participating in the UKNEQAS Haematinics external quality assurance scheme. A total of 283 laboratories returned data from 14 method groups. The mean recovery of added standard varied from 68.5% to 157.2%. Recoveries for most methods were within the acceptable range of 90–110% but two of the larger methods had more extreme recoveries of 157.2% (Abbott Architect) and approximately 70% (Bayer immuno 1).The variable recovery of some methods may reflect differences in antibody specificity and the use of two different international standards for primary calibration (spleen and liver ferritin). Current commercially available kits for ferritin are standardized against the 1st and 2nd international standard. Despite the differences in recovery observed intermethod comparison on monthly UKNEQAS surveys generally show close intermethod agreement with the all lab trimmed mean (ALTM) The biases of the methods relative to the ALTM do not seem consistent with either the standard used for primary calibration or the recovery of the 3rd international standard. The use of a single international standard is normally recommended for primary method calibration, but the variable results obtained by this study call into question the suitability of the third international standard for this purpose.

Method	*n*	Mean % recovery 94/572	95% CI	Mean % recovery 94/572	95% CI	Standard used to calibrate
Abbott architect	38	157.2	151.8–162.7	156.8	151.5–162	Nk
Abbott axsym	18	103.8	97.5–110.1	104.7	98.6–110.9	80/602 liver
Abbott IMX	8	103.1	98.3–107.9	103.2	100.3–106.2	Nk
Autodelphia	5	108.7	93.7–136.2	117.1	101.6–146.5	Nk
BayerImmuno1	14	69.0	67.6–70.5	75.5	74.0–77	80/602 liver
Beckman access	49	106.4	104.4–108.4	109.7	108.1–111.3	80/578 spleen
BayerACS180	7	107.6	96.8–118.4	111	101.2–120.7	Nk
Bayer Centaur	63	103.9	102.2–105.5	107.7	105.8–109.6	80/602 liver
DPC Immulite 1	4	89.1	86.5–90.5	85.3	74.8–87.8	Nk
DPC Imm2000	37	96.3	93.9–98.6	96.8	94.8–98.8	80/578 spleen
Olympus 600	2	73.8	69.8–77.8	86.8	85.2–88.4	Nk
Roche E170	21	93.1	88.5–97.7.	92.4	87.8–97.0	80/602 liver
Roche Elecsys	13	87.9	80.2–95.7	89.9	85.2–94.5	Nk
Tosoh AIA	4	68.5	66.7–70.6	69.3	65.8–71.9	Nk

Samsung Bioepis Exhibit No. 1002
Page 5083

196

Evaluation of DiffMaster Octavia for use on Wright–Geimsa stained blood smears

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Introduction: Differential count of leucocytes in peripheral blood is traditionally performed manually with a light microscope. A Swedish company, CellaVision AB, provides a computerized system with decision support. The system, DiffMaster Octavia, automatically collects and preclassifies images of blood cells on a stained blood smear, presenting them to the user in an organized manner. The system has predominantly been used in laboratories analysing May–Grunwald–Giemsa stained blood smears. Since no major study on Wright–Giemsa staining smears has been performed with the system, the Haematology Department, Royal Hallamshire Hospital performed an evaluation based on the NCCLS H20-A protocol.

Methodology: Differential counts (200 leucocytes/smear) were performed on Wright–Giemsa stained smears in duplicate for each sample, using both manual light microscopy and the DiffMaster Octavia. Two examiners analysed one slide each per sample. The evaluation assessed accuracy, imprecision and clinical sensitivity/specificity. The imprecision evaluation was calculated both as intraassay and interassay variation. Interassay variation was calculated using the duplicate samples. Intraassay variation was determined for both methods in a separate study by analysing the same slides five times each by five Biomedical Scientists.

Results: Regression analysis showed no systematic difference between the methods. Imprecision was in general lower for DiffMaster Octavia. The clinical sensitivity and specificity evaluation showed equivalent results.

Conclusions: The results demonstrated that DiffMaster Octavia contributes to a higher quality in the haematology laboratory. The system makes analysis less susceptible to its inherent subjective variation. The DiffMaster Octavia clearly has the potential to move blood film morphology into a new era. Automated classification is dependent on smear and stain quality, there is more work to be done on this, when optimized, results are excellent. It provides an excellent reference for teaching and with higher classification rates it will undoubtedly have a role in managing morphology workload of large hospitals.

197

Introduction of an integrated care pathway for neutropenic sepsis

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Protocol driven care for the management of neutropenic sepsis is widely practised. With the perception of increased numbers of neutropenic patients being referred directly to A + E outside of 'office hours' an integrated care pathway was developed for the management of suspected neutropenic sepsis. The aim being to improve awareness, communication and documentation for junior doctors and nurse practitioners and to provide detailed guidance in the management of suspected neutropenic sepsis. The notes of all neutropenic patients admitted as an emergency to the inpatient haemato-oncology unit were retrospectively reviewed for the 6-month period beginning January 2003. There were 38 evaluable episodes and the care pathway was used in 18 (47%). All patients were receiving myelosuppressive chemotherapy-22 of 38 were NHL, two myeloma, two AML and 12 other solid tumour patients. Twenty-five patients were 7–14 d post-chemotherapy and 20 of those (80%) had grade 4 neutropenia. Eighteen (47%) patients were referred by their GP. Patients presented out of hours in 19 (50%) cases. Fourteen patients (37%) were seen by a doctor within 30 min of presentation. Of the 33 acute neutropenic episodes seven patients (21%) received antibiotics within 60 min, nine (27%) waited 60–120 min and 17 patients waited more than 2 h. As a result of this survey we aim to further educate both patients and GPs to phone earlier and not delay their admission until after office hours. We plan develop a direct nurse to on-call team referral pathway to further reduce treatment delays with the aim of delivering the first dose of antibiotics within 1 h of arrival.

198

The use and abuse of haematology investigations in a young stroke population

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Triggered by awareness of inconsistency and gross overuse of haematological investigation in medical patients, we conducted a retrospective study of 92 young (<65 years) patients with acute ischaemic stroke. All were objectively diagnosed. TIA and other causes of stroke were excluded. We reviewed and compared practice on an acute stroke unit (ASU) with management in a general medical ward (GM). The populations were similar; ASU, 50 patients, 58% men, mean age 57 years and GM 42 patients, 52% men, mean age 55 years. Notably, coagulation screens were performed in 46% and 40% of cases respectively (excluding subjects on warfarin). Other uninformative tests were often requested in both ASU and GM settings such as LFTs in 96% and 90%, and CRP in 82% and 79% respectively. Testing for MPD and antiphospholipid syndrome (APS) is indicated in this population as a positive diagnosis would alter prognosis and management. While most patients had a full blood count, few had appropriate investigation of APS either in hospital or following discharge. LA testing was performed on 6% ASU and 14.4% GM cases and anti-cardiolipin in 8% and 7% of cases respectively. The mean age of those tested for APA was lower than the overall population. Inherited thrombophilia was tested for in 10% of ASU and 4.7% of GM cases. The study confirms our anecdotal observations. Coagulation screens are performed inappropriately, often leading to additional irrelevant investigations. Cases of APS are probably overlooked. Testing for heritable thrombophilia was not overused. There is a clear need to ensure that testing is carried out to detect disorders that change management and that tests that are not clinically indicated are minimized.

199

Genetic screening for haemachromatosis – a cautionary tale

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Haemachromatosis is a common autosomal recessive genetic disorder of iron metabolism. Several single nucleotide polymorphisms (SNPs) within the HFE gene have been reported which are associated with the disease. C282Y was the first SNP reported with approximately 80% of patients with hereditary Haemachromatosis worldwide being homozygous for the polymorphism. Other SNPs including H63D and S65C have also been associated with the haemachromatosis phenotype. In our laboratory we had been routinely screening patients for the two common SNPs C282Y and H63D using a multiplex PCR (Stott *et al*,

Samsung Bioepis Exhibit No. 1002
Page 5084

Clinical Chemistry 1999;45:3, 426–428). However, conflicting results with another laboratory led us to investigate this method. In our laboratory two family members appeared homozygous for H63D but another laboratory had found these individuals to be heterozygous. Subsequent sequence analysis revealed heterozygosity for H63D and in addition heterozygosity for S65C. The presence of the S65C polymorphism appears to interfere with the BbrPI restriction enzyme site thus resulting in the anomaly. To determine the prevalence of the S65C genotype and its impact on our mutliplex method we re-analysed 250 patients for H63D and S65C by restriction fragment length polymorphisms (RFLP) with bcl-1 and hinf-1 restriction enzyme digests respectively. H63D was wild type in 79% of patients with 19% showing both alleles and 2% with homozygous genotype. S65C was wild type in 95.2% of patients with 4.8% heterozygous and no homozygous patients. There were five (2%) patients that showed compound heterozygosity for both H63D and S65C. On review all of these five cases had been mistakenly genotyped as homozygous for H63D. This clearly shows that the presence of the S65C polymorphism interferes with the BbrPI restriction enzyme digest leading to anomalous reporting of the H63D genotype. This study indicates that using the multiplex method described may lead to a potential error rate of up to 2% and our current practise is to screen for all three mutations.

200

Results of bone marrow biopsy patient satisfaction survey at the Royal Bournemouth Hospital

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The 'Safety of Medazolam sedation for Bone Marrow Biopsy (BMB)' audit, presented last year at this meeting, yielded alarming results. These prompted us to set a standard of 'no routine medazolam usage for BMB'. A patient satisfaction survey was therefore constructed to assess levels of pain experienced by patients undergoing BMB to enable other analgesic approaches to be addressed. Patients less than 65 years of age who were having a BMB under local anaesthetic were targeted to complete the questionnaire. Thirty questionnaires were completed over a 3-month period. Questions included number of patient BMBs, a pain scale for patient completion and whether additional analgesia prohibiting driving after the procedure would be acceptable to patient. The results indicated that for 80% patients this was their only BMB; 80% patients indicated some level of discomfort on the pain scale of 1–10 but only 7% indicated a pain level >5. Only 23% patients indicated that they would like further analgesia. All these patients accepted the inability to drive after such medication. We therefore propose a proforma for patient assessment prior to BMB and oral analgesia in addition to local anaesthetic in selected cases. The full questionnaire and results will be presented.

201

The use of anagrelide for control of thrombocythaemia at the regional haematology unit, Belfast City Hospital

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Anagrelide is currently available off license for the control of elevated platelet counts caused by primary marrow pathology. We wished to review the indications, side-effects and response in patients treated at BCH for control of elevated platelet counts with anagrelide.

Method: We accessed pharmacy records from January 2000 and identified 14 patients who had the relevant data collected retrospectively.

Results: Fourteen patients (2M:12F) with a mean age 61.5 years (range 27–82). Twelve of these had essential thrombocythaemia, one had polycythaemia vera and one had a primary myeloproliferative disorder 'NOS'. All but one patient had previously been treated with hydrozyurea (HU); one continues to be treated with both anagrelide and HU, while a further young female opted to be treated solely with interferon-α. Anagrelide was used as a second line agent for failure of adequate control with HU and also for those younger patients. Side-effects included headache, palpitations, fluid retention, diarrhoea and nausea, all of which declined over time suggesting long-term tolerability. Two patients required concomitant prescribing of recombinant erythropoietin. Only one patient suffered a thrombohaemorrhagic event, which occurred on HU alone with a platelet count over 500. The range of dose to maintain a normal platelet count was from 500 mcg to 7 mg daily in divided doses, taking on average 4.5 months to achieve a platelet count less than 400.

Discussion: The MRC PT1 trial comparing anagrelide and aspirin to hydroxyurea and aspirin recently reported an increase in serious adverse events in the anagrelide arm (6 out of 400 showed progressive myelofibrosis). It was recommended that patients switch from anagrelide to HU.

Conclusion: This cohort has been maintained on anagrelide as a second line agent or due to their young age with overall good control of the platelet counts and an acceptable side-effects profile.

202

Results of an open-label study of high-dose liposomal amphotericin B (AmBisome) in the treatment of confirmed fungal infections in high-risk patients

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Invasive fungal infections remain a significant cause of morbidity and mortality in immunocompromised patients. Liposomal Amphotericin B (AmBisome) has shown response rates of around 40–60% in the treatment of invasive filamentous fungal infections. The pharmacodymnamic properties of AmBisome suggest that it may be of greater efficacy at higher doses. We conducted a single arm, open label, multicentre trial to evaluate the efficacy of a high-dose regimen of AmBisome. Patients with proven or probable invasive fungal infection, as defined by the EORTC-MSG criteria, were treated with AmBisome 10 mg/kg/d for 5 d, followed by AmBisome 3 mg/kg/d for 9 d. A complete (CR) or partial (PR) response was considered a successful outcome. Thirty-one patients have been recruited to date and we report the results from the first 17 patients. There were nine male and eight female patients, aged 8–68 years (median – 36 years). Fourteen patients had an underlying haematological malignancy, nine (53%) had relapsed disease and 11 underwent stem cell transplantation (related allogeneic – six, MUD – two, autologous – three). Ten patients had proven infection diagnosed by histology (four patients) and culture (*Aspergillus* spp-5; Rhizopus Arrhizus-1) while seven had probable disease. At 14 d, the overall response rate to treatment was 68.7% (11/16 – data unavailable for one patient). Two patients had CR (12.5%) and nine patients had PR (56.2%) to treatment. All patients treated to day 14 were continued on AmBisome. The median treatment duration was 29 d (range 2–192 d). Survival rate at 12 weeks was 58.8% (10/17) with only two fungal related deaths reported. Nephrotoxicity rate (defined as an increase in creatinine greater than two times baseline) was 13%; in line with other studies using lower doses of AmBisome.

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Samsung Bioepis Exhibit No. 1002
Page 5085

High-dose AmBisome treatment is efficacious and well tolerated in the management of proven or probable invasive fungal disease.

203

Communication with patients diagnosed with haematological malignancy. On behalf of the Regional Multiprofessional Audit Group

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In 2000 a regional oncology audit was carried out to identify patients and carers perspectives of cancer services in Northern Ireland in an aim to monitor recommendations set out by the Calman-Hine report in 1995 and the Campbell report in 1996. A subsequent audit in 2002 was performed to assess patients diagnosed with haematological malignancy. The report recommended equity of care, cancer services as patient centres, patients and carers views to be paramount and performance of clinical audit to measure effectiveness of the service provided. The questionnaire had been developed for the accreditation of cancer services in the Trent region audit and was adapted to include questions on Hickman line insertion. Five main areas were covered as follows; diagnosis, treatment, follow-up, quality of information sources and carers experiences. A total of 178 outpatients attending Belfast City Hospital Trust, Ulster and Community Hospitals Trust and United Hospital Trust completed a questionnaire in an interview type setting with the help of clinical audit staff. Informed consent was obtained. The project was supported by a grant from the Regional Multiprofessional Audit Group. Satisfaction scores were calculated for each main area and identified that patients were most satisfied with the explanation they received of necessary investigations, diagnosis and treatment and the ability to contact healthcare staff when required. There was less satisfaction with referral from the general practitioner, time spent waiting at the clinic before diagnosis, privacy during surgery and radiotherapy and explanation of side-effects of surgery. The Likert scale was used to indicate how good patients felt the information they had received was. Written information on diagnosis and treatment and complementary therapies. Carers were not satisfied in over 20% of cases with the opportunity to speak to the doctors about their concerns. Comparison of the previous regional audit of oncology patients and those diagnosed with haematological malignancy revealed similar areas of strength and weakness, but overall haematology patients were more satisfied with the care they received.

204

Peripheral blood leucocyte and lymphocyte subpopulations in elderly Kuwaiti subjects

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Immune senescence and alteration in the haematological parameters are thought to accompany advancing age. This may result in altered immune surveillance and alterations in the leucocyte populations, and may be important in the development of cancers and in the morbidity and mortality from infection that occur with relative higher frequency in the elderly. In the present study, we determined the relative and absolute numbers of immunocompetent cells, including CD2, CD3, CD19, CD4, CD8, CD56, HLADR, CD45RO, and CD45RA cells in the blood of 58 elderly Kuwaiti subjects (27 females, 31 males, average age 67.8 ± 6.9 years) and compared them with values found in 127 healthy adult subjects in the same population (63 females, 64 males, average age 27.9 ± 9.2 years). Hb levels in the elderly subjects was significantly lower when compared to adult controls ($P = 0.019$), while white blood cells (WBC) and absolute lymphocyte counts were comparable in the two groups. Absolute and relative CD2 and CD45RA cells, and absolute CD3 cells were not significantly different in the two groups. However, CD4, CD19, CD56, and relative CD3 and CD8 cells were significantly lower in the elderly compared to controls ($P < 0.001$, 0.002, 0.035, 0.001 and 0.001 respectively). In contrast, HLADR and total CD45RO cells were significantly higher in the elderly ($P < 0.001$). The CD4 to CD8 ratio was significantly lower in the elderly subjects ($P = 0.002$), and CD4 cells showed progressive decline with age. The results show alteration in both the B and T lymphocyte populations in older subjects. We speculate that these changes may be linked to some diseases associated with ageing.

205

Pseudothrombocytopenia (PTCP) apparent *in vitro* in ethylene diamine tetra acetic acid (EDTA), sodium citrate and heparin anticoagulants but not by flow cytometric analysis using CD61 antibody

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In vitro platelet aggregation due to ethylene diamine tetra acetic acid (EDTA)-dependent PTCP is usually reversible when repeated in sodium citrate or heparin. We would like to report the second case of PTCP in the world in which EDTA, sodium citrate and heparin failed in preventing platelet aggregation. Platelet counts using CD61 antibody revealed a more reliable result. An 80-year-old with no past history of bleeding problems was referred for investigation of thrombocytopenia prior to an elective herniorrhaphy and transurethral resection of benign prostatic hypertrophy. He had a past history of hypertension. He was on ramipril, aspirin, frusemide and tamsulosin. His Hb and WBC were normal and the platelet count was 7000/μl in EDTA, 2000/μl in citrate and 5000/μl in heparin. A blood film showed platelet clumping and no other abnormalities. Serum electrophoresis revealed an IgG-λ paraprotein level of 6 g/l. A bone marrow aspiration and biopsy showed no increase in megakaryocytes or plasma cells. Skeletal survey was normal. Rheumatoid factor, anti-nuclear and anti-cardiolipin antibodies were negative. His CD61 platelet count using a flowcytometry was 108 000/μl. His Platelet Function Analyser closure time and clotting profiles were normal. He underwent surgery uneventfully. The proposed mechanism in PTCP is an EDTA mediated IgG and/or IgM antibody which reacts with hidden cryptic antigens on platelet glycoprotein IIb/IIIa. We have not been able to explain the phenomenon in our patient. Since the failure to recognize EDTA-dependent PTCP may result in inappropriate treatment, identification of this is important. Measurement using CD61 in this situation is essential.

206

Blood film reports: how often are they acted upon?

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It is familiar to all haematologists that advice provided with blood films is sometimes not followed, but there is little data available on the extent of non-concordance. We present a variety of methods for

Samsung Bioepis Exhibit No. 1002
Page 5086

investigating this and the results of a local audit. Blood film reports from a 2% sample of in-patient and clinic FBC requests from a major Edinburgh teaching hospital over a 2-week period included a request to telephone to register receipt of results. A 20% sample of non-responders was studied in greater detail to investigate reasons for non-concordance. Additionally, blood film reports issued over a 1-month period suggesting that a further sample for haematinic assays be sent were followed up to see if such a sample was subsequently received. Only 20/173 (12%) of study reports were registered as having been received. There were no replies from FBC samples with normal counts. Response rates were equally poor in all hospital departments. The detailed analysis of 38 non-responders suggested that the level of non-concordance was not explained by rapid discharge or movement of patients or by non-receipt of printed results. Out of 20 suggestions for further tests, only three samples (15%) were subsequently requested, despite blood being drawn again from 12 of the patients for other tests. None of the patients had had the relevant tests performed in the previous 2 years. This study suggests that non-concordance with blood film reports is may be unexpectedly extensive and may frequently lead to clinically indicated tests not being performed. The study has prompted investigation of the presentation of results and of ward organization factors, and we suggest this is an important area for audit in other hospitals.

207

Variability in platelet dose and incremental response in haematology patients in the UK

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Morbidity and mortality due to haemorrhage in patients with thrombocytopenia has been markedly reduced since the introduction of platelet transfusion. Practice varies considerably and the optimal dose is not known. This study aimed to provide information about the current variability in adult transfusion doses (ATD) of platelets and the response achieved in patients with bone marrow failure due to treatment or disease. Platelet concentrates from three transfusion centres processed over a 6-week period were assessed for volume, platelet concentration and dose. A total of 3326 platelet concentrates were evaluated 1474 apheresis and 1852 derived from whole blood donations. The mean (range) volume of a pack was 206 (149–332) and 277 (172–361) ml for apheresis and pooled platelets respectively. The mean platelet concentrations were 1291 (233–2461) and 1092 (512–1791) $\times 10^9$/l. The mean number of platelets per unit was 265 (45–472) and 300 (158–439) $\times 10^9$. Twenty-three patients (12M, 11F, age 19–65 years) received 75 single and six double ATD platelet transfusions: seven AML, two ALL, four myeloma, two aplastic anaemia, one CML, one MDS (RAEB), one Hodgkins disease and five NHL. For single ATD platelet transfusions the mean dose of platelets was 274 (186–370) $\times 10^9$/unit, 3.8 (1.9–7.1) $\times 10^9$/kg patient body weight. The mean platelet increment at 20–24 h post-transfusion was 14 (−6–77) $\times 10^9$/l. For double ATD platelet transfusion, the mean platelet dose was 511 (373–593) $\times 10^9$/unit, 7.7 (5.3–9.6) $\times 10^9$/kg patient body weight. These transfusions yielded a mean platelet increment of 27 (0–79) $\times 10^9$/l. This study confirms considerable variability in the transfused dose. Clinical factors, notably infection and drug therapy, affect the incremental response to platelet transfusion, making it difficult to determine the optimal dose of platelets using this parameter. A better measure of the effectiveness platelet dose is haemorrhage. An international multicentre study to address this is in progress.

208

SHOT 6th Annual Report – errors remain the predominant finding

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The 6th SHOT report again showed a preponderance of 'incorrect blood component transfused' events, an increase of 21% over the previous 12 months. Almost all hospitals participate in SHOT, but over 50% submitted no incident reports, suggesting that transfusion hazards remain under-recognized and under-reported. Two-thirds of errors occurred in clinical areas, the commonest being failure to carry out the bedside check. One-third of errors occurred in hospital laboratories; 40% of 'wrong blood' events involved multiple errors. There were 32 reports of ABO incompatible red cell transfusions resulting in the deaths of two patients and major morbidity in four. Two patients suffered major morbidity due to ABO incompatible FFP. Twenty-one patients (of whom two died) received unnecessary transfusions on the basis of wrong blood count results. Nineteen RhD negative patients (including three young females) inadvertently received RhD positive transfusions, 13/19 due to laboratory errors. Special transfusion requirements were not met in 83 patients, 60 of whom were put at risk of TA-GvHD. Failure or inability to access patients' transfusion records and lack of communication in shared care were contributory factors. SHOT made the following recommendations: (1) Resources must be available to hospitals for improvements in transfusion safety. (2) Appropriate use of blood components must be strenuously promoted. (3) Blood administration training should be implemented and competency testing developed. (4) Blood should only be prescribed by authorized clinicians. (5) Blood transfusion teaching must be included in all relevant curricula. (6) Hospital transfusion laboratory staffing must be sufficient for safe practice. (7) Electronic aids to transfusion safety should be assessed and developed at national level. (8) Policies should be developed for communicating special transfusion needs where care is shared. (9) There is a need for a national body, with relevant expertise and resource, to advise government on priorities for improvements in transfusion safety.

209

Oral vitamin K does not affect vitamin K-dependent coagulation factors in normal individuals

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Recent data suggest that use of vitamin K in the peri-operative period reduces blood losses and transfusion requirements. The only likely mechanism, which could explain these observations is that vitamin K administration leads to augmentation of the levels of vitamin K dependent factors. We tested this hypothesis in a randomized, double-blind study comparing changes in levels of factor II, VII, IX and X in healthy volunteers given either 10 mg of oral vitamin K or placebo. Twenty healthy volunteers (10 men) had samples taken for assays of factors II, VII, IX and X before and 24 h after receiving 10 mg of vitamin K (Konakion MM) or a placebo. The groups were similar in age (38.7 years, SD 2.3: 41.1 years SD 3.8 respectively) and sex (five men in each group). All assays were performed by standard one-stage procedure on an Electra 1800C coagulometer. Analysis of change in coagulation factor levels, comparing the recipients of vitamin K with placebo, by two-tailed *t*-test, showed no significant difference for any factor. In well-nourished volunteers administration of vitamin K does not augment coagulation factor levels and therefore would not be expected to

Samsung Bioepis Exhibit No. 1002
Page 5087

reduce peri-operative blood loss and transfusion requirement (Barnette *et al*, *Acta Anaesthesiol Scand* 1997;41:78; Kaplon *et al*, *J Heart Lung Transplant* 1999;18:346).

210

The incidence and aetiology of anti-c, anti-E and anti-Kell antibodies in antenatal samples across West Yorkshire from January 2000 to June 2002: time to review blood selection guidelines for transfusion to females?

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Strategies to reduce haemolytic disease of the newborn (HDN) have concentrated on preventing the formation of anti-D and managing pregnancies complicated by anti-D. With the success of such work, the relative importance of other antibodies implicated in HDN has increased. This study looked at the incidence of anti-c, anti-E and anti-Kell in women attending antenatal clinics across West Yorkshire. Case notes were then reviewed to ascertain the incidence of blood product transfusion prior to the discovery of the antibody, indication for transfusion if transfused, parity when the antibody was discovered, and the presence of other antibodies. Overall, there were 26 cases of anti-c, 21 cases of anti-E and 23 cases of anti-Kell, involving a total of 58 women in 10 different hospitals in the study period. Notes were located for 53 (91%) of these women, accounting for 64 of the total 70 cases. Thirty-four cases (53%) had received at least one blood product transfusion prior to the discovery of the antibody. Transfusion related to an obstetric or gynaecological problem in 24 of the 27 cases for which an indication was recorded. There was no significant difference in parity or the presence of other antibodies between the previously transfused and the non-transfused group. Although uncommon, anti-c, anti-E and anti-Kell may all cause HDN. In particular, anti-c and anti-Kell may cause severe, life-threatening HDN. The high rate of prior transfusion suggests that the antibodies were induced as a consequence of transfusion in many of the cases, and were therefore potentially preventable. The question of whether females requiring transfusion should receive Rh and Kell matched blood products has been debated previously. However, given the advances in laboratory technology and data computerization, as well as the prevailing medico-legal climate, it may well be prudent to review current practice.

211

An audit of blood issue fridge activity

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We recently installed an automatic system for storing cross-matched blood prior to collection (DiaMed's Blood Audit and Release System). This system allows analysis of blood collection activity in our hospital. For seven consecutive days the following activities were recorded: load (L) – cross-matched unit loaded, temporary removal (TR) – unit removed for transfusion or storage in another facility, stock return (SR) – unused unit returned and permanent removal (PR). The number of units specified for each patient was also documented. A total of 2031 activities were recorded during this period, 58% of which occurred during 06.00–18.00 Monday–Friday and 28% was error data (separate analysis). A total of 645 units were loaded, of which only 455 (70.5%) subsequently underwent TR. A further 114 units (17.7%) were SR. Of single unit TR, a median of 5.4 (range 0–15) were removed in any 6-h period and occurred equally during all time periods; whereas multiple unit TR were predominantly 06.00–18.00 Monday–Friday. SR activity (114 units) occurred almost exclusively 06.00–12.00 Monday–Friday. Average PR was 46 units/d (range 29–82) with a peak of 82 units on Thursday 06.00–12.00. 81.15% of PR activity was 06.00–12.00 Tuesday–Saturday. Overall, there is a high cross-match workload. The data suggest that a total of 47.2% of units are not transfused following cross-match, with 29.5% never even removed from the fridge. The high rates of SR and PR on mornings following elective surgical lists suggest that excess numbers of units are requested for surgery, which is confirmed by returns data. Single unit TR occurs at all times; this implies single unit transfusions are given 24 h a day despite the likelihood that many of these could be avoided during patients' sleeping hours. Data from this system should allow better rationalization and planning of cross-match requests.

212

Defective high shear platelet function among platelet donors

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As single donor plateletpheresis concentrates now account for about 40% of platelet concentrates in the UK, the platelet quality of individual donations is becoming increasingly important. The aim of this study was to investigate the prevalence of defective platelet function among 100 healthy UK platelet donors on three consecutive donation days. High shear platelet function testing was performed by the PFA-100 within citrated blood samples taken prior to plateletpheresis. Abnormal CADP closure times (CTs)were obtained in an average of 23% of donations but with no severe abnormalities > 200 s. Abnormal CEPI CTs were obtained in an average of 32% of donations with 14% between 200 and >300 s. An average of 16% donors were presenting with prolonged CEPI CTs only, indicative of an aspirin-like pattern. Of these 9% of all donors were presenting with a severe aspirin-like defect with prolonged CEPI CTs between 200 and 300 s and with the majority appearing transient in nature. An average of 4% of all donors were presenting with maximal CEPI CTs of >300 s. Platelet dysfunction as detected by the PFA-100 is surprisingly common within a voluntary platelet donor population. The majority of CEPI defects appeared transient in nature and are indicative of surreptitious intake of cyclooxygenase inhibitors (e.g. aspirin). Identification of defective platelet function within donors provides a simple means to eradicate defective platelets from being clinically utilized.

Samsung Bioepis Exhibit No. 1002
Page 5088

Oral Communication

Presidents Free Communications

213

Ximelagatran, an oral direct thrombin inhibitor, compared with current standard therapy for acute, symptomatic deep vein thrombosis, with or without pulmonary embolism: the THRIVE Treatment Study

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Ximelagatran (Exanta™, AstraZeneca), an oral direct thrombin inhibitor, has a rapid onset of action and predictable pharmacokinetics without food or alcohol interactions and a low potential for drug interactions. In this international, randomized, double-blind, double-dummy trial, 2489 patients with acute deep vein thrombosis (DVT), of whom 37% had locally confirmed pulmonary embolism (PE), received oral ximelagatran 36 mg bid for 6 months or s.c. enoxaparin 1 mg/kg bid for a minimum of 5 d followed by warfarin (target INR 2.0–3.0) for 6 months. The aim was to determine whether ximelagatran is non-inferior to enoxaparin/warfarin in preventing recurrent venous thromboembolism (VTE), by comparing Kaplan–Meier estimates of the cumulative risk of an event at 6 months. At baseline, bilateral compression leg ultrasonography and ventilation-perfusion lung scanning were performed. An independent committee adjudicated all VTE recurrences, bleeding events and mortality. Ximelagatran was administered to 1240 patients and enoxaparin/warfarin to 1249. Demographics of both groups were similar (mean age, 57 ± 16 years; 53% men). Comparison of the outcome of clinical endpoints between the two treatment groups demonstrated non-inferiority of ximelagatran versus enoxaparin/warfarin (table). Incidence of serum alanine aminotransferase elevations (>3 times the upper limit of normal) was 9.6% and 2.0% for patients receiving ximelagatran and enoxaparin/warfarin respectively. During 6 months of treatment, oral ximelagatran, administered in fixed doses of 36 mg bid without coagulation monitoring, was non-inferior to enoxaparin/warfarin in preventing recurrent VTE in patients with acute DVT with or without PE. Ximelagatran was associated with a favourable outcome with respect to major bleeding.

Event	Ximelagatran, *n* (%*)	Enoxaparin/warfarin, *n* (%*)	Absolute difference ximelagatran–enoxaparin/warfarin %* (95% CI)
Recurrent VTE (ITT)	26 (2.1)	24 (2.0)	+0.2** (−1.0; +1.3)
Recurrent VTE (OT)	23 (2.0)	17 (1.5)	+0.5 (−0.6; +1.6)
Major bleeding (OT)	14 (1.3)	26 (2.2)	−1.0 (−2.1; +0.1)
Recurrent VTE and/or major bleeding (OT)	37 (3.2)	43 (3.7)	−0.5 (−2.0; +1.0)
All-cause mortality (ITT)	28 (2.3)	42 (3.4)	−1.1 (−2.4; +0.2)
All-cause mortality (OT)	7 (0.7)	10 (0.9)	−0.2 (−1.0; +0.5)

*Estimated cumulative risk; **rounded figure. CI, confidence interval; ITT, intention-to-treat; OT, on-treatment.

214

Polycythaemia vera and pregnancy: a review of treatment and outcome of 16 pregnancies in 8 women

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Polycythaemia vera (PV) is a rare condition in women of childbearing age, the literature to date comprises only 20 case reports of pregnancies. We report 16 pregnancy outcomes in eight women, age 17–38 years, who had PV during pregnancy. The pregnancies were divided into standard antenatal care (group A) or intervention relating to their PV (aspirin 75 mg, ±LMWH, vitamin C and E, with uterine artery Doppler assessment) group B. In group A there was only 1/7 live births; this was an elective delivery at 34/40 due to placental insufficiency and foetal distress. First trimester miscarriage occurred in three (42%), The remaining three (42%) resulted in still-birth, at 25 and 29 weeks. Two were complicated by intrauterine growth retardation (IUGR) and oligohydramnios with placental infarcts in one. The third was a twin pregnancy also affected by IUGR, twin 1 was dead on ultrasound scanning and twin 2 died within 24 h of caesarean section. All nine patients in group B received aspirin; four had venesection to attain packed cell volume (PCV) < 0.45 and four received prophylactic LMW heparin (two antenatally). There were eight live births (89%), seven at term (78%), and one first trimester miscarriage (11%). Pregnancies with PV are high-risk with increased incidence of first trimester miscarriage, stillbirth, IUGR and premature delivery. Although maternal outcome is good, foetal outcome may be poor. There was no correlation between PCV, platelet count or results of thrombophilia screening and pregnancy outcome. Patients who received aspirin 75 mg, regular monitoring ± LMW heparin had a good outcome with 89% live births compared with 14% in the standard antenatal care group. A prospective study is required to develop treatment further and substantiate initial findings.

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Samsung Bioepis Exhibit No. 1002
Page 5089

215

Genomic breakpoint cloning and a functional assay of DNA topoisomerase II cleavage reveal translocation mechanism underlying formation of t(15;17) in therapy-related acute promyelocytic leukaemia

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Mechanisms underlying formation of chromosomal translocations that represent a critical step in leukaemogenesis through the generation of oncogenic chimaeric fusion proteins, are poorly understood. However, a significant proportion of AML cases arise following exposure to chemotherapeutic agents targeting DNA topoisomerase II, e.g. etoposide and mitoxantrone. For acute promyelocytic leukaemia (APL), which is characterized by the t(15;17)(q22;q12-21) generating the PML-RARα oncoprotein, recent studies have highlighted an increase in secondary cases following exposure to mitoxantrone for treatment of prior breast carcinoma. Drugs targeting DNA topoisomerase II act by damaging DNA by disrupting the cleavage-religation equilibrium, increasing the concentration of DNA topoisomerase II covalent complexes; however, their precise role in the pathogenesis of secondary leukaemias has not been firmly established. We characterized the genomic breakpoint junctions in five cases of therapy-related APL (t-APL). Induction of DNA topoisomerase II cleavage complexes by various chemotherapeutic DNA topoisomerase II inhibitors was examined in the normal homologues of the *PML* and *RARA* breakpoint sequences using an *in vitro* assay that entails trapping of the cleavage complexes and mapping of the cleavage sites at the sequence level. All four cases of mitoxantrone-related t-APL occurring following breast carcinoma treatment were found to have genomic breakpoints clustered within a 7-bp region of *PML* intron 6, consistent with a damage hotspot and corresponding precisely to a functional DNA topoisomerase II cleavage site induced by mitoxantrone. Functional DNA cleavage sites induced by etoposide and its metabolites were also identified at *PML* and *RARA* breakpoint junctions in a further case of t-APL arising following etoposide. Analysis of breakpoint junctions revealed regions of micro-homology and minimal processing, consistent with involvement of the non-homologous end-joining (NHEJ) pathway. This study directly implicates DNA topoisomerase II in mediating DNA cleavage at translocation breakpoints in t-APL, with the t(15;17) being generated through aberrant repair mediated by the NHEJ pathway.

216

T-cell-mediated colitis and bone destruction in a murine model of autoimmunity are both driven by RANK/RANK LIGAND interactions and modulated by recombinant Fc-OPG

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Deregulation and hyper-activation of T cells is linked to bone destruction in auto-immune and malignant diseases such as ATLL due to the expression of RANKL. We have studied Interleukin 2-deficient (IL2−/−) mouse model of autoimmunity to assess the role of RANKL in bone and other organs targeted by T-cell mediated inflammation. IL2−/− mice spontaneously develop a haematopoietic disorder characterized by lymphoid hyperplasia with an infiltrate of activated T cells in bone, spleen, lymph nodes, and particularly the gut, resulting in severe colitis. Bone physiology was evaluated in IL2−/− mice and controls at 4, 7, and 9 weeks of age corresponding to; no clinical disease, moderate, and severe disease respectively. Femora and tibia were assessed by X-ray, densitometry, dynamic and static histomorphometry, and TRAP staining. Bone-marrow mRNA was assessed by Taqman RT-PCR for RANK/RANKL/OPG expression and serum for RANKL/OPG by ELISA. Purified (>93%) IL2−/− T cells were adoptively transferred into Rag1−/− mice to assess reproducibility of bone disease. An IL2−/−/TCR β−/− hybrid animal was generated to assess if IL2 acted on bone solely via CD4 T cells. Once bone disease in IL2−/− mice was described, animals were treated, alongside controls, with Fc-OPG.

Results: IL2−/− mice develop significant osteopenia between 4 and 9 weeks of age ($P < 0.001$); have abnormal bone formation ($P < 0.01$), have raised osteoclast numbers ($P = 0.041$), OPG/RANK/RANKL mRNA levels, and serum RANKL ($P = 0.007$). Bone disease and colitis can be transferred to Rag1−/− mice using IL2−/− T cells ($P = 0.001$), IL2−/−/TCR−/− mice are disease free. Fc-OPG reverses bone disease evident in IL2−/− mice ($P < 0.001$) and prevents colitis. This anti-inflammatory effect is associated with loss of colonic dendritic cells.

Conclusion: Our results demonstrate that activated T cells induce bone disease and colitis in the IL2−/− mouse, the methods by which pathology is produced, and an effective treatment. We also demonstrate a novel anti-inflammatory role of osteoprotegerin and its mechanism of action (Fc-OPG kindly supplied by Amgen Inc.).

217

Diagnosis and outcome prediction for childhood acute leukaemia by gene expression profiling

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Successful treatment of patients with leukaemia depends on early identification of patients at risk for disease relapse. Risk stratification has achieved this. Although the majority of patients are cured, the

Samsung Bioepis Exhibit No. 1002
Page 5090

mechanism of relapse is poorly understood. Drug resistance plays a major role in recurrence of disease and is predictive for outcome. To address disease relapse and to develop more accurate stratification, we have prospectively analysed the gene expression pattern of leukaemic blasts with the use of oligonucleotide microarrays for classification purposes and to unravel genes involved in drug resistance. Children diagnosed with acute leukaemia at the Royal London, GOS and UCL Hospitals have been included in this study from February 2002 onwards. Ethical approval and written consent were obtained. The expression profile of diagnostic leukaemic cells was determined using oligonucleotide microarrays (U133A, Affymetrix). Data was analysed with Micro Array Suite 5.0. Concurrently, chemosensitivity was determined in these blasts, using the ATP assay. The LC50 for all drugs used was calculated. We classified patients as either sensitive or resistant. We used GeneSpring 5.0.3 to analyse the microarray data. Statistical group comparison discriminated expression profiles between patients with ALL and AML. Disease phenotype was predicted in three patients with unclassified leukaemia. Similarly, we have defined molecular signatures for subtypes of ALL, e.g. hyperdiploidy, TEL-AML1, T-ALL and E2A-PBX1. Next we correlated expression data with drug chemosensitivity profiles in order to define outcome predictors. Gene sets were identified that are predictive for sensitivity to Dexamethasone, Vincristine, and Asparaginase. This kind of analysis also provides us with an insight into the mechanism of drug resistance, which has the potential to identify new drug targets. In conclusion, we find expression analysis an accurate diagnostic tool in classification of childhood acute leukaemia. Correlation with chemosensitivity and treatment response promises to add a prognostic feature to this technology.

218

Haploidentical transplantation – a single centre experience

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In children without matched related or unrelated donors, haploidentical family members are the only source of stem cell graft. However, haploidentical haematopoietic stem cell transplantation (HSCT) is usually associated with poorer outcomes. We present 35 consecutively treated paediatric patients (19 males, 16 females) using a fludarabine-based regimen consisting of cyclophosphamide, anti-lymphocyte globulin and busulphan or total body irradiation. They received mobilized peripheral blood stem cells (PBSC) haploidentical stem cell grafts from parental (31) or sibling (three) donors. Twenty-eight children underwent HSCT for underlying malignant diseases [relapsed ALL (14), relapsed AML (five), JMML (two), MDS (three), Haemophagocytic lymphohistiocytosis (HLH) (three), biphenotypic leukaemia (one)] and seven for underlying non-malignant diseases [Fanconi anaemia (three), primary immunodeficiencies (two), osteopetrosis (one), adrenoleukodystrophy (one)]. The median follow up for this patient group was 17 months (1–55). We observed engraftment in 100% patients with rejection in 2.8%. Acute graft-versus-host disease (GvHD) was observed in 30% patients but only two children needed treatment. Of the 33 evaluable patients, 100 d survival was observed in 30 (90%). Opportunistic infections were seen in 43% patients, of which 26% succumbed to their infections. The infections were CMV (six), HSV (one), RSV (two), EBV (one), Mycobacterium kansasii (one), HZV (two), Aspergillosis (three), Adenovirus (one), Parainfluenzae type 3 (one). The latter four patients died. At the time of last follow up, 60% patients were alive, well and disease free. We observed 14 deaths: eight (relapse or disease progression), one (VOD), one (radiation toxicity) and four (infection related). We conclude that using fludarabine-based conditioning regimen and CD34-selected PBSC graft, engraftment is achievable in majority of children receiving haploidentical donors, with low incidence of severe GvHD. Infection-related deaths were relatively low and exclusively in malignant diseases. Relapse remains the major cause of treatment failure and novel approaches like donor lymphocyte infusions to prevent disease recurrence are urgently needed.

Oral Communication

Haematological Malignancies

219

Circulating BCR-ABL specific CD8$^+$ cells are detectable in chronic myeloid leukaemia patients and healthy subjects

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The fusion oncoprotein BCR-ABL that characterizes chronic myeloid leukaemia (CML) is a leukaemia-specific neoantigen, which may be immunogenic *in vivo*. KQSSKALQR and GFKQSSKAL peptide sequences spanning the b3a2 BCR-ABL junction have binding affinity for HLA-A3 and B8 respectively, and we have shown the presence of KQSSKALQR on the CML cell surface. Here we investigate circulating BCR-ABL specific CD8 positive T cells directed against the BCR-ABL junction using fluorochrome-labelled tetramers of HLA-A3 with KQSSKALQR and of HLA-B8 with GFKQSSKAL. Three groups consisting of HLA-A3 and/or HLA- B8 positive healthy donors, CML patients with the relevant HLA type and the b3a2 transcript and a negative control group that did not possess the relevant HLA types and/or transcript were studied. Fresh peripheral blood mononuclear cells were isolated from subjects and stained with the relevant tetramer and anti-CD8 and assessed by FACS analysis. Eight of 12 CML patients studied serially on at least three occasions had BCR-ABL junction specific CD8$^+$ T cells. Specific T cells were more likely to be found in patients with a low leukaemic burden as assessed by quantitative real time PCR ($P = 0.03$). Three of 18 healthy donors had BCR-ABL junction specific T cells. No specific T cells were detected in any of the negative control subjects. An in-vitro dendritic cell protocol was used to expand BCR-ABL specific CTL and assessed by radiochromium release and tetramer. Radiochromium release confirmed specific CTL expansion in two of seven healthy donors and one of six CML patients whilst tetramer assessment demonstrated specific CD8$^+$ T cells in three of seven and two of six subjects respectively. Failure to detect these tetramer positive cells by radiochromium release may represent the lower sensitivity of this assay or a genuine dysfunctional CD8$^+$ T-cell phenotype. The occurrence of specific T cells in some healthy donors might represent an immune response to occult BCR-ABL rearrangements.

220

Rapid and quantitative detection of mutations of the FLT3 gene in AML

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The most common single mutation observed in approximately one-third of AML cases is an activating mutation of the tyrosine kinase receptor FLT3. The presence of the mutation is associated with adverse prognosis in terms of increased risk of disease relapse. The majority of mutations (25–35% of AML patients) occur as an internal tandem

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Samsung Bioepis Exhibit No. 1002
Page 5091

duplication (ITD) within the juxtamembrane region. A further 3–7% of patients have a mutation within the tyrosine kinase domain (TKD) of FLT3. The identification of the type of mutation, and population size, may have prognostic implications. We have used the Agilent 2100 Bioanalyser to assay PCR reactions for either the ITD or the TKD mutations. The 'chips' used within the Bioanalyser separate RNA or DNA through glass etched micro channels filled with gel matrix, nucleic acid is stained and laser detected. The PCR primers and protocol for the ITD analysis were as previously published (Kottaridis *et al*, *Blood* 2001;98), but within our laboratory the reactions were analysed on the Bioanalyser using a DNA 500 chip. We detected the presence of an ITD in 27% of the 125 AML samples, with the clone population varying from 1.1% to 98%. A separate PCR reaction using primers flanking the TKD domain was used, in combination with an ECO RV or Hinf I (Smith *et al*, MTAL Workshop, 2003) digestion, to detect and quantitate the different mutations. Distinctive digestion and/or heteroduplex patterns were observed that identified D835, D835del, I836del or D839G mutation. In total, TKD mutations were observed in 16% (13.6% D835 mutations, 2.4% I836del, 0% D839G) of samples examined, with clone population from 3.9% to 46.3%. The D835/6 mutation rate was higher than previously reported and may be due to the efficiency and sensitivity of the 2100 BioAnalyser for the analysis of FLT3 mutations.

221

Expression of AML1-ETO in human CD34$^+$ cells blocks granulocytic differentiation and increases self-renewal

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The t(8;21) translocation that encodes the AML1-ETO fusion protein (aka RUNX1-CBF2T1), is one of the most frequent translocations in AML. Although its role in leukaemogenesis is unclear, the fusion gene is believed to dysregulate the expression of genes that are important for normal differentiation and proliferation of haemopoeitic progenitors. Here, we describe the effect of AML1-ETO on granulocyte and monocyte development. Expression of AML1-ETO in human CD34$^+$ cells was achieved by retroviral infection of these cells using a vector co-expressing GFP. The effect of AML1-ETO on myeloid growth and development was initially studied in bulk liquid culture, in the presence of IL-3, SCF and G/GM-CSF. Exogenous expression of AML1-ETO in these cells had an initial inhibitory effect on myeloid growth ($P < 0.01$). Despite this, AML1-ETO expressing cells continued to proliferate >80 d, resulting in a 5×10^3-fold expansion over that observed for control cells (which stopped growing around day 28). In addition, expression of AML1-ETO was able to suppress granulocytic colony formation by 45% when compared to control cultures. In contrast, there was no observed decrease in monocytic colony forming efficiency in cells expressing AML1-ETO. Morphologically, granulocyte terminal differentiation in AML1-ETO expressing cells was inhibited by 83% ($P < 0.01$) giving rise to a build-up of early to intermediate granulocytes, which maintained this appearance throughout the prolonged period of expansion. Phenotypically, these cells were GFP$^+$, CD13$^+$, M-CSF-R$^-$ and showed developmental irregularities such as the failure to lose expression of CD34$^+$ or significantly upregulate CD11b in the same manner as control cells ($P < 0.01$). However, there was no significant difference in CD14 expression in monocytic cells expressing AML1-ETO (GFP$^+$, CD13$^+$, M-CSF-R$^+$). Taken together, these results suggest that expression of AML1-ETO suppressed the differentiation of granulocytic cells and allowed extensive self-renewal to take place supporting a causal role for t(8;21) translocations in leukaemogenesis.

222

The relationship between *FLT3* mutation status, biological characteristics and outcome in patients with acute promyelocytic leukaemia

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The majority of cases of acute promyelocytic leukaemia (APL) are characterized by the t(15;17) leading to formation of the PML-RARα fusion protein. Evidence to date suggests that PML-RARα is insufficient for leukaemogenesis, although the precise nature of cooperating events implicated in generating the full disease phenotype remain uncertain. Potential candidates include activating mutations of FLT3, which could confer a survival and proliferative advantage thereby complementing the differentiation block induced by PML-RARα. The aetiological and clinical significance of *FLT3* mutations in APL is not firmly established. We studied 157 cases of *PML-RARA* positive APL (median age 37 years) treated with ATRA and anthracycline-based chemotherapy in the MRC AML 10/12 trials. *FLT3* mutation was detected in 75/157 cases (48%): 60 length mutations (ITDs), 18 activation loop mutations (D835/I836), including three cases with ITD+D835. Presence of ITDs was correlated with M3v subtype, bcr 3 *PML* breakpoint and expression of reciprocal *RARA-PML* transcripts. The latter association could indicate that expression of the reciprocal fusion product, which has been proposed to contribute to genomic instability predisposes to FLT3 ITD formation, or alternatively FLT3 ITD and t(15;17) could arise through common mechanisms. Microarray analysis using Affymetrix U133A GeneChips undertaken in 25 cases revealed distinct gene expression profiles between cases with ITDs, D835 and wild-type FLT3, suggesting differences in downstream targets according to the nature of the FLT3 mutation. There was a trend towards poorer prognosis in APL with FLT3 mutations (57% survival at 5 years versus 69% wild type, $P = 0.08$), reflecting a higher incidence of induction death (21% vs. 8%, $P = 0.03$); although there was no evidence of effect for *FLT3* mutation status on outcome after stratification for WBC. Nevertheless, the high frequency of activating mutations of FLT3 in cases with WBC above 10×10^9/l (40/51, 78%) provides a rationale for future evaluation of FLT3 inhibitors as a component of induction therapy particularly in this higher risk group of patients.

223

Haemorrhagic cystitis and BK virus: association and treatment

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Objectives: To determine the association between BK virus and haemorrhagic cystitis (HC) and to ascertain the effectiveness of Cidofovir in the treatment of BK-associated HC.

Method: We conducted a prospective study of 44 patients undergoing chemotherapy, PBSCT and BMT. The association between HC and BK virus was studied by monitoring the urinary excretion of BK virus using electron microscopy and polymerase chain reaction. Urine dipstick analysis was performed daily on all patients.

Samsung Bioepis Exhibit No. 1002
Page 5092

Result: HC occurred in eight of 44 (18.1%) patients (seven had allogeneic PBSCT/BMT and one had chemotherapy). BK viruria (>1 positive sample) was detected in all the eight (100%) patients with HC and in six (16.7%) of the remaining 36 patients without HC. BK viruria was detected in 14 of 44 (31.8%) patients undergoing various treatments. Eight of 14 (57%) developed HC whilst none of the 30 BK-negative patients developed this complication. The occurrence of HC and BK viruria was related to the source of the PBSC/BM ($P < 0.0123$). HC occurred in seven of 17 (41.2%) allogeneic PBSCT/BM recipients whilst none of the eight autologous PBSCT recipients developed HC. Among the 17 recipients of allogeneic PBSCT/BMT, BK viruria was detected in 12 (70.6%) patients. Seven of the 12 (58.3%) patients developed HC. Occurrence of BK viruria (but not HC) was also shown to be related to the primary disease ($P = 0.0046$). However, the incidence of HC and BK viruria were unrelated to treatment regimes and occurrence of graft-versus-host-disease. The association between HC and BK was shown to be statistically significant ($P < 0.0001$). Treatment of four of the eight patients with HC associated with BK viruria with Cidofovir resulted in resolution of HC and a reduction in the BK virus load. None of these patients demonstrated any side-effects to Cidofovir.

Conclusion: We conclude that there is a significant association between BK virus and HC. In the event of failed conservative measures, Cidofovir may be used to treat BK-associated HC. However, patients should be monitored closely for side effects to this drug.

224

Sodium valproate, when combined with all-trans retinoic acid, induces apoptosis and histone acetylation in primary blasts from patients with acute myeloid leukaemia (AML)

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There is increasing evidence that the abnormal patterns of gene expression documented in AML can be associated with perturbations in chromatin modelling, raising the possibility that modulators of chromatin structure may be of therapeutic benefit. We and others have examined the potential use of sodium valproate (SV), a potent histone deacetylase inhibitor, in AML. Although SV is able to enhance the response of AML blasts to ATRA *in vitro* previous studies have not correlated the cellular response with changes in histone acetylation and methylation. Blasts from eight patients with AML were exposed to SV (1 mmol/l) and ATRA (100 nmol/l) at levels of both drugs, which are achievable clinically. Treatment with either drug alone had little impact on survival or differentiation of primary AML blasts. In contrast combined therapy with both SV and ATRA resulted in a marked reduction in the numbers of surviving cells together with an increased proportion of cells in the sub-G1 gate on cell cycle analysis, indicative of induced apoptosis. Immunophenotypic analysis demonstrated that myeloid differentiation markers were induced in cells surviving exposure to both agents for 5 d. Histone acetylation was studied after electrophoretic separation of histones and immunostaining with antibodies for specific histone acetylation events. Treatment with 1 mmol/l SV was associated with increased acetylation, which was detectable by 1 h and sustained for 18 h. Concurrent changes in histone methylation were observed. Treatment with ATRA did not alter histone acetylation patterns when used either alone or in combination with SV. In summary, our results confirm that combined SV and ATRA treatment of AML blasts results in both reduced cell survival and a shift towards a more mature postmitotic phenotype. These events are associated with SV mediated alterations in patterns of histone acetylation. These data provide the basis for an ongoing Phase II trial of combined SV and ATRA therapy in patients with relapsed AML.

Oral Communication

Cellular & Molecular Biology

225

Epigenetic inheritance and leukaemogenesis: lessons from the M-CSF receptor gene

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Complex epigenetic mechanisms direct the inheritance of information from mother to daughter cells, which consequently determines specific patterns of gene expression. It is now appreciated that deregulation of these mechanisms is a recurring feature in the establishment and maintenance of oncogenic transformation. We have studied epigenetic aspects of the regulation of the human M-CSF receptor gene (c-FMS), as it is a target for AML1 and AML1-ETO. We have shown that the promoter and elements within the second intron (FIRE) bind transcription factors *in vivo* and macrophage differentiation leads to increased DNaseI hypersensitivity over FIRE. Using chromatin immunoprecipitation assays we have shown that in t(8;21) cells, the two elements within the second intron, FIRE and FIRE-1kb, are targets for AML1/ETO, which closely follows HDAC1 recruitment. Leukaemic t(8;21) cell lines express low levels of c-FMS mRNA, yet displayed strong DNaseI hypersensitivity over the intronic elements. We showed that AML1-ETO repressed FIRE activity, and this associated with increased methylation and decreased acetylation of histone H3 lysine 9 at key regulatory elements. DMS *in vivo* footprinting experiments of c-FMS cis-regulatory elements with leukaemia patient samples showed that CD34+ blast cells had transcription factor complexes bound at FIRE independent of the presence of t(8;21). This did not induce DNA cytosine methylation, as the presence of AML1-ETO did not associate with increased CpG methylation at any c-FMS regulatory element. Despite having a bound complement of transcription factors resembling highly expressing macrophages, the leukaemic cells, however, showed DNA topology more closely resembling non-expressing Hela cells, as evidenced by *in vivo* UV footprinting. It appears, therefore, that binding AML1-ETO to the c-FMS second intron leads to repression of gene expression through 'active' repression rather than assembly of the gene into epigenetically silent heterochromatin. We postulate that this may contribute to the monocytic differentiation block observed in these leukaemias.

226

Inhibition of the internal tandem duplicated FLT3 receptor by tyrosine kinase inhibitor PKC412 alters DNA repair kinetics and down-regulates DNA repair enzymes, RAD51, DNA PK, PARP, and MSH2

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The presence of the FLT3 receptor ITD mutation confers a worse DFS and OS in AML. Increased relapse risk may result from upregulated

Samsung Bioepis Exhibit No. 1002
Page 5093

DNA repair following chemotherapeutic agent exposure. The mutated FLT3 receptor signals via STAT5, a transcriptional activator for genes including the DNA repair enzyme RAD51. We investigated whether inhibition of FLT3 would alter DNA repair following subtoxic doses of daunorubicin. Cell lines MV4-11 (homozygous for FLT3 ITD) and HL60 (FLT3 wild type (WT) and six-patient AML samples (three ITD, three WT) were incubated with PKC412 for 24 h, then exposed to cytostatic doses of daunorubicin. DNA damage was assessed by comet assay before and during 24 h of repair. In comparison with controls MV4-11 and one-third ITD patient samples showed loss of early (within 1 h) repair when treated with PKC412, however this was not seen in HL60 or WT patient samples. Phosphorylated STAT5 and total cellular phosphotyrosine were measured by flow cytometry. PKC412 incubation resulted in reduction in phosphoSTAT5 and Phosphotyrosine in MV4-11 (39.5% and 65% respectively) with no response in HL60s. This was confirmed in patient samples. Mean reduction for ITDs 50.5% and 11.1%; and for WTs 0% vs. 5.8% respectively. Quantitative PCR was performed on cDNA made from RNA extracted from cell line and patient samples before and after incubation with PKC412. RAD51, DNA PK, PARP, and MSH2 levels were down-regulated in MV4-11s in contrast to HL60s. RAD51 and DNA PK also down-regulated, in ITD but not WT patient samples. In summary incubation with PKC412 resulted in a decrease in early DNA repair following sublethal DNA damage by daunorubicin at doses which down-regulated phosphoSTAT5 and total phosphotyrosine levels, and which resulted in a reduction in DNA repair enzyme mRNA levels. This would suggest that FLT3 mutations might confer their poor prognosis in part via upregulation of DNA repair following cytotoxic drug exposure.

227

An SCL +19 enhancer targets haematopoietic progenitors and endothelium in the mouse embryo and postnatal mice

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The stem cell leukaemia (SCL) gene is a basic loop-helix transcription factor with a critical role in normal haematopoiesis and angiogenesis. The SCL gene is normally expressed in haematopoietic stem cells, mast cells, erythroid cells, megakaryocytes and endothelium. Aberrant expression of the SCL gene leads to T-cell acute lymphoblastic leukaemia, whereas SCL−/− mice die due to the absence of haematopoiesis. Hence, temporal and spatial regulation of SCL expression is essential. Our laboratory has previously characterized a 5.5 kb enhancer located 3′ of the SCL transcription start site, which is capable of targeting the expression of β-galactozidase (lacZ) reporter gene to embryonic endothelium and haematopoietic stem cells in the foetal liver and the bone marrow. Subsequent experiments showed that a 641-base pair core enhancer gave an identical pattern of lacZ expression in the embryo. However, it was unclear if this element (later referred to as the +19 enhancer) was capable of maintaining reporter gene expression into the adulthood, since no lacZ activity was observed in postnatal mice. Using a transgenic construct, which contains a eukaryotic reporter gene, human placental alkaline phosphatase, we show that the +19 enhancer targets haematopoietic progenitors and endothelium in the embryo and in the adult mouse. Phenotypic analysis of the transgene-positive cells in the foetal liver and the bone marrow by fluorescence-activated cell sorting demonstrates that they are present within the c-kit-bright population. It is therefore likely that the +19 enhancer targets haematopoietic stem cells. Long-term reconstitution experiments are currently underway to confirm this hypothesis.

228

Differential HOX gene expression in differentiating myeloid cell lines

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In mammalian cells the structure of chromatin dictates the accessibility of chromosomal DNA to transcription factors. Regulation of the acetylation and methylation status of core histone proteins results in local chromatin remodelling. In neoplastic cells this causes the silencing of genes encoding anti-oncogenic transcription factors. Aberrant expression of transcription factors, e.g. the PML-RARα fusion protein, recruit transcriptional repressors including histone deacetylases (HDACs) which condense chromatin into inaccessible units. *Hox* genes are master regulators of developmental processes and play a crucial role in haematopoiesis. Global down-regulation of *Hox* gene expression has been reported in PML-RARα patients, suggesting that *Hox* genes are direct targets of the particular HDAC-repressor complex associated with this fusion protein. A real-time PCR approach was undertaken to evaluate the short-term effects of two HDAC inhibitors, trichostatin A (TSA), and sodium valproate (NaV) alone and in combination with all-trans retinoic acid (ATRA) in a panel of leukaemic cell lines (HL-60, NB4, KG1a, K562, Kasumi-1, Mono Mac 6 and U937). Up-regulation of *CD86* was used as a marker of differentiation, and histological studies were performed to confirm the differentiating capacity of these treatments. *CD86* was found to be up-regulated significantly in the HL60, Mono Mac 6 and NB4 cell lines (>15-fold) within 8 h of combined TSA plus ATRA treatment. NaV plus ATRA induced greater expression of a number of *Hox* genes from the *HOXB* and *HOXD* cluster (particularly *B1*, *D8* and *D10*) in comparison with TSA plus ATRA treatment, suggesting these genes are repressed by an HDAC-containing complex in the normal state. Other *Hox* genes remained unaffected by the treatments, including *HOXA9*, indicating a degree of specificity of these treatments. Clinical trials involving HDAC inhibitors with differentiation agents are currently ongoing and monitoring of *Hox* gene expression during such treatments may prove beneficial.

229

CD8-independent, MHC class I-restricted antigen recognition by murine CD4 T cells via transferred T-cell receptors

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CD4 T helper cells can play a critical role in the anti-tumour immune response. Cytokines secreted by CD4 T cells can have a direct effect on tumour cells and provide help for CTL priming and effector function. In this study we tested if it was possible to generate MHC-class I-restricted helper T cells by retroviral TCR gene transfer into CD4 lymphocytes. We used a TCR that recognizes the influenza virus A nucleoprotein (NP366–379) peptide in the context of murine D^b MHC class I. Murine splenocytes were isolated from

Samsung Bioepis Exhibit No. 1002
Page 5094

C57Bl6 mice ($H2^b$) and activated with conconavalin A and IL-7, and after 48 h transduced with the pMX-TCRα-IRES-TCRβ retroviral vector. The transduced splenocytes were then cultured in the presence of IL2 for a further 48 h before staining with anti-murine CD4, CD8 and V-β-11 antibodies and sorting into CD4 V-β-11 positive and CD8 V-β-11 positive populations. Sorted cells were expanded for a further 48–72 h prior to functional assays. The sorted TCR-transduced cells were tested for γ-interferon secretion in response to BM-derived dendritic cells (DCs) pulsed with NP peptide, an irrelevant peptide (MDM100) or no peptide. Secreted γ-interferon was measured by ELISA. The results are shown in the table below. Antigen-specific γ-interferon secretion was observed by both CD8-sorted cells (100% purity by FACS) and CD4 sorted cells (99.93% purity). The results indicate that it is feasible to generate CD4 helper T cells that are specific for peptide epitopes presented in the context of MHC class I.

Effector cells	Peptide (10 μmol/l)	Gamma Interferon (ng/ml) at +6 h	Gamma interferon (ng/ml) at +24 h
CD8 – F5TCR	NP	32.07	185.4
CD8 – F5TCR	MDM100	0.04	0.236
CD8 – F5TCR	No peptide	0.07	0.364
CD4 – F5TCR	NP	2.04	178.5
CD4 – F5TCR	MDM100	0.09	0.082
CD4 – F5TCR	No peptide	0.161	0.156

230

JUNE-1 is a novel gene involved in erythroid differentiation

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The process of erythropoiesis is initiated by the action of erythropoietin (EPO) on erythroid progenitors, inducing a complex cascade of reactions leading to red blood cell development. In addition to erythroid progenitors, receptors for EPO have been found on tumour cells; an important finding, since EPO is used to treat anaemia in cancer patients. However, relatively little is known about genes that are regulated by EPO. The aim of our group is to investigate genes induced by EPO to give a fuller understanding of the downstream effects of this hormone in cellular proliferation and differentiation. Using differential display technology we identified a novel EPO induced gene that we have named *JUNE-1*. We have cloned full-length cDNAs encoding human and murine *JUNE-1*. The intron/exon structure of the gene has been analysed, and its chromosomal localization with respect to known polymorphic markers determined. Murine *JUNE-1* mRNA is expressed in all tissues examined so far, including tissues involved in haematopoiesis, such as spleen and kidney. The mRNA expression pattern of human *JUNE-1* is under investigation, and it has so far been shown to be highly expressed in a variety of leukaemia and lymphoma cell lines. A monoclonal antibody to human *JUNE-1* is in development, which will be used in protein expression and functional studies. Several potential binding sites for haematological transcription factors have been identified in the promoter region. The C-terminal region contains a *p*lant *h*omeo *d*omain (PHD), indicating a possible role in DNA or chromatin binding, with putative actions in transcriptional regulation or chromatin remodelling. This is supported by the presence of a nuclear targeting motif in the protein sequence. These observations are being further explored using assays such as yeast-two-hybrid and ChIP analysis, to further delineate the functions of *JUNE-1* in processes including erythroid differentiation, proliferation, and tumour biology.

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Samsung Bioepis Exhibit No. 1002
Page 5095

Annex 1

Pfizer Inc's opposition statement against EP 1 720 571 B1 (ALEXION PHARMACEUTICALS, INC.)
"Method of treating hemolytic disease"

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1 Introduction

This constitutes opponent Pfizer Inc's statement under Rule 76 (c) EPC, namely the statement of the extent to which European Patent number EP 1 720 571 B1 ("the Patent") is opposed and the grounds on which the opposition is based as well as an indication of facts, evidence and arguments presented of these grounds.

The following documents are introduced into the proceedings:

	Document	**Publication date**
D1	Hillmen et al, The New England Journal of Medecine 2004;350:552-9	Feb 5, 2004
D2	Medscape Medical News, Dec 13, 2002, Jane Salodof MacNeil	Dec 2002
D3	Hillmen et al, British Journal of Hematology 121 (Suppl. 1) 87-95, 2003, Abstract 257	April 2003
D4	Hillmen et al, Blood. 100(11). November 16, 2002. Abstract No. 154	Nov 2002
D5	Hillmen et al, Journal of The American Society of Hematology 102(11), 2003, Abstract 1858	Nov 2003
D6	Rother et al, Molecular Immunology. 40(2003), p 197, Abstract 54	Sept 2003
D7	Rosse et al, American Society of Hematology – Education program, January 2004 (2004-01), pages 48-62	Jan 2004
D8	Biotechnology News, 17 DEC 2004, 24(30), p.10	Dec 2004
D9	U.S. Patent No. 6,355,245	2002
D10	Thomas et al, Molecular Immunology, Vol. 33, No. 17/18, pages 1389-1401, 1996	1996
D11	Drugs of the Future 2004, 29(7): 673-676	July 2004
D12	Hillmen et al, British Journal of Haematology, 125 (Suppl.1),1-72, Abstract 5	April 2004
	Decision	
	T1979/09	Sept 2011

All the claims of the Patent are opposed.

The grounds on which the Patent is opposed are:
Article 100(c)/Article 123(2) EPC: Added subject-matter
Article 100(a)/Article 54 EPC: Lack of novelty
Article 100(a)/ Article 56 EPC: Lack of inventive step
Article 100(b)/Article 83 EPC: Insufficiency of disclosure

Samsung Bioepis Exhibit No. 1002
Page 5096

2- The patent

2.1 Formalities

The Patent was granted on European Patent Application No.05712607.0, which was derived from PCT Patent Application No. PCT/US2005/003225 ("the PCT Application" or "the Application as filed"), which was filed on Feb 03, 2005 ("the Filing Date") and published as WO2005/074607. Priority was claimed from US Patent Application No. 10/771,552 ("Priority Application") filed on Feb 03, 2004 ("Claimed Priority Date").

2.2 The Claims

There are eight claims in the Patent, two of which are independent claims, namely claims 1 and 5. The remaining claims are all dependent, directly or indirectly, on at least one of the independent claims.

3- Requests

It is requested that the Patent be revoked in its entirety.

Oral proceedings are requested if the Patent cannot be revoked on the basis of the written submissions alone. If oral proceedings take place and either the Patentee or any other party chooses to speak in French or German, simultaneous translation into English is requested.

4- Added subject matter (Art 100(c)/Art 123(2) EPC)

According to Art.123(2) EPC a European patent application may not be amended in such a way that it contains subject-matter which extends beyond the content of the application as filed. It is therefore, according to Art.100(c) EPC, a ground of opposition that a European patent contains subject-matter that extends beyond the content of the application as filed. According to the Guidelines for examination (H-IV, 2.2), this legal principle is to be interpreted to mean that "An amendment should be regarded as introducing subject-matter which extends beyond the content of the application as filed, and therefore unallowable, if the overall change in the content of the application (whether by way of addition, alteration or excision) results in the skilled person being presented with information which is not directly and unambiguously derivable from that previously presented by the application, even when account is taken of matter which is implicit to a person skilled in the art".

The combination of features in claims 1 and 5 of the opposed patent is not directly and unambiguously disclosed by the application as filed, thereby contravening Art.123(2) EPC.

Claims 1 and 5 of the opposed patent were introduced into the application by way of amendment and contain, in combination, the following features:

(1) the use of eculizumab
(2) the treatment of a patient afflicted with paroxysmal nocturnal hemoglobinuria
(3) intravenous administration
(4) administration of 600 mg each week for four weeks

(5) administration of 900 mg a week later
(6) administration of 900 mg biweekly thereafter
(7) a breakthrough in serum hemolytic activity when the patient is treated under a dosing schedule according to (4) to (6)
(8) administration of 900 mg every 12 days

There is no mention in the claims of the application as filed of any of features (2) to (8), these claims relating to the treatment of hemolytic disease in general terms and not mentioning any features relating to the route of administration or dosage regimen. Therefore, to be allowable according to Art.100(c) EPC, the combination of features (1)-(8) must be directly and unambiguously derivable from the description.

The description discloses feature (2) on page 11, lines 5-6: "The present disclosure relates to a method of treating paroxysmal nocturnal hemoglobinuria ("PNH") and other hemolytic diseases in mammals". Within this broad umbrella, the description contemplates the treatment of a variety of different diseases and symptoms such as abdominal pain, fatigue, dyspnea, insomnia, hemoglobinuria, anemia, erectile dysfunction, dysphagia, thrombosis (page 23, lines 7-17).

As for feature (1), it is disclosed that a large number of compounds of varying pharmacology can be used in this method. These compounds bind to or otherwise block the generation and/or activity of one or more complement components (page 11, lines 24-25). From this broad class, anti-C5 antibodies are described as particularly useful (page 12, lines 1-3) and h5G1.1-mAb (eculizumab) is described as a particularly useful anti-C5 antibody (page 18, lines 12-20).

Regarding feature (3), the selected compound can be administered via a number of routes, one of which is "intravenous infusion by injection" (page 20, lines 4-8). However, the term "intravenous administration" as used in the claim, covers both injection into a vein using a hypodermic needle and the use of a catheter and therefore has a broader meaning than "intravenous infusion by injection".

There is no disclosure in the general description of the invention of any specific dosage or dosage regimen. On the contrary, it is disclosed that the dose selected should vary considerably depending on the compound selected, the manner of administration, the particular symptoms of the patient, the overall health, condition, size and age of the patient, and the judgment of the prescribing physician (page 19, lines 17-23; page 20, lines 1-3). The general teaching of the application, therefore, is that the dose to be administered should be chosen by the physician on a patient-by-patient basis.

In fact, the only basis for features (4)-(8) comes from the specific Example of the application as filed presented on pages 32-39. In this specific, detailed example, eleven patients from a particular patient group (PNH patients that were transfusion-dependent and hemolytic) were treated weekly with a 600 mg intravenous infusion of eculizumab for four weeks, then with 900 mg eculizumab one week later (by an unspecified route) and then with 900 mg eculizumab on a bi-weekly basis (page 32, lines 5-16; also by an unspecified route). Two of the patients exhibited breakthrough in serum hemolytic activity during this biweekly phase of the treatment which was controlled by reducing the dosing interval from fourteen to twelve days (page 35, lines 8-17).

Samsung Bioepis Exhibit No. 1002
Page 5098

There is therefore no unambiguous disclosure of the claimed invention in the description. Rather, the features of the claim are drawn separately and variously from different parts of the disclosure, some of which are contradictory, requiring the creation of intermediate generalisations and/or selections.

The subject-matter of claim 1 is not directly and unambiguously derivable from the general part of the description since:

(a) It is necessary to select a pharmacological class (anti-C5 antibody) from a list of such classes (compounds that bind to or otherwise block the generation and/or activity of one or more complement components); a specific compound (eculizumab) from a list of compounds within that class; the disease PNH as the hemolytic disease to be treated from a list of several diseases; and a route of administration (intravenous) from several possible routes.

(b) There is no disclosure of "intravenous administration" in general terms, only the disclosure of "intravenous infusion by injection".

(c) There is no disclosure of any specific dosage regimen, only the disclosure that dosage will vary depending on the exact clinical situation to be addressed.

The subject-matter of claim 1 is not directly and unambiguously derivable from the Example since:

(a) the example is a specific disclosure that contains several other essential features that are missing from claim 1. In particular, the patients in this study were transfusion-dependent and hemolytic – this is a specific patient sub-group and the invention has not been shown to work in other sub-groups. Claim 1 therefore represents a generalisation of the actual teaching of the Example. Moreover, the specific meaning ascribed to the term "transfusion-dependent" on page 32, lines 7-9 should be specified in the claim. Likewise, the infusion time of "approximately 30 minutes" (page 32, lines 12-14) is not specified in the claim, despite being an essential feature of the Example. Finally, the breakthrough occurred in the extension study between weeks 12 and 64 (page 32 lines 17-20; page 35, lines 8-9) whereas the claim is not so limited and covers treatment regimens where the shorter 12 day treatment cycle is initiated in the initial 12 week acute phase of treatment.

(b) In the Example the patients received "a weekly 600 mg intravenous infusion" of eculizumab which implies (i) the drug was administered at time points one week apart and (ii) a catheter-based intravenous infusion was used to administer the drug. The corresponding features of the claim are "intravenous administration" which covers the use of a catheter and a direct injection and administration of "600 mg each week for four weeks" which implies that the drug was administered once within each of four successive calendar weeks but not necessarily at weekly intervals.

(c) In the Example, the route by which the drug was administered following the initial four week dosing is not specified whereas in the claim the route is specified as the intravenous route.

It is furthermore not appropriate to combine features from the general part of the description and the Example since:

Samsung Bioepis Exhibit No. 1002
Page 5099

(a) This would create an intermediate generalisation – the features of the Example are detailed and specific and associated with each other and to take one or more out of this narrow context and combine it with features of the invention described in general terms would create embodiments of the invention which were not contemplated in the application as filed.

(b) The general part of the description and the Example are not compatible teachings in that the general part of the description clearly contemplates a treatment in which the dosage is selected on a patient-by-patient basis according to the exact clinical situation presented whereas the Example teaches that the same, specific dosage should be administered to each patient regardless of the particular symptoms, overall health, condition, size and age of the patient.

Therefore, claims 1 and 5 of the patent include subject matter which is not directly and unambiguously derivable from the description or claims of the application as filed and are not allowable according to Art.123(2) and Art.100(c) EPC. The dependent claims, which all contain the features of claims 1 and 5, also contravene these provisions.

5- Lack of novelty (Article 100(a)/Art 54 EPC)

5.1 – The subject matter of claim 1 and 5 is not entitled to the priority

According to Art.87(1) EPC, the priority of an earlier application may be claimed if it discloses "the same invention". According to the Guidelines for Examination (F-VI, 1.3) and G2/98, this is to be interpreted to mean that the subject matter of a claim in a European patent may enjoy the priority of a previous application only if the skilled person can derive the subject-matter of the claim directly and unambiguously, using common general knowledge, from the previous application as a whole.

The combination of features in claims 1 and 5 of the opposed patent is not directly and unambiguously disclosed in the priority application (US patent application No. 10/771,552 filed on 03 February 2004), in contravention of Art.87(1). The claims are therefore not entitled to the priority date.

Claims 1 and 5 of the opposed patent contain, in combination, the following features:

(1) the use of eculizumab
(2) the treatment of a patient afflicted with paroxysmal nocturnal hemoglobinuria
(3) intravenous administration
(4) administration of 600 mg each week for four weeks
(5) administration of 900 mg a week later
(6) administration of 900 mg biweekly thereafter
(7) a breakthrough in serum hemolytic activity when the patient is treated under a dosing schedule according to (4) to (6)
(8) administration of 900 mg every 12 days

There is no mention in the claims of the priority application of any of features (2) to (8), these claims relating to the treatment of hemolytic disease in general terms and not mentioning any

Samsung Bioepis Exhibit No. 1002
Page 5100

features relating to the route of administration or dosage regimen. Therefore, for the priority claim to be valid, the combination of features (1)-(8) must be directly and unambiguously derivable from the description.

The description of the priority application discloses feature (2) on page 4, lines 13-14: "Paroxysmal nocturnal hemoglobinuria ("PNH") and other hemolytic diseases are treated in accordance with this disclosure". Within this broad umbrella, the description contemplates the treatment of a variety of different diseases and symptoms such as hemoglobinuria, anemia, fatigue, low red blood cell count, erectile dysfunction, dysphagia and thrombosis (page 22, lines 12-17).

As for feature (1), it is disclosed that a large number of compounds of varying pharmacology can be used in this method. These compounds bind to or otherwise block the generation and/or activity of one or more complement components (page 11, lines 7-9). From this broad class, anti-C5 antibodies are described as particularly useful (page 11, lines 9-11) and h5G1.1-mAb (eculizumab) is described as a particularly useful anti-C5 antibody (page 17, line 23 – page 18, line 8).

Regarding feature (3), the selected compound can be administered via a number of routes, one of which is "intravenous infusion by injection" (page 19, lines 15-18). However, the term "intravenous administration" as used in the claim, covers both injection into a vein using a hypodermic needle and the use of a catheter and therefore has a broader meaning than "intravenous infusion by injection".

There is no disclosure in the general description in the priority application of any specific dosage or dosage regimen. On the contrary, it is disclosed that the dose selected should vary considerably depending on the manner of administration, the particular symptoms of the patient, the overall health, condition, size and age of the patient, and the judgement of the prescribing physician (page 19, lines 12-14). The general teaching of the application, therefore, is that the dose to be administered should be chosen by the physician on a patient-by-patient basis.

In fact, the only basis for features (4)-(8) comes from the specific Example of the invention presented on pages 30-35. In this specific, detailed example, eleven patients from a particular patient group (PNH patients that were transfusion-dependent and hemolytic) were treated weekly with a 600 mg intravenous infusion of eculizumab for four weeks, then with 900 mg eculizumab one week later (no route of administration specified) and then with 900 mg eculizumab on a bi-weekly basis (page 30, lines 6-16; no route of administration specified). One of the patients exhibited breakthrough in serum hemolytic activity during this biweekly phase of the treatment which was controlled by reducing the dosing interval from fourteen to twelve days (page 32, lines 13-19).

There is therefore no unambiguous disclosure of the claimed invention in the description. Rather, the features of the claim are drawn separately and variously from different parts of the disclosure, some of which are contradictory, requiring the creation of intermediate generalisations and/or selections.

The subject-matter of claim 1 is not directly and unambiguously derivable from the general part of the description since:

Samsung Bioepis Exhibit No. 1002
Page 5101

(a) It is necessary to select a pharmacological class (anti-C5 antibody) from a list of such classes (compounds that bind to or otherwise block the generation and/or activity of one or more complement components); a specific compound (eculizumab) from a list of compounds within that class; the disease PNH as the hemolytic disease to be treated from a list of several diseases; and a route of administration (intravenous) from several possible routes.

(b) There is no disclosure of "intravenous administration" in general terms, only the disclosure of "intravenous infusion by injection".

(c) There is no disclosure of any specific dosage regimen, only the disclosure that dosage will vary depending on the exact clinical situation to be addressed.

The subject-matter of claim 1 is not directly and unambiguously derivable from the Example since:

(a) the example is a specific disclosure that contains several other essential features that are missing from claim 1. In particular, the patients in this study were transfusion-dependent and hemolytic – this is a specific patient sub-group and the invention has not been shown to work in other sub-groups. Claim 1 therefore represents a generalisation of the actual teaching of the Example. Moreover, the specific meaning ascribed to the term "transfusion-dependent" on page 30, lines 8-9 should be specified in the claim. Likewise, the infusion time of "approximately 30 minutes" (page 30, lines 13-14) is not specified in the claim, despite being an essential feature of the Example.

(b) In the Example the patients received "a weekly 600 mg intravenous infusion" of eculizumab which implies (i) the drug was administered at time points one week apart and (ii) a catheter-based intravenous infusion was used to administer the drug. The corresponding features of the claim are "intravenous administration" which covers the use of a catheter and a direct injection and administration of "600 mg each week for four weeks" which implies that the drug was administered once within each of four successive calendar weeks but not necessarily at weekly intervals.

(c) In the Example, the route by which the drug was administered following the initial four week dosing is not specified whereas in the claim the route is specified as the intravenous route.

It is furthermore not appropriate to combine features from the general part of the description and the Example since:

(a) This would create an intermediate generalisation – the features of the Example are detailed and specific and associated with each other and to take one or more out of this narrow context and combine it with features of the invention described in general terms would create embodiments of the invention which were not contemplated in the application as filed.

(b) The general part of the description and the Example are not compatible teachings in that the general part of the description clearly contemplates a treatment in which the dosage is selected on a patient-by-patient basis according to the exact clinical situation presented whereas the Example teaches that the same, specific dosage

Samsung Bioepis Exhibit No. 1002
Page 5102

should be administered to each patient regardless of the particular symptoms, overall health, condition, size and age of the patient.

Therefore, claims 1 and 5 of the patent include subject matter which is not directly and unambiguously derivable from the priority application and are not entitled to the priority date. The validity of these claims is consequently to be considered as of the filing date of 03 February 2005.

5.2 – Features of claims 1 and 5 as granted

Claim 1 as granted contains the following features:

(1) the use of eculizumab
(2) the treatment of a patient afflicted with paroxysmal nocturnal hemoglobinuria
(3) intravenous administration
(4) administration of 600 mg each week for four weeks
(5) administration of 900 mg a week later
(6) administration of 900 mg biweekly thereafter
(7) a breakthrough in serum hemolytic activity when the patient is treated under a dosing schedule according to (4) to (6)
(8) administration of 900 mg every 12 days

The same numbering can be applied to claim 5 which comprises the same features as claim 1. The above numbering of the features of claims 1 and 5 will be used in the following sections.

5.3 – D1 (Hillmen et Al, 2004) anticipates the subject matter of claims 1 to 8

D1 published on Feb 5, 2004 and is therefore part of the state of the art under Art 54(2) EPC against claims which are not entitled to the priority date.

- Claims 1 and 5

On page 552, paragraph entitled "Methods", D1 describes a trial where 11 patients with PNH **(2)** received infusions **(3)** of Eculizumab **(1)** (600mg) every week for 4 weeks **(4)**, followed one week later by a 900-mg dose **(5)** and then by 900 mg every other week **(6)** through week 12.

On page 555, last sentence to page 556, first sentence, D1 describes one patient from the trial in whom eculizumab level fell below 35µg per milliliter at week 12, hemolytic activity returned **(7)** and lactate deshydrogenase levels increased transiently. The dosing frequency was increased from 900 mg every 14 days to 900 mg every 12 days **(8)**, reestablishing complete complement blockade during the ongoing 12-month extension study (data not shown).

Therefore, D1 anticipates claims 1 and 5 which lack novelty.

Samsung Bioepis Exhibit No. 1002
Page 5103

- 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

Samsung Bioepis Exhibit No. 1002
Page 5104

completed a 3-month pilot study receiving 600 mg Eculizumab by intravenous infusion over 30 min weekly for four doses and then 900 mg every other week."

D4 (Hillmen et al) is an abstract published on Nov 16, 2002 and is therefore part of the state of the art under Art 54(2) EPC against all claims. D4 discloses that "Ten transfusion-dependent PNH patients have been entered into an ongoing open label Pilot study of eculizumab and all patients will have completed the study by November 2002. Patients receive 600 mg eculizumab by intravenous infusion over 30 minutes weekly for 4 doses and then 900 mg every other week".

D6 (Rother et Al) is an abstract published in Sept 2003 and is therefore part of the state of the art under Art 54(2) EPC against all claims. D6 discloses that "Eleven transfusion dependent PNH patients have completed an open-label pilot study of eculizumab. Patients received 600 mg eculizumab by intravenous infusion over 30 min weekly for four doses, followed by 900 mg 1 week later, and then 900 mg every other week for the remainder of the trial."

It was therefore very well established at the priority date that Eculizumab could be used for treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein eculizumab is to be administered intravenously to the patient under the following dosing schedule: 600 mg each week for four weeks; 900 mg 1 week later and 900 mg on a biweekly basis.

6.2 Reducing the dosing interval to prevent potential breakthrough in patients treated with 900mg Eculizumab biweekly was known before the priority date.

D5 (Hillmen et al) is an abstract published on Nov 16 2003 and is therefore part of the state of the art under Art 54(2) EPC against all claims.

D5 relates to the results of the extension of the study disclosed in D4. On lines 5 to 8, D5 discloses that,

> *"We previously reported that eculizumab was very effective at acutely controlling intravascular hemolysis in a 3-month Pilot study of transfusion-dependent PNH patients. We now report that all 11 patients from the initial study entered an extension trial of 900mg eculizumab by i.v. infusion over 30 minutes every other week for one year."*

The pilot study mentioned in the above paragraph is the pilot study discussed lines 8 to 10 of D4. This is consistent with the disclosure of D4, which also mentions that the patients who have completed the pilot study have all entered into an extension study which results are reported in D5.

Therefore the patients from the study discussed in D5 have received 600 mg eculizumab by intravenous infusion over 30 minutes weekly for 4 doses and then 900 mg every other week during the pilot study disclosed in detail in D4. Then they entered into the extension study and continued to receive 900 mg eculizumab by i.v. infusion over 30 minutes every other week (see D5, l.7-8).

On page 510a, 1st col., l.8-12, D5 discloses that

> *"Two patients experienced transient breakthroughs in complement activity associated with increased LDH, hemoglobinuria and mild symptoms, these resolved immediately upon next dose of eculizumab and further breakthroughs were prevented by a slight decrease in the dosing interval."*

It was therefore very well established at the priority date that breakthroughs in serum hemolytic activity observed in patients afflicted with PNH and treated with Eculizumab under a dosing schedule according to items (i) to (iii) of claim 1 as granted could be prevented by slightly decreasing the dosing interval.

The difference between the subject matter of claim 1 and the disclosure of D5 is that the reduced dosing interval is 12 days.

The effect of this difference is to prevent further breakthrough in serum hemolytic activity in patients which exhibit such breakthrough when treated under a dosing schedule according to items (i) to (iii) of claim 1 as granted.

The objective technical problem to be solved is to identify a reduced interval allowing prevention of further breakthrough in serum hemolytic activity in said patients.

The man skilled in the art, starting from D5 and aiming at reducing the interval between two 900mg doses would have tried different intervals shorter than 14 days and would have determined very easily that a 12 days interval is suitable for preventing further breakthrough in serum hemolytic activity in patients exhibiting breakthrough when treated under a dosing schedule according to items (i) to (iii) of claim 1 as granted. As shown in figure 5 of the patent, this is exactly what has been done during the trial described in the examples of the patent. Figure 5 shows the effect of the dosing interval on hemoglobinuria in one of the two patients exhibiting breakthrough of hemolysis activity. When Eculizumab is administered every 14 days, hemoglobinuria is observed before the next dose is administered (see Fig.5 urine grade at day 13). The dosing interval was reduced to 13 days and hemoglobinuria was still observed on the last day of the interval (see Fig.5 urine grade at day 41). Therefore, the dosing interval was reduced to 12 days and no further hemoglobinuria event is observed in Fig.5.

The skilled person, observing a breakthrough in hemolytic activity in a patient administered with 900mg every 14 days, would have applied the teaching of D5 and reduced the dosing interval until it reaches the 12 days dosing interval such that further breakthrough were prevented. Indeed such a reduction in dosing interval represents a routine modification.

We make reference to T1979/09. Section 7 of the reasons of the decision concern an auxiliary request which included a dosage regimen. Reasons 7.3.1 states that:

> *"It is well known that in designing dosage regimen the two major parameters that can be adjusted are the dose itself, i.e. the quantity of drug administered, and the dosing frequency, i.e. the time interval between doses."*

Samsung Bioepis Exhibit No. 1002
Page 5106

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

Samsung Bioepis Exhibit No. 1002
Page 5107

h5G1.1 HuG4 is also discussed on page 1396 (paragraph bridging col.1 and 2) and on page 1399, col.2. However, the exact sequence of the humanized antibody is not disclosed in D10.

In view of the above, none of the patent, D9 or D10 provides a detailed description of the structure of h5G1.1 mAb or Eculizumab.

Therefore, the man skilled in the art would not be able to carry out the invention as he would not be able to prepare the specific antibody Eculizumab, which is an essential feature of the claims 1 to 8.

Therefore claims 1 to 8 are not sufficiently disclosed in the sense of Article 83 EPC.

7.2 Claims 1 to 8 contravene Article 83 because the invention is not disclosed in a manner sufficiently clear and complete.

Claim 1 as granted comprises the feature that the "patient exhibits breakthrough in serum hemolytic activity when treated under a dosing schedule according to items (i) to (iii)".

The term "breakthrough in serum hemolytic activity" is neither defined in claim 1 nor in the general disclosure of the application as filed. This term is used in the example section (see page 35, lines 9-10) and in particular in connection with two patients who did not sustain levels of Eculizumab necessary to consistently block complement. Again, no definition of this term is provided in the example. On page 35, lines 11 to 15, the application as filed discloses that "breakthrough of complement blockade resulted in hemoglobuniria, dysphagia and increased LDH and AST which correlated with the return of the hemolytic activity". These events (hemoglobinuria, dysphagia and increased LDH and AST) seem to be associated to some extent with serum hemolytic activity. However, it is not clear if one, two, three or four of these events are necessary and/or sufficient to characterize a breakthrough according to claim 1.

Claim 1 does not provide any indication relative to the characterization of "breakthrough in serum hemolytic activity". In the absence of additional guidance from the description, the skilled person would not be able to determine when a patient exhibits breakthrough in serum hemolytic activity and as a consequence would not be able to determine if and when the dosing interval should be reduced to 12 days.

Therefore, the skilled person would not be able to carry out the subject matter of claims 1 to 8 which therefore contravene Art.83 EPC.

Samsung Bioepis Exhibit No. 1002
Page 5108

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ROYAUME UNI

Date
07.05.2018

Reference	Application No./Patent No.
	16195355.9 - 1112 / 3167888
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

Summons to attend oral proceedings pursuant to Rule 115(1) EPC

You are hereby summoned to attend oral proceedings arranged in connection with the above-mentioned European patent application.

The matters to be discussed are set out in the communication accompanying this summons (EPO Form 2906).

The oral proceedings, which will not be public, will take place before the Examining Division

on 05.02.19 at 09.00 hrs at the EPO,
PschorrHöfe, Bayerstr. 34, 80335 Munich

No changes to the date of the oral proceedings can be made, except on serious grounds (see OJ EPO 2009, 68). If you do not appear as summoned, the oral proceedings may continue without you (R. 115(2) EPC, see also OJ EPO 2008, 471).

Your attention is drawn to Rule 4 EPC, regarding the language of the oral proceedings, and to Special edition No. 3 OJ EPO 2007, L.1, concerning the filing of authorisations for company employees and lawyers acting as representatives before the EPO.

The final date for making written submissions and/or amendments (R. 116 EPC) is 04.01.19.

The actual room number as well as the waiting room numbers will be given to you by the porter in the foyer at the above EPO address.

Parking is available in the underground car park, accessible only via the entrance "Grasserstrasse 2/6". On presentation of the summons to oral proceedings at one of the porters' lodges in the PschorrHöfe, the parking ticket will be revoked.

Please note that the oral proceedings will always take place as scheduled unless you are otherwise informed. The EPO will generally not send a reminder or a further communication prior to the date of oral proceedings.

1st Examiner:	2nd Examiner:	Chairman:
Collura A	Terenzi C	Orlando M

Registered letter with advice of delivery
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Samsung Bioepis Exhibit No. 1002
Page 5109

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Application No.: 16 195 355.9

Preparation for oral proceedings - Instructions to Support Service

Oral proceedings are to be held in connection with the above patent application

1. The matters to be discussed are set out in the annex (Form 2906)

2. Dispatch the summons using Form 2008/2310 and Form 2906 for the parties to attend on:

Day 05.02.2019 Time 09:00

Archibus

Conference Room 9999 booked

ORAL 01, 02, 03 and 05 coded

02.05.18 m
Date Initials

2.1 Parties' submissions in preparation for the oral proceedings, if any, should be made no later than

1 month(s)

before the date of the oral proceedings
(transfer to Form 2008.1 /2310.1)

2.2 Encode ORAL(04)

coded

02.05.18 m
Date Initials

2.3 Dispatch Form 2008.7 /2310.7 to division

02.05.18 m
Date Initials

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3. ☐ Arrange for the following special equipment to be provided in the conference room:

..... Date Initials

4. Return the dossier to primary examiner after dispatching the summons

..... Date Initials

26.04.18
Date

Orlando, Michele	Terenzi, Carla	Collura, Alessandra	.....
Chairman	2nd examiner	1st examiner	Legal member

Enclosure(s):

Samsung Bioepis Exhibit No. 1002
Page 5111

For the Examining Division

Annexes:
Confirmation of receipt (Form 2936)
Communication (EPO Form 2906)

Samsung Bioepis Exhibit No. 1002
Page 5112

The examination is being carried out on the **following application documents**

Description, Pages

1-4, 6-43, 45-55	as originally filed			
44	received on	10-07-2017	with letter of	10-07-2017
5	filed in electronic form on			16-01-2018

Sequence listings, SEQ ID NO

1-10	received on	10-07-2017	with letter of	10-07-2017

Claims, Numbers

1, 2	filed in electronic form on	16-01-2018

Drawings, Sheets

1/4-4/4	as originally filed

Reference is made to the documents already cited in the course of the procedure.

Moreover, the applicant should be aware of documents D8 and D9 filed by Third Parties on 12.12.17 which may be discussed in the course of the procedure and of WO2005/074607 (D10) which is the publication pamphlet of EP1720571, referred to in D9.

1. Articles 76(1) and 123(2) EPC

The present application does not meet the requirements of Article 76(1) EPC for the following reasons.

The applicant amended page 5 of the description of application as filed (which is the same as the description of th e parent application), and introduced a limitation to the length of the sequence of the indicated SEQ ID NO:3 and SEQ ID NO:4.

The applicant also amended the claims and introduced the same limitation to the length of the SEQ ID NO:4 in present claim 1.

Samsung Bioepis Exhibit No. 1002
Page 5113

In particular, instead of the full length of the sequences 3 and 4 provided in the application, the specific embodiments disclosed on page 5 and claimed in claim 1 are presently directed to:

(i) an antibody having a heavy chain variable region consisting of SEQ ID NO:1 and a light chain variable region consisting of residues 23-109 of SEQ ID NO: 3; and

(ii) an antibody (as claimed) comprising a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of residues 23 to 214 of SEQ ID NO:4.

In his letter dated 16.01.2018, the applicant explained that the amendments that he had carried out were to be considered the correction of an obvious mistake which met the requirements of Rule 139 EPC.

As already stated in the official communication from the examining division dated 17.11.17, it cannot be excluded by the skilled reader that the SEQ ID NO: 3 and 4 as filed in the parent application and in the application as originally filed were actually those for which protection was sought.

Moreover, in the original and parent application no disclosure was present of the fact that sequences 3 and 4 did not have to include the leader peptide.

For the reasons given above, the examining division concludes that the subject-matter of the present application does not meet the requirements of Articles 76(1) and 123(2) EPC.

2. Article 54 EPC

2.1 Eculizumab is considered to have been available to the public before the filing date of the present application

a) In the official communication dated 17.11.17, the examining division expressed its opinion on the fact that, since D1-D5 are all publications referring to eculizumab and since in none of these documents it was stated that the product had not been available for public use or that the working groups using it had been bound to a confidentiality agreement with Alexion Pharmaceuticals, it had to be concluded that the structure of the antibody was available to the public at the time of filing of the present application.

Moreover, the examining division pointed out that any of the scientists working with eculizumab could have performed a sequence listing on the antibody with the general knowledge available in the art. Reference was also made to D7, which disclosed most of the sequence of the light and heavy chains of 5G1.1 (which is eculizumab, see D4) and explained how the sequence was found.

b) On 12.12.17, Third Party observations concerning the present application were received by the EPO.

In the letter it was pointed out that the patentee himself, in opposition proceedings for a granted patent relating to eculizumab (i.e. EP1720571 filing date 03.02.2005; priority date 03.02.2004, in which the applicant was the patentee) had stated that the sequence for eculizumab had been submitted to Chemical Abstracts Services (CAS) and entered STN database on 14.02.1999 (see also the document D6 cited in the European Search Report).

In particular the applicant, at the time of the opposition proceedings against EP1720571, had argued against the objection of lack of sufficiency of disclosure raised by the opponent and based on the allegation that the complete structure of eculizumab was neither disclosed in the application as filed nor in any of the prior art documents cited by the opponent.

c) With a letter dated 16.01.2018, the applicant provided his arguments in response to the objections raised both by the examining division and by Third Parties.

The applicant acknowledged that on 14.02.1999 the sequence of eculizumab was entered in CAS Registry, but that the sequence was not correct.

In support of his arguments, the applicant provided the correspondence between Mr Saxe, who was the primary intellectual property counsel at Alexion Pharmaceuticals, and Mr Bishop, from the CAS Registry database. From this correspondence, it can be seen that Alexion had filed a sequence of eculizumab which contained some mistakes and that the corrected sequence was substituted to the wrong one in May 2009.

The applicant also:

(i) provided evidence of the fact that the marketing approval from the FDA for eculizumab was received by Alexion on 16.05.2007, i.e. after the filing date of the present application;

(ii) stated that when eculizumab was made available for clinical trials, all physicians and participants were required to maintain Alexion's confidential and/or proprietary information in strict confidence. For that reason the skilled person couldn't have obtained and analysed eculizumab to determine its structure; and

(iii) affirmed that the third party observation filed on 12.12.2017 were merely circumstantial and did not provide evidence that eculizumab was publically available at the date of filing of the application.

The examining division considered all the arguments provided in respect to the disputed question of whether eculizumab was publically available or not at the filing date of the present application.

The opinion of the examining division is the following:

1. The fact that Alexion has received the marketing authorisation for eculizumab from the FDA on 16.03.2007 does not necessarily mean that the antibody was not available to the public or that is structure was not known before the filing/priority of the present application.

2. It is clear from Mr. Saxe's declaration that Alexion Pharmaceuticals had intended to submit the correct sequence of eculizumab to CAS and that the incorrect sequence was submitted only inadvertently.

This means that (i) there was no interest in keeping the substance secret after the date of submission to CAS and (ii) anybody to whom the correction of the mistake in the sequence would not have been obvious could have contacted Alexion Pharmaceuticals Inc. to obtain the correct sequence.

Even if it were now purported that Alexion Pharmaceuticals would have never disclosed the correct sequence to anybody who had asked them for it, that would not amount to a valid defence since under the legal principle of "*nemo potest venire contra factum proprium*" Alexion Pharmaceuticals would be bound to its declared intention to lay the compound open under the CAS rules in order to have other patents be regarded as sufficiently disclosed.

The statement from the applicant acting as patentee in EP1720571 (see D9, page 14, item 5.1.2) confirms the fact that he considered the structure of eculizumab to have been disclosed at the filing date of the present application. To be noted is also the fact that the date of the applicant's statement in D9, namely the 30.12.2013, is later than the date in which the correction to the eculizumab structure was requested to CAS.

3. The many patent and non-patent literature documents published before the priority date and relating to the use of eculizumab can be regarded as evidence that the compound was well-known and available to the public before the priority date of the patent application. Among these documents, different published patent applications belonging to the applicant can be seen (see for example D8 filed by Third Party on 12.12.2017 and in particular WO2005/074607 published on 18.08.2005, which is the international publication pamphlet of EP1720571).

4. In respect to the statement of the applicant that the physicians and participants to the studies referred to in the prior art were bound to an agreement of secrecy and confidentiality, a proof of the confidentiality agreement between the company and each participant/physician involved in the studies should be provided.

For the reasons given above, the examining division concludes that the structure of eculizumab was available to the public at the filing/priority date of the present application.

2.2 The present application lacks novelty in view of the disclosure of D1-D5

As already pointed out in the European Searching Opinion and in the official communication from the examining division dated 17.11.17, the disclosures of D1-D5 teaching eculizumab and its use in the treatment of paroxysmal nocturnal hemoglobinuria are considered novelty destroying for the subject-matter of the claimed invention.

2.3 The present application also lacks novelty over the disclosure of D10

The examining division considers that the present application also lacks novelty over the disclosure of D10, which is a prior art document by Alexion Pharmaceuticals relating to eculizumab, for which the applicant explicitly stated that the structure of the antibody had been made available to the public on 14.02.1999.

3. Article 56 EPC

The present application does not meet the requirements of Article 56 EPC, because the subject-matter of the claims 1-2 is not inventive.

As a matter of fact, claims which are not novel cannot be considered to involve any exercise of an inventive step.

4. Article 83 EPC

The attention of the applicant is drawn to the fact that in case a negative conclusion on Articles 123(2) and 76(1) EPC and a positive conclusion on Article 54 EPC will be taken by the examining division in the further prosecution of the case (in particular at the oral proceedings), an objection under Article 83 EPC would be likely to arise.

In the description of the invention (see page 5 last paragraph), the applicant stated that eculizumab is a specific embodiment of the described invention.

In the application as originally filed, as well as in the parent application, sequences 3 and 4 were disclosed for identifying respectively the light chain variable region and the light chain of eculizumab.

On 16.01.2018, the applicant amended the description of the invention and the subject-matter of the claims and stated that the original application referred to the wrong sequences 3 and 4, which erroneously included the leader peptide.

The application has therefore been amended in order to assign a different meaning than one originally meant to the SEQ ID NO:3 and the SEQ ID NO:4.

Therefore, in the situation identified above, following the statements of the applicant in his letter dated 16.01.18, i.e. that (i) the sequences filed with the original application were not those identifying the structure of eculizumab, and (ii) that the structure of eculizumab was not available to the public at the date of filing of the present application, it would follow that the present application was not sufficiently disclosed at the relevant date.

Indeed the skilled person, at the filing date, wouldn't have known which structure was to be assigned to eculizumab.

5. Procedure

It is not at present apparent to the examining division which part of the application could serve as basis for a new and allowable set of claims.

Refusal of the application under Art. 97(2) EPC should therefore be expected.

However, since the applicant has requested oral proceedings, a summons is dispatched with the present communication.

Any item with respect to novelty and inventive step as well the fulfilment of any requirements of the EPC which will become relevant in the course of the procedure will be discussed during oral proceedings.

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mation about drugs and patient-specific drug information to other pharmacists, other health-care professionals, and patients as appropriate.

15. Initiation of and participation in drug and drug-related (e.g., medication administration devices) research, including formal clinical drug investigations.

ASHP believes that pharmacists have a responsibility to communicate advances in the development and delivery of these and other clinical pharmacy services to the health-care community and the public through appropriate publications, presentations, and programs.

ASHP statement on unit dose drug distribution

Am J Hosp Pharm. 1989; 46:2346

The unit dose system of medication distribution is a pharmacy-coordinated method of dispensing and controlling medications in organized health-care settings. The unit dose system may differ in form, depending on the specific needs of the organization. However, the following distinctive elements are basic to all unit dose systems: medications are contained in single unit packages; they are dispensed in a form that is as ready-to-administer as possible; for most medications, not more than a 24-hour supply[a] of doses is delivered to or available at the patient-care area at any time.[1,2]

Numerous studies concerning unit dose drug distribution systems have been published over the past several decades. These studies indicate categorically that unit dose systems, with respect to other drug distribution methods, are (1) safer for the patient, (2) more efficient and economical for the organization, and (3) a more effective method of utilizing professional resources.

More specifically, the inherent advantages of unit dose systems over alternative distribution procedures are

1. A reduction in the incidence of medication errors.
2. A decrease in the total cost of medication-related activities.
3. A more efficient usage of pharmacy and nursing personnel, allowing for more direct patient-care involvement by pharmacists and nurses.
4. Improved overall drug control and drug-use monitoring.
5. More accurate patient billing for drugs.
6. The elimination or minimization of drug credits.
7. Greater control by the pharmacist over pharmacy workload patterns and staff scheduling.
8. A reduction in the size of drug inventories located in patient-care areas.
9. Greater adaptability to computerized and automated procedures.

In view of these demonstrated benefits, ASHP considers the unit dose system to be an essential part of drug distribution and control in organized health-care settings in which drug therapy is an integral component of health-care delivery.

[a] In long-term-care facilities, a larger supply of medication (e.g., 48 or 72 hours) may be acceptable.

References

1. Summerfield MR. Unit dose primer. Bethesda, MD: American Society of Hospital Pharmacists; 1983.
2. ASHP technical assistance bulletin on hospital drug distribution and control. *Am J Hosp Pharm.* 1980; 37: 1097–103.

Approved by the ASHP House of Delegates, June 5, 1989. Approved by the ASHP Board of Directors, November 16, 1988. Supersedes earlier versions approved by the ASHP House of Delegates on June 8, 1981, and by the Board of Directors on April 19, 1975, and November 13–14, 1980.

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Samsung Bioepis Exhibit No. 1002
Page 5119

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Reports

2286 **Robotic system for i.v. antineoplastic drug preparation: Description and preliminary evaluation under simulated conditions**
Dennis D. Cote and Mark G. Torchia

2294 **Factors influencing pharmacists' selection of their first practice setting**
Edmund A. Carter and Richard Segal

2300 **System for voluntary reporting of adverse drug reactions in a university hospital**
Thomas E. Vorce-West, Lynda Barstow, and Bill Butcher

2304 **Accuracy and time requirements of a bar-code inventory system for controlled substances**
Kim Crawford Dinklage, Sara J. White, Julie C. Lenhart, and Harold N. Godwin

2308 **National survey of critical-care pharmaceutical services**
Joseph F. Dasta, Richard Segal, and Anthony Cunningham

2313 **Evaluation of five procedures for measuring nonstandard doses of nifedipine liquid**
Wendy J. Rosen and Cary E. Johnson

2318 **Effect of primary fluids and dilutional volumes on the delivery of cefazolin sodium by a membrane infusion device**
Milap C. Nahata, Diane E. Durrell, and Marcia A. Miller

2321 **Stability of famotidine 20 and 40 mg/L and amino acids in total parenteral nutrient solutions**
Linda Bullock, Joseph F. Fitzgerald, Melvin R. Glick, Robert B. Parks, Joseph G. Schnabel, and Bruce G. Hancock

2326 **Stability of famotidine 20 and 50 mg/L in total nutrient admixtures**
Linda Bullock, Joseph F. Fitzgerald, and Melvin R. Glick

2329 **Stability of famotidine 20 and 40 mg/L in total nutrient admixtures**
Jose B. Montoro, Leonor Pou, Pilar Salvador, Carmen Pastor, and Santiago M. Cano

Note

2333 **Long-term stability of famotidine 20 mg/L in a total parenteral nutrient solution**
Joseph E. DiStefano, Francis P. Mitrano, Richard J. Baptista, Mary M. Der, Anthony P. Silvestri, John D. Palombo, and Bruce R. Bistrian

Primer

2336 **Situational theory of leadership**
David J. Waller, Steven R. Smith, and John T. Warnock

ASHP Reports

2342 **ASHP statement on the pharmacist's role with respect to drug delivery systems and administration devices**

2344 **ASHP statement on the pharmacist's role in clinical pharmacokinetic services**

2345 **ASHP statement on the pharmacist's clinical role in organized health-care settings**

2346 **ASHP statement on unit dose drug distribution**

2347 **ASHP guidelines on selecting pharmaceutical manufacturers and suppliers**

AIDS Facts

2349 AS-101 . . . AIDS hotlines for the deaf . . . Preventing pathogen transmission via respiratory resuscitation devices . . . Efficacy of low-dose zidovudine therapy

2351 Classified Advertising

2388 CP Digest

2398 Current Literature

Journal References . . . Book Reviews: *Training Manual for Intravenous Admixture Personnel, Fourth Edition* (Hunt), Clyde Buchanan . . . *USP DI, Ninth Edition* (United States Pharmacopeial Convention, Inc.), Lucinda G. Miller . . . Books Received

2404 Advertising Index

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Samsung Bioepis Exhibit No. 1002
Page 5120

AMERICAN JOURNAL OF HOSPITAL PHARMACY

VOLUME 46 NUMBER 11 NOVEMBER 1989

NO 7 8

clinical career ladders

robotic system for i.v. drug preparation

factors influencing selection of first practice setting

voluntary reporting of adverse drug reactions

bar-code inventory system for controlled substances

national survey of critical-care pharmaceutical services

situational leadership

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Samsung Bioepis Exhibit No. 1002
Page 5121

PUBLIC SUMMARY DOCUMENT
Product: Eculizumab, solution concentrate for I.V. infusion, 300 mg in 30 mL, Soliris®
Sponsor: Alexion Pharmaceuticals Australasia Pty Ltd
Date of PBAC Consideration: March 2009

1. Purpose of Application

The submission sought a Section 100 (Highly Specialised Drugs Program) PBS listing, or a recommendation for inclusion on the Life Saving Drugs Program (LSDP) for treatment of paroxysmal nocturnal haemoglobinuria (PNH).

Highly Specialised Drugs are medicines for the treatment of chronic conditions, which, because of their clinical use or other special features, are restricted to supply to public and private hospitals having access to appropriate specialist facilities.

Life Saving Drugs Program
The Commonwealth Government provides funds under an appropriation item established for the specific purpose of assisting access to expensive and lifesaving drugs accepted by the PBAC as clinically effective, but not available as pharmaceutical benefits because of a failure to meet cost effectiveness criteria. Financial assistance for such drugs is approved in accordance with specified eligibility criteria and subject to certain conditions as agreed by the Ministers for Health and Finance.

2. Background

At the July 2008 meeting, the PBAC rejected the application for Section 100 listing of eculizumab for the treatment of patients with PNH on the basis of an unacceptably high and highly uncertain estimated cost per additional death avoided over a 2-year period.

The PBAC also rejected this application for consideration for the LSDP. The Committee agreed that eculizumab may meet the criteria for the LSDP for an as yet unidentified subgroup of patients with PNH, but that it was not possible to identify this subgroup at the present time. The Committee noted the sponsor' s indication that it was working on a set of eligibility criteria to identify a population of patients that would benefit most from treatment with eculizumab.

The Public Summary Document for eculizumab from the July 2008 meeting can be accessed from
http://www.health.gov.au/internet/main/publishing.nsf/Content/211923B8041DA9D2CA2574EF00060D0C/$File/Eculizumab%20Final%20PSD%20Alexion%20Pharmaceuticals%20Inc.pdf

3. Registration Status

Eculizumab was TGA registered on 20 March 2009 for the treatment of patients with paroxysmal nocturnal haemoglobinuria (PNH) to reduce haemolysis.

4. Listing Requested and PBAC's View

Section 100 (Highly Specialised Drug)
Private hospital authority required
Treatment of patients with paroxysmal nocturnal haemoglobinuria to reduce haemolysis.
or

Samsung Bioepis Exhibit No. 1002
Page 5122

Inclusion on the Life Saving Drugs Program for treatment of paroxysmal nocturnal haemoglobinuria to reduce haemolysis.

For PBAC's view see Recommendation and Reasons.

5. Clinical Place for the Proposed Therapy

Paroxysmal nocturnal haemoglobinuria is a clonal haemopoietic stem cell disorder that is extremely rare, progressive and life-threatening. Complement mediated intravascular haemolysis is the central mechanism responsible for morbidities and mortality in PNH including thromboembolism, renal dysfunction, pulmonary hypertension, severe anaemia and disabling fatigue.

Currently, therapeutic management of PNH is supportive and mainly addresses the treatment of anaemia and prevention of thrombotic events.

Eculizumab would provide a treatment option for patients with PNH to reduce haemolysis, however it is not curative for the underlying disease.

6. Comparator

The submission nominated supportive care as the main comparator. The comparator was accepted, as previously, by the PBAC as appropriate.

7. Clinical Trials

The submission presented the same clinical trial evidence as in the submission to the July 2008 PBAC meeting. One 26 week randomised placebo controlled trial (TRIUMPH), two uncontrolled single arm eculizumab studies (52 week SHEPHERD and 12 week PILOT) and the E05-001 extension study (median duration 22 months) were presented.

The trials published at the time of submission are as follows:

Trial/First author	Protocol title/ Publication title	Publication citation
Randomised trial		
C04-001 (TRIUMPH) Hillmen P, et al.	The Complement Inhibitor Eculizumab in Paroxysmal Nocturnal Haemoglobinuria.	N Engl J Med 2006; 355 (12):1233-1243.
Supportive non-randomised trials		
C04-002 (SHEPHERD) Brodsky R, et al.	Multicentre Phase 3 Study of the Complement Inhibitor Eculizumab for the Treatment of Patients with Paroxysmal Nocturnal Haemoglobinuria.	Blood 2008; 111 (4):1840-1847.
C02-001 (Pilot Study) & 2 extension studies Hillmen P ,et al	Effect of Eculizumab on Hemolysis and Transfusion Requirements in Patients with Paroxysmal Nocturnal Hemoglobinuria.	N Engl J Med 2004, 350:552-559.
Hill A, et al.	Sustained response and long-term safety of eculizumab in paroxysmal nocturnal haemoglobinuria	Blood 2005; 106:2559-65

Trial/First author	Protocol title/ Publication title	Publication citation
E05-001 (Phase 3 Common Extension Trial) Hillmen P , et al.	Effect of the Complement Inhibitor Eculizumab on Thromboembolism in Patients with Paroxysmal Nocturnal Haemoglobinuria.	Blood 2007; 110 (12):4123-8.

8. Results of Trials

At the July 2008 meeting the PBAC considered that the direct evidence from the TRIUMPH trial to establish the superiority of eculizumab in clinical effectiveness was limited to transfusions avoided and Hb stabilisation, and claimed benefits in terms of fewer thromboembolic events, fewer deaths and improved kidney function were not adequately demonstrated.

The re-submission presented new data regarding the effect of eculizumab on kidney function and damage of other end-organs. The key additional *post hoc* analyses of severe morbidity events of individual end-organs before and during eculizumab treatment in extension study E05-001 are summarised below:

End-organ	Pre-treatment (1683 pt-years)		During treatment (382 pt-years)		P-value	Relative rate reduction	*Absolute difference (events/100 pt-yrs*
	No. of events	Events/ 100 pt-yrs	No. of events	Events/ 100 pt-yrs			
Brain	29	1.72	4	1.05	NS	39%	*0.67*
Liver	52	3.09	8	2.10	NS	32%	*0.99*
GI	66	3.92	3	0.79	Sig	80%	*3.13*
Kidney	71	4.22	8	2.10	Sig	50%	*2.12*
TE	124	7.37	5	1.31	Sig	82%	*6.06*
Compo-site*	271	16.10	28	7.34	Sig	54%	*8.76*

* Composite Severe Morbidity Event included non-overlapping events in the 5 most common systems - brain, liver, GI, renal and thrombosis

The proportions of patients with events were not reported, except for pre-treatment thromboembolic events, 31.8% (62/195). The claim of long-term improvement in end-organ function and therefore survival with eculizumab treatment is based on post-hoc before and after comparisons in the uncontrolled E05-001 study with median treatment duration of 22 months.

At the July 2008 meeting, the PBAC was not satisfied that the comparative toxicity of eculizumab and BSC was adequately elucidated, as two non-fatal cases of meningococcal infection occurred despite immunisation in the clinical trial/studies and longer term safety data were very limited.

No new toxicity data were presented in the re-submission except for one additional death reported in study E05-001 and the inclusion of PSUR 2 (10 reported deaths and 1 fatal meningococcal infection case during the 6-month reporting period).

9. Clinical Claim

The re-submission claimed that eculizumab is therapeutically superior to best supportive care for the treatment of PNH to reduce morbidity and mortality with similar safety issues.

For PBAC's view see Recommendation and Reasons.

10. Economic Analysis

The economic evaluation was updated, using the proposed new effective price and 3-year mortality rates for patients receiving eculizumab (from the included eculizumab trial/studies) and BSC (derived from de Latour 2008 study). In the previous submission the incremental cost effectiveness ratio (ICER) was estimated to be greater than $200,000 per additional death avoided over a two year period, whilst in the re-submission the incremental cost effectiveness ratio (ICER) was also estimated to be greater than $200,000 per additional death avoided over a three year period

In the previous submission, neither costs nor outcomes were discounted. In the current re-submission, only costs but not outcomes were discounted.

11. Estimated PBS Usage and Financial Implications

The financial cost per year to the PBS was estimated to be $10-$30 million in Year 5
The likely number of patients per year was estimated to be less than 10,000 in Year 5.

The PBAC considered the utilisation of eculizumab for PNH was also uncertain as the extent of under diagnosis of PNH in Australia is unclear and there was uncertainty in the applicability of the UK incidence and prevalence to the Australian population

12. Recommendation and Reasons

The comparator of best supportive care (BSC) was accepted, as previously, by the PBAC as appropriate. The submission presented the same clinical trial evidence as in the submission to the July 2008 PBAC meeting. One 26 week randomised placebo controlled trial (TRIUMPH), two uncontrolled single arm eculizumab studies (52 week SHEPHERD and 12 week PILOT) and the E05-001 extension study (median duration 22 months) were presented. The Committee considered that uncertainty remained in the difference in mortality of patients receiving eculizumab and those receiving best supportive care and in the magnitude of the absolute benefit of eculizumab over best supportive care in reduction of thromboembolic events. The effectiveness of eculizumab in preserving end-organ function was also not clear as improvements were based on post-hoc before and after comparisons in the uncontrolled E05-001 extension study and there was still concern about the lack of long-term clinical effectiveness data.

The PBAC considered that the evidence presented was not sufficient to support the claim of similar safety of eculizumab to best supportive care due to the lack of long term placebo controlled trials. The increased risk of meningococcal infection with eculizumab treatment remained a concern, especially the increased risk of contracting meningococcal strain B infection for which there is no vaccine available. Although the submission claimed that eculizumab reduced PNH related fatigue, the potential for patients to experience fatigue as an adverse effect of treatment with eculizumab was noted, as fatigue was acknowledged in the submission as a moderate to severe symptom of PNH which causes significant patient distress.

The Committee considered that, with an annual drug cost approaching $500,000 per year, the incremental cost per additional death avoided was unacceptably high and uncertain. The utilisation of eculizumab for PNH was also uncertain as the extent of under diagnosis of PNH

Samsung Bioepis Exhibit No. 1002
Page 5125

in Australia is unclear and there was uncertainty in the applicability of the UK incidence and prevalence to the Australian population.

The PBAC noted the advice from the Highly Specialised Drugs Working Party that again supported listing of eculizumab under the Highly Specialised Drugs Program subject to meeting clinical and cost effectiveness criteria.

However, the PBAC rejected listing eculizumab on the PBS on the basis of an unacceptably high and highly uncertain cost-effectiveness.

In relation to the criteria for inclusion on the Life Saving Drugs Program (LSDP), the Committee accepted that PNH may shorten the lifespan of some patients suffering from the disease and that the submission's proposed link between treatment with eculizumab resulting in the reduction of thromboembolic events and an improved lifespan of some patients with PNH was not unreasonable (Criterion 2). The PBAC accepted eculizumab is safe to use in patients with bone marrow dysfunction and chronic kidney disease and hence that Criterion 4 for the LSDP was met. The Committee considered that eculizumab may be clinically effective for some patients with PNH, and hence that the drug meets Criterion 5 of the LSDP. With regard to Criterion 10 of the LSDP, the PBAC considered that the proposed funding algorithm did not clearly identify those patients with PNH who would most benefit from treatment with eculizumab, however that criteria for initiation and continuation of treatment with eculizumab could be developed to identify those patients with PNH who would benefit most from treatment with eculizumab.

The PBAC therefore considered that eculizumab meets the criteria for inclusion on the LSDP but that specific requirements for initiation and continuation of treatment would need to be developed by independent experts to identify those patients with paroxysmal nocturnal haemoglobinaemia (PNH) who would benefit most from treatment with eculizumab.

The PBAC noted that although eculizumab meets the criteria for the LSDP, the inclusion of eculizumab on LSDP would mean a new direction for that program, as the disease being treated is not a rare inherited enzyme deficiency, for which the LSDP was established. The PBAC also noted the steep escalation of costs associated with recent applications for consideration of drugs under the LSDP.

The PBAC noted that the submission meets the criteria for an Independent Review.

13. Context for Decision

The PBAC helps decide whether and, if so, how medicines should be subsidised in Australia. It considers submissions in this context. A PBAC decision not to recommend listing or not to recommend changing a listing does not represent a final PBAC view about the merits of the medicine. A company can resubmit to the PBAC or seek independent review of the PBAC decision.

14. Sponsor's Comment

The Sponsor agrees that eculizumab is an appropriate drug to be listed and funded under the Life Savings Drug Program. It is Alexion's intent to work closely with the LSDP and the Health Ministry to find rapid solutions for access to funded eculizumab therapy for patients at highest risk from this ultra-rare, destructive, progressive and life threatening disease of PNH.

Samsung Bioepis Exhibit No. 1002
Page 5126

We look forward to creating a unique and sustainable funding model for the LSDP and eculizumab in PNH, for all stakeholders; from Government to patients.

Samsung Bioepis Exhibit No. 1002
Page 5127

EP 16 19 5355.9
Alexion Pharmaceuticals, Inc.
Our Ref.: P2913 EP/3

DECLARATION REGARDING DETERMINING THE STARTING RESIDUE OF THE ACTIVE LIGHT CHAIN SEQUENCE OF ECULIZUMAB

I, Bruce Andrien, Director, Early Assay Development, of Alexion Pharmaceuticals, Inc., of 100 College Street, New Haven, CT 06510, United States of America, make the following factual declaration concerning the signal peptide and n-terminal amino acid residue of the light chain of eculizumab:

1) I am Director, Early Assay Development, of Alexion Pharmaceuticals, Inc. ("Alexion"), as shown in my curriculum vitae, a copy of which is **attached** herewith and marked Exhibit A.

2) As also shown in my curriculum vitae, I have a Ph.D. from the University of California, San Diego in Engineering Physics. My Ph.D. training focused on designing materials and optimizing scientific instrumentation including mass spectrometers for use in determining accurate molecular weights of materials. Since my Ph.D. was completed in 1991, my career has been devoted to application of increasingly more precise methods for determining the molecular weight, protein sequence and chemical signature information of large molecular weight proteins in the biotechnology industry. In my 10 year role at Analytica of Branford, I developed electrospray sources and mass spectrometers for analyzing peptides, proteins and monoclonal antibodies under the supervision of John B. Fenn, the recipient of the 2002 Nobel Prize in Chemistry for the development of electrospray for the analysis of biological macromolecules.

3) Through my work at Alexion over the past 16 years, I have become very familiar with the protein sequences and structures of antibodies.

4) I understand that EP 16 17 2335.8 is a European patent application whose parent international application was filed on March 16, 2007.

5) I understand the pending claim 1 of EP 16 17 2335.8 reads as follows:

Samsung Bioepis Exhibit No. 1002
Page 5128

Claim 1. A pharmaceutical composition comprising an antibody that binds C5 or an active antibody fragment thereof for use in treating a patient suffering from paroxysmal nocturnal hemoglobinuria (PNH), wherein the antibody binds C5 or an active fragment thereof comprises a heavy chain and a light chain, wherein the heavy chain consists of SEQ ID NO:2 and the light chain consists of SEQ ID NO:4.

6) I understand that SEQ ID NO: 4 in EP 16 17 2335.8 reads as follows:

MDMRVPAQLLGLLLLWLRGARCDIQMTQSPSSLSASVGDRVTITCGASENIYGALNWYQQKPGK
APKLLIYGATNLADGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQNVLNTPLTFGQGTKVEIKRT
VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLS
STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

7) I was asked to determine whether one skilled in the art of antibody structure would have immediately recognized that the light chain identified by SEQ ID NO:4 includes the leader peptide of the light chain, and whether one of skill in the art could have easily determined where the leader peptide ends and the active fragment begins in SEQ ID NO:4.

8) Upon being shown the light chain of SEQ ID NO:4, I immediately recognized three features that indicated the leader peptide was included. The first two features were that the first residue was a methionine (M) and that this first methionine residue was followed by 21 amino acid residues, most of which are hydrophobic. Methionine is encoded in DNA as the ATG start codon and first amino acid in eukaryotic proteins. Methionine, is therefore, also the starting residue of signal peptides expressing the light chain of antibodies, but is cleaved off with the entire signal peptide in the cell from light chains as the multi-chain protein folds and is assembled into a fully formed antibody. The presence of Met as the first amino acid present in SEQ ID NO:4, combined with the length and amino acid content of this peptide, is characteristic of signal (leader) peptides of immature proteins and indicated the signal peptide was still present.

The third feature was that the entire peptide sequence of the light chain was 236 residues, which is longer than the average light chain of a mature antibody which ranges from about 211-217 amino acids. See Janeway *et al.*, Immunobiology, 5th

Samsung Bioepis Exhibit No. 1002
Page 5129

Edition New York, Garland Science 2001. Again, this factor reinforced to me that the signal peptide was present in SEQ ID NO:4. The signal peptide is naturally present on the N-terminus of immature antibody proteins and this fact is well-known and established amongst scientists who work with antibodies.

9) People familiar with the field of antibody sequences know that antibody sequences are usually numbered according to the "Kabat" numbering scheme. This is because the Kabat's database, which began in 1969, but was first published in 1976, was one of the oldest biological sequence databases maintained and free to the public. Kabat's database was also unique because it contained protein sequence alignments. Kabat's database was published in printed form in five growing editions between 1976 and 1991. Kabat's database continued online for a while after the last printed version in 1991 at http/kabatdatabase.com, but because the information contained in the database has grown with our ability to sequence more easily, it has increased to be more than five times larger than the last printed version containing 2387 pages. The database is still used today by practitioners in the art, but contains a convenient user interface. See Kabatman at www.bioinf.org.uk. To answer this question, I consulted an in-house copy of the iconic 2387 page, last printed version of "Sequences of Proteins of Immunological Interest" Volume 1, 5th Edition, published in 1991 by the U.S. Department of Health and Human Services and compiled by Elvin A. Kabat *et al.*, which is also known as NIH Publication No. 91-3242. See attached Exhibit B.

10) In the Table of Contents on page (iii) for "Sequences of Proteins of Immunological Interest" Volume 1, 5th Edition, the page indicated to show "Signal Sequences of Light Chains" for "Human Kappa Light Chains" is page 1. See Exhibit C. The active fragment sequences of "Human Kappa Chains Subgroup I" are indicated to be shown on page 103. See Exhibit C.

11) As directed by the Table of Contents in Exhibit C, on page 1, top table, **for 22-residue signal peptides** (1, 2, 5-11, 15, 16, & 18 sequences) of human kappa light chains the first three residues (sequences in columns) are predominantly Met-Asp-Met or MDM in single letter code, just as in the beginning of SEQ ID NO:4. See Exhibit D. The numbering system on the left column of this page in the top table uses a negative sign

in front of the signal peptide residue numbers; therefore Met-Asp-Met corresponds to -22 to -20. See Exhibit D. The final residues in **signal peptides having a 22 amino acid length** as shown in the top table on page 1 are predominantly Arg-Cys (RC), numbered as -2 and -1, just as in SEQ ID NO:4. Although there are three occurrences of Lys-Cys (KC) residues as -2 and -1 in **the 22 residue signal peptides** in the top table on page 1 of Exhibit D, the pattern is always a positively-charged residue followed by Cys (C) at the -1 position, just as in position 22 of SEQ ID NO:4.

12) As described above, the active peptide sequences for human kappa light chains begin on page 103 of "Sequences of Proteins of Immunological Interest" Volume 1, 5th Edition, published in 1991. Pages 103-108 are included as Exhibit E. The numbering system of Framework 1 is again on the left side of the page and is numbered downward from 0 to 23, for example, for Framework 1 (FR1). It is well-known to scientists working in the field of antibodies that Framework 1 is the N-terminus of the active antibody light chain. Here, even in 1991, it was evident that Asp-Ile-Gln-Met (DIQM) were the first 4 residues of an active human kappa light chain subgroup 1. See Exhibit E. Human kappa light chain subgroup I was examined first because it was known that subgroup 1 outnumbers each of the other subgroups in usage. See Solomon et al., American Journal of Pathology 137(4) 855-862 (1990).

I found that in SEQ ID NO:4, immediately following the 22-residue signal peptide, is the clear and unambiguous start site to the active antibody fragment, i.e., DIQM at positions 23-26. The vast majority of human kappa light chains catalogued in 1991 started with this sequence. The statistics on page 108 of Exhibit E show the number of full and/or partial sequences of kappa light chains known in 1991 that began with D (118/125), followed by I (120/123), followed by Q (112/123) and followed by M (109/124). Therefore, for all of the reasons above, I conclude that one skilled in the art in 2007 would have immediately recognized that the signal peptide is included in SEQ ID NO:4 and would have also found it straightforward and simple to delineate the signal peptide from the active N-terminus of the light chain in SEQ ID NO:4, as starting with (DIQM).

Samsung Bioepis Exhibit No. 1002
Page 5131

DECLARED at New Haven, CT
United States of America

This 23 Day of May 2017

Bruce A. Andrien Jr.

Bruce A. Andrien Jr., Ph.D.

Witness signature

J. Darrell Fontenot Ph.D., J.D.,
Sr. Dir., Patent Counsel,
Alexion Pharmaceuticals, Inc.
100 College Street, New Haven, CT, 06510,
United States of America

Witness Name, Capacity and Address

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: January 19, 2017
Electronic Signature for Jill Gorny Sloper, Esq.: /Jill Gorny Sloper, Esq./

EP 16 19 5355.9
Alexion Pharmaceuticals, Inc.
Our Ref.: P2913 EP/3

Docket No.: AXJ-114BUSCN3
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Leonard Bell *et al.*

Application No.: 15/260,888

Filed: September 9, 2016

For: TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

Confirmation No.: 2558

Art Unit: 1644

Examiner: J. L. Rogers

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION PURSUANT TO 37 CFR 1.132 BY DR. LEONARD BELL

I, Leonard Bell, M.D., a co-inventor of the subject invention, declare that:

1. I founded Alexion Pharmaceuticals, Inc. and served as its Chief Executive Officer from 1992 until 2015. In that role, I was closely involved in the development of eculizumab and in the administration and monitoring of all clinical trials involving eculizumab.

2. Prior to March 15, 2007, which is the date the subject patent application was filed, eculizumab was not available to the public. In order to be approved for marketing, eculizumab was used in clinical trials before March 15, 2007, but only under strict control and conditions of confidentiality. Patients in clinical trials of eculizumab received intravenous infusions of eculizumab at hospitals and infusion clinics and never themselves possessed samples of eculizumab. Researchers and staff involved in clinical trials of eculizumab were not permitted to use or distribute eculizumab for

purposes other than conducting the clinical trials, and were strictly accountable to Alexion for the inventory of eculizumab and for information about eculizumab.

3. Moreover, prior to March 15, 2007, the complete structure of eculizumab was not disclosed to the public. For example, the Hillmen reference (Hillmen et al., N. ENGL. J. MED., 350, pp. 552-59 (2004)) does not disclose any parts of the sequence of eculizumab and the '245 patent (U.S. Pat. No. 6,355,245) does not disclose or even hint at the unique non-naturally occurring, protein-engineered heavy chain (including the CH1-hinge-CH2-CH3 regions) of eculizumab.

4. Nor does the Thomas reference (Thomas et al., MOLECULAR IMMUNOLOGY, 33 (17/18), pp. 1389-1401 (1996)) describe eculizumab. The Thomas reference describes an antibody comprising the humanized variable region of eculizumab, but does not disclose or hint at the unique non-naturally occurring, protein-engineered heavy chain of eculizumab, which confers important functional effects (as discussed below). Rather, the antibody described in the Thomas reference contains a *naturally-occurring* IgG4 heavy chain. The antibody described in the Thomas reference therefore is not eculizumab. It is both structurally and functionally different from eculizumab.

5. As shown below, a sequence alignment of the heavy chain constant regions and hinge of eculizumab (shown in bold on the top line below) and that of the antibody described in the Thomas reference (shown on the bottom line below) demonstrates that the two antibodies are structurally very different, with fourteen different amino acid residues in the constant regions (underlined and highlighted) (four different residues in the CH1 domain, six different residues in the hinge region and four different residues in the CH2 domain):

```
-----------------------------CH1-------------------
ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
```

```
------------CH1-------------------------------|Hinge-
FPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVERKCCV
FPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGP

-Hinge--|-----------------CH2------------------
ECPPCPAPP-VAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDP
PCPSCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDP

---------CH2-----------------------------------------
EVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKC
EVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKC

--------CH2-------|-------------CH3---------------
KVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKG
KVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKG

---------------------------CH3---------------------
FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNV
FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNV

---------CH3-------------
FSCSVMHEALHNHYTQKSLSLSLGK
FSCSVMHEALHNHYTQKSLSLSLGK
```

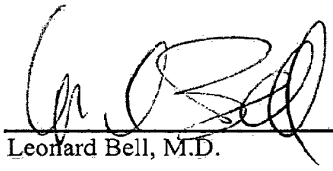
6. Also, the functional properties of eculizumab and the antibody described in the Thomas reference are very different. For example, eculizumab does not bind as strongly to Fcγ receptors as the Thomas antibody. Binding to the Fcγ receptors initiates and amplifies immune responses that are undesirable for an antibody intended to shut down the terminal complement pathway. Thus, engineering eculizumab to minimize binding to these receptors provided important advantages over the antibody described in the Thomas reference.

7. Eculizumab also has a much longer plasma half-life than the antibody described in the Thomas reference. It is advantageous for an antibody like eculizumab to remain in the circulation longer so that it does not need to be administered to patients as frequently.

8. Accordingly, as demonstrated by comparative data between eculizumab and the antibody described in the Thomas reference, the non-natural, protein-engineered, heavy chain of eculizumab reduces effector function activation, minimizes immunogenicity, and contributes to a longer half-life of eculizumab compared to the antibody described in the Thomas reference. These properties were surprising and unpredictable, and made eculizumab a better drug candidate than the antibody described in the Thomas reference.

9. In sum: the complete structure of eculizumab was not available to the public on or before March 15, 2007; eculizumab was not available to the public or being used by the public prior to that time; the unique protein-engineered heavy chain of eculizumab was not described in the Hillmen reference, the Thomas reference or the '245 patent; and the unique protein-engineered heavy chain of eculizumab could not be derived from the combined disclosures of the Hillmen reference, the Thomas reference and/or the '245 patent.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or of any patent issued thereon.



Leonard Bell, M.D. 1/18/2017 Date

SEQUENCE LISTING

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<120> TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

<130> P2913 EP/3

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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
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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

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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
        115                 120                 125
Ser Val Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr
    130                 135                 140
Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160
Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175
Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190
Val Pro Ser Ser Asn Phe Gly Thr Gln Thr Tyr Thr Cys Asn Val Asp
        195                 200                 205
His Lys Pro Ser Asn Thr Lys Val Asp Lys Thr Val Glu Arg Lys Cys
    210                 215                 220
Cys Val Glu Cys Pro Pro Cys Pro Ala Pro Pro Val Ala Gly Pro Ser
225                 230                 235                 240
Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg
                245                 250                 255
Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser Gln Glu Asp Pro
            260                 265                 270
Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala
        275                 280                 285
Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr Tyr Arg Val Val
    290                 295                 300
Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr
305                 310                 315                 320
Lys Cys Lys Val Ser Asn Lys Gly Leu Pro Ser Ser Ile Glu Lys Thr
                325                 330                 335
Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu
            340                 345                 350
Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys
        355                 360                 365
Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
    370                 375                 380
Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp
385                 390                 395                 400
Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr Val Asp Lys Ser
                405                 410                 415
Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala
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Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Leu Gly Lys
        435                 440                 445
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            20                  25                  30
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Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80
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
            100                 105
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            20                  25                  30
Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80
Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn Thr Pro Leu
                85                  90                  95
Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
            100                 105                 110
Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
        115                 120                 125
Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
    130                 135                 140
Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
145                 150                 155                 160
Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
                165                 170                 175
Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
            180                 185                 190
Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
        195                 200                 205
Phe Asn Arg Gly Glu Cys
    210
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1               5
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1 5 10 15
Asp

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Gln Asn Val Leu Asn Thr Pro Leu Thr
1 5

TABLE OF CONTENTS

INTRODUCTION....xiii
REFERENCES TO INTRODUCTION....lxxiii

SIGNAL SEQUENCES OF LIGHT CHAINS

HUMAN KAPPA CHAINS....1
HUMAN LAMBDA CHAINS....5

MOUSE KAPPA CHAINS....7
MOUSE LAMBDA CHAINS....17

MISCELLANEOUS KAPPA CHAINS....19
MISCELLANEOUS LAMBDA CHAINS....21

SIGNAL SEQUENCES OF HEAVY CHAINS

HUMAN....23

MOUSE....29

MISCELLANEOUS....40

SIGNAL SEQUENCES OF T-LYMPHOCYTE RECEPTOR

HUMAN ALPHA CHAINS....45
HUMAN BETA CHAINS....49
HUMAN GAMMA CHAINS....53
HUMAN DELTA CHAINS....55

MOUSE ALPHA CHAINS....57
MOUSE BETA CHAINS....61
MOUSE GAMMA CHAINS....65
MOUSE DELTA CHAINS....67

MISCELLANEOUS ALPHA CHAINS....69
MISCELLANEOUS BETA CHAINS....71

SIGNAL SEQUENCES OF RELATED PROTEINS

BETA-2-MICROGLOBULINS....73

MAJOR HISTOCOMPATIBILITY ANTIGENS CLASS I....75

I REGION ANTIGENS CLASS II....82

COMPLEMENT....89

T-CELL SURFACE ANTIGENS....91

INTEGRINS....94

MISCELLANEOUS PROTEINS....96

VARIABLE REGION LIGHT CHAIN SEQUENCES

HUMAN KAPPA CHAINS SUBGROUP I....103
HUMAN KAPPA CHAINS SUBGROUP II....113
HUMAN KAPPA CHAINS SUBGROUP III....118
HUMAN KAPPA CHAINS SUBGROUP IV....128

HUMAN LAMBDA CHAINS SUBGROUP I....131
HUMAN LAMBDA CHAINS SUBGROUP II....135
HUMAN LAMBDA CHAINS SUBGROUP III....139
HUMAN LAMBDA CHAINS SUBGROUP IV....144
HUMAN LAMBDA CHAINS SUBGROUP V....146
HUMAN LAMBDA CHAINS SUBGROUP VI....148

MOUSE KAPPA CHAINS SUBGROUP I....151
MOUSE KAPPA CHAINS SUBGROUP II....163
MOUSE KAPPA CHAINS SUBGROUP III....190
MOUSE KAPPA CHAINS SUBGROUP IV....201
MOUSE KAPPA CHAINS SUBGROUP V....208
MOUSE KAPPA CHAINS SUBGROUP VI....239
MOUSE KAPPA CHAINS SUBGROUP VII....257

MISCELLANEOUS MOUSE KAPPA CHAINS....259

MOUSE LAMBDA CHAINS....263

RAT KAPPA CHAINS....271

RAT LAMBDA CHAINS....274

RABBIT KAPPA CHAINS....276

RABBIT LAMBDA CHAINS....290

CHICKEN LAMBDA CHAINS....293

MISCELLANEOUS KAPPA CHAINS....303

MISCELLANEOUS LAMBDA CHAINS....306

MISCELLANEOUS LIGHT CHAINS....308

EP 16 19 5355.9
Alexion Pharmaceuticals, Inc.
Our Ref.: P2913 EP/3

Exhibit A

BRUCE A. ANDRIEN, Jr., Ph.D.

7 Finch Lane, Guilford, CT 06437, (203) 457-0067
E-Mail: bldandrien@comcast.net

CAREER EXPERIENCE:

Alexion Pharmaceuticals, Incorporated, Cheshire, Connecticut; Director Early Assay Development; July 2015 - Present.

Development responsibilities

- Developed processes to support Research's ever expanding needs including testing of proteins within the following areas: Biacore, HPLC, LOC, IEF, Mass Spectrometry, and biological assays.
- Lead the Process Development and Analytical Sciences Quality Improvement Team
- Lead the Process Development and Analytical Sciences Bi-Paratopic team
- Supported four diligences teams
- Supported three early research teams
- Provide response to regulatory agency questions

Alexion Pharmaceuticals, Incorporated, Cheshire, Connecticut; Director Analytical Sciences; July 2014 – July 2015.

GMP Compliance responsibilities

- Provide analytical support of CMC submissions, address comments from regulatory agencies regarding test methods and validation
- Direct and coordinate the transfer of release and stability test methods into Quality Control or to contract laboratories worldwide to support release testing of commercial drug product
- Provide support and troubleshooting to Quality Control groups for analytical test methods. Support includes assisting in assay invalids and/or OOS investigations, aberrant trends or special studies, etc.
- Manage, delegate, and prioritize GMP release and stability testing for all new pre-clinical and early stage (Phase 1 and Phase 2) clinical products.
- Direct formulation development activities, oversee design, execution, and analysis of formulation development studies.
- Manage qualification and validation of analytical methods for early stage clinical products in addition to existing commercial products.
- Manage in-process testing and coordinate outsource testing for new pre-clinical and early stage (Phase 1 and Phase 2) clinical products.
- Coordinate quarterly review meetings and provide updates, and generate reports for early stage (Phase 1 and Phase 2) clinical products.
- Generate or revise specifications for early stage (Phase 1 and Phase 2) clinical products
- Maintain appropriate compliance within area of responsibility with regards to current SOPs, validation / qualifications, and training. Represent the area functions as required during audits by external agencies and auditors, as well as by internal auditors
- Establish systems within area of responsibility to ensure compliance, good lab practices and efficiency

Development responsibilities

- Direct the development, improvement, qualification, and validation of analytical testing of proteins. Tests within area are: HPLC, SDS-PAGE, Western blots, IEF, ELISAs, biological assays and protein determinations.
- Generate and oversee product comparability protocols for early stage (Phase 1 and Phase 2) clinical products.
- Oversee the analytical support of cell culture and purification development groups in support of new clone or media optimizations and improvements to purification processes.
- Work with Purification Development to plan and coordinate formulation development studies, including generating and executing formulation stability protocols, compiling data, trending and presenting to all facets of the organization.

Other Responsibilities:

- Train, coach, and mentor Analytical Sciences laboratory personnel by working with employees to develop performance objectives and to monitor performance against the objectives.
- Interact with Alexion Pharmaceuticals personnel within Development, and other departments as required to meet objectives; interact with Alexion Pharmaceuticals business partners and technical representatives as required to meet objectives
- Represent Analytical services on Task Force or Planning Committees as assigned

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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.

Samsung Bioepis Exhibit No. 1002
Page 5144

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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.

Samsung Bioepis Exhibit No. 1002
Page 5145

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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)

Samsung Bioepis Exhibit No. 1002
Page 5146

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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)

Samsung Bioepis Exhibit No. 1002
Page 5147

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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).

Samsung Bioepis Exhibit No. 1002
Page 5148

BRUCE A. ANDRIEN, Jr., Ph.D.
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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).

Samsung Bioepis Exhibit No. 1002
Page 5151

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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.

Samsung Bioepis Exhibit No. 1002
Page 5152

BRUCE A. ANDRIEN, Jr., Ph.D.

7 Finch Lane, Guilford, CT 06437, (203) 457-0067
E-Mail: bldandrien@comcast.net

AWARDED PATENTS:

Title: "Nucleic Acid Encoding Anti-C5 Antibodies having Improved Pharmacokinetics"
Inventors: Bruce A. Andrien, Jr.; Douglas L. Sheridan; and Paul P. Tamburini
Patent No.: 9,206,251 B2
Date of Patent: Dec. 8, 2015

Title: "Methods of Treating C5 Mediated Complement-Associated Conditions with Anti-C5 Antibodies having Improved Pharmacokinetics"
Inventors: Bruce A. Andrien, Jr.; Douglas L. Sheridan; and Paul P. Tamburini
Patent No.: 9,107,861 B1
Date of Patent: Aug. 18, 2015

Title: "Anti-C5 Antibodies having Improved Pharmacokinetics"
Inventors: Bruce A. Andrien, Jr.; Douglas L. Sheridan; and Paul P. Tamburini
Patent No.: 9,079,949 B1; WO 2015/134894 A1; New Zealand 631007; European 15 710 396.1
Date of Patent: Jul. 14, 2015; 06Mar. 2015; 02Feb2016; 06.03.2015

Title: "Mass Spectrometry with Multipole Ion Guides"
Inventors: Craig M. Whitehouse, Bruce A. Andrien, Jr.; and Gulcicek; Erol E.
Patent No.: 6,753,523; 6,987,264; and 7,189,967
Date of Patent: June 22, 2004

Title: "Atmospheric Pressure Ion Source"
Inventors: Craig M. Whitehouse, Bruce A. Andrien Jr., Michael A. Sansone, Clement Catalano
Patent No.: 6,593,568 B1
Date of Patent: July 15, 2003

Title: "Direct Flow Injection Analysis Nebulization Electrospray and APCI Mass Spectrometry"
Inventors: Bruce A. Andrien Jr., Fred Banks, James Boyle
Patent No.: 6,458,597 B1; 6,858,437 B2; and 7,364,913
Date of Patent: October 1, 2002; and Feb 22, 2005

Title: "Curved Introduction for Mass Spectrometry"
Inventors: Bruce A. Andrien Jr., Michael A. Sansone, Allan G. Burt, Craig M. Whitehouse
Patent No.: 6,326,616 B1 and 6,573,494 B1
Date of Patent: December 4, 2001 and June 3, 2003

Title: "Multiple Sample Introduction Mass Spectrometry"
Inventors: Bruce A. Andrien Jr., Craig M. Whitehouse, Shida Shen, Michael A. Sansone,
Patent No.: 6,207,954 B1 and 6,541,768 B2
Date of Patent: Mar 27, 2001 and April 1, 2003

Title: "Disposable Microtip Probe for Low Flow Electrospray"
Inventors: Bruce A. Andrien Jr., Craig M. Whitehouse, Michael A. Sansone, Denise M. Sauro, Glenn P. Whitehouse
Patent No.: 6,127,680 and 6,337,480 B1
Date of Patent: October 3, 2000 and January 8, 2002

Title: "Time-Of-Flight Mass Spectrometry from Surfaces"
Inventors: Craig M. Whitehouse, Bruce A. Andrien Jr.
Patent No.: 6,040,575 and 6,204,500 B1 and 6,600,155 B1
Date of Patent: March 21, 2000 and Jul 29 ,2003

Title: "Multipole Ion Guide Trap Time-of-Flight Mass Spectrometry with MS/MS^n Analysis Capability"
Inventors: Craig M. Whitehouse, Thomas Dresch, and Bruce A. Andrien Jr.
Patent No.: 6,011,259
Date of Patent: January 4, 2000

Samsung Bioepis Exhibit No. 1002
Page 5153

BRUCE A. ANDRIEN, Jr., Ph.D.

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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.

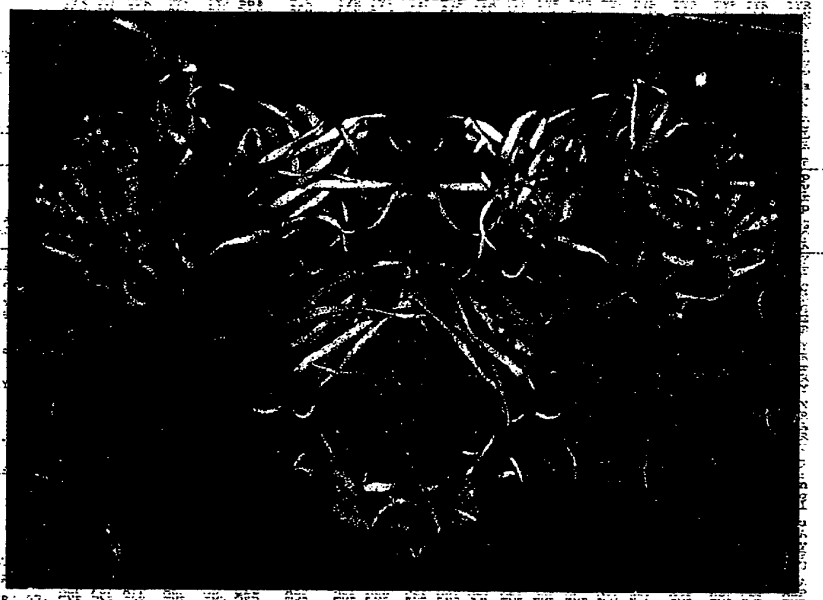
Samsung Bioepis Exhibit No. 1002
Page 5154

Exhibit B

SEQUENCES OF PROTEINS OF IMMUNOLOGICAL INTEREST

VOL. I

FIFTH EDITION



1991

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
National Institutes of Health

SEQUENCES OF PROTEINS OF IMMUNOLOGICAL INTEREST

FIFTH EDITION

Tabulation and Analysis of
Amino Acid and Nucleic Acid Sequences of Precursors,
V-Regions, C-Regions, J-Chain, T-Cell Receptors for Antigen,
T-Cell Surface Antigens, β_2-Microglobulins,
Major Histocompatibility Antigens, Thy-1, Complement,
C-Reactive Protein, Thymopoietin, Integrins, Post-gamma Globulin,
α_2-Macroglobulins, and Other Related Proteins

1991

Elvin A. Kabat, Tai Te Wu*, Harold M. Perry†,*
Kay S. Gottesman‡, and Carl Foeller†

*Depts. of Microbiology, Genetics and Development, and Neurology, Cancer Center/Institute of Cancer Research, College of Physicians and Surgeons, Columbia University, New York, NY 10032 and the National Institute of Allergy and Infectious Diseases, and the Office of the Director National Institutes of Health, Bethesda, MD 20892.

*Depts. of Biochemistry, Molecular Biology, and Cell Biology, and Engineering Sciences and Applied Mathematics and Biomedical Engineering, Northwestern University, Evanston, IL 60208 and the Cancer Center, Northwestern University Medical School, Chicago, IL 60611

†BBN Systems and Technologies, 10 Moulton Street, Cambridge, MA 02138

‡Formerly with BBN. Present address Laboratory for Applied Research in Academic Information, William H. Welch Medical Library, The Johns Hopkins University, Baltimore, Md 21205

The collection and maintenance of this data base is sponsored under grant 5R01 AI-125616 to E.A. Kabat of Columbia University by the following components of the National Institutes of Health, Bethesda, MD 20892:

Office of the Director
National Center for Research Resources
National Cancer Institute
National Institute of Allergy and Infectious Diseases
National Institute of Diabetes, Digestive and Kidney Diseases
National Institute of General Medical Sciences
National Library of Medicine

Work with the PROPHET software package is supported by a subcontract from Columbia University to BBN Systems and Techologies, Cambridge, MA 02138

U.S. DEPARTMENT OF HEALTH
AND HUMAN SERVICES

Public Health Service
National Institutes of Health

NIH Publication No. 91-3242

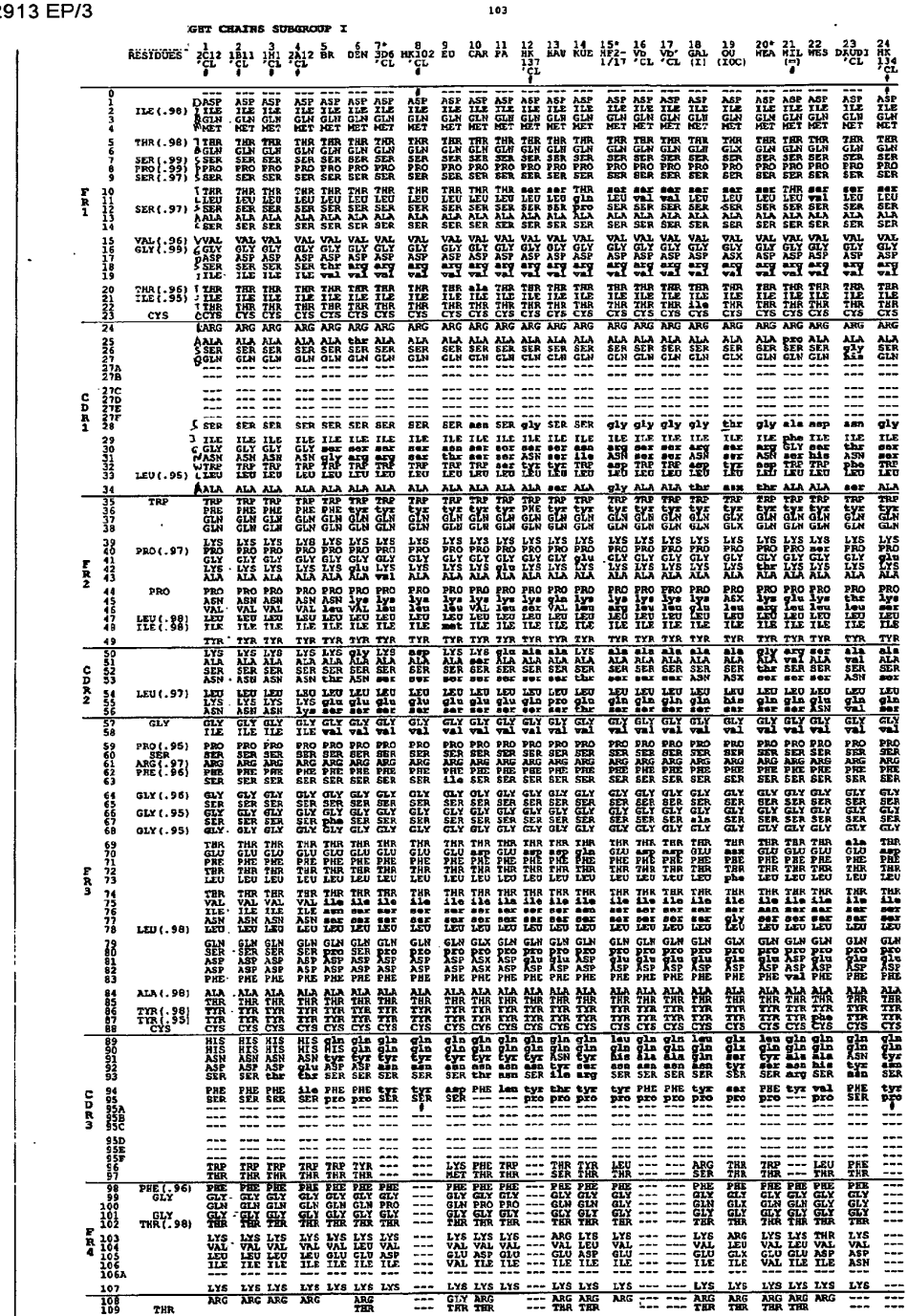
Exhibit D

HUMAN KAPPA LIGHT CHAINS

INVARIANT RESIDUES	1 HK101 /CL	2 HK 117 /CL	3 VKI-Cne1 /CL	4 HuVH CAMP /CL	5 VKI-2I /CL	6 3D6 /CL	7 V108 /CL	8 Vb /CL	9 Vb' /CL	10 HK102 /CL	11 2A12 /CL	12 1R1 /CL	13 2C12 /CL	14 1B11 /CL	15 Vd /CL	16 Va' /CL	17 Ve /CL	18 V13 /CL	19 GM 607 /CL	20 A3 /CL	21 RPMI 6410 /CL #	22 A5 /CL	23 A19 /CL
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-23		---	---	---		---	---	---	---	---	---				---	---	---	---		---	---	---	---
-22		MET	MET	---	---	THR	MET	MET	MET	MET	MET	MET			MET	MET	---	MET		---	---	---	---
-21		ASP	ASP	---	---	ASP	ASP	GLU	ASP	ASP	ASP				ASP	ASP	---	ASP		---	---	---	---
-20	MET(.98)	MET	MET	MET	---	VAL	MET	MET	MET	MET	MET	MET			MET	MET	MET	MET		MET	MET	MET	MET
-19		ARG	ARG	ARG	MET	ARG	ARG	ARG	ARG	MET	ARG	ARG			ARG	ARG	ARG	ARG		ARG	ARG	ARG	ARG
-18		VAL	VAL	ALA	GLY	ALA	VAL	VAL	VAL	VAL	VAL	VAL			VAL	VAL	VAL	VAL		LEU	LEU	LEU	LEU
-17		LEU	LEU	PRO	TRP	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO		PRO	PRO	PRO	PRO		PRO	PRO	PRO	LEU
-16		ALA	ALA	THR	SER	THR	ALA	ALA	ALA	ALA	ALA	ALA			ALA	ALA	ALA	ALA		ALA	ALA	ALA	ALA
-15		GLN	GLN	GLN	CYS	GLN	GLN	GLN	GLN	GLN	GLN	LEU	LEU		GLN	GLN	GLN	GLN		GLN	GLN	GLN	GLN
-14		LEU	LEU	LEU	ILE	LEU	LEU	LEU	LEU	LEU	LEU	LEU			LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU
-13		LEU	LEU	LEU	ILE	LEU	LEU	LEU	LEU	LEU	LEU	LEU			LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU
-12		GLY	GLY	GLY	LEU	GLY	GLY	GLY	GLY	GLY	GLY	GLY			GLY	GLY	GLY	GLY		GLY	GLY	GLY	GLY
-11		LEU	LEU	LEU	PHE	LEU	LEU	LEU	LEU	LEU	LEU	LEU			LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU
-10	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU			LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU
-9		LEU	LEU	VAL	VAL	GLY	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU	LEU		MET	MET	MET	MET
-8	LEU(.95)	LEU	LEU	LEU	ALA	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU
-7	TRP(.95)	CYS	CYS	TRP	THR	TRP	TRP	TRP	THR	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP		VAL	VAL	ILE	TRP
-6		PHE	PHE	LEU	ALA	LEU	LEU	LEU	PHE	PHE	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		VAL	VAL	ILE	VAL
-5		PRO	PRO	PRO	THR	PRO	PRO	ARG	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO		SER	PRO	PRO	PRO
-4		GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY
-3		ALA	ALA	ALA	VAL	ALA	ALA	ALA	SER	SER	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	SER	SER	SER	SER	SER
-2		ARG	ARG	ARG	HIS	ARG	LYS	ARG	ARG	ARG	LYS	LYS	LYS	LYS	LYS	ARG	ARG	ARG	SER	SER	SER	SER	SER
-1		CYS	CYS	CYS	SER	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	GLY	GLY	GLY	ALA	GLY

	24 NG9 'CL	25 HIC (HI) 'CL #	26 HAH 14.1 'CL #	27 HAH 14.2 'CL #	28 HAH 16.1 'CL #	29 NOV 'CL #	30 8E10 'CL #	31 7H3 'CL #	32 Humkv 325 'CL	33 EV1-15 'CL	34 IARC/ BL41 'CL	35 Humkv 305 'CL	36 LS1 'CL	37 LS2 'CL	38 LS4 'CL	39 LS5 'CL	40 LS6 'CL	41 LS7 'CL	42 LS8 'CL	43 Vg 'CL	44 Vh 'CL	45 CLL 'CL #	46 Hum Xy328 'CL #	47 Hum Kables 'CL #
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-20		MET	MET	MET	MET				MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET		
-19		GLU	GLU	GLU	GLU	GLU			GLU	GLU	GLU	GLU	GLU	GLU	GLU	GLU	GLU	GLU	GLU	GLU	GLU	GLU		
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-15		GLN	GLN	GLN	GLN	GLN			GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN		
-14		LEU	LEU	LEU	LEU	LEU			LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		
-13		LEU	LEU	LEU	LEU	LEU			LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		
-12		PHE	PHE	PHE	PHE	PHE			PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE		
-11		LEU	LEU	LEU	LEU	LEU			LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		
-10		LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		
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-5		PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO		
-4	VAL	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP
-3	PRO	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR
-2	SER	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR
-1	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLT	GLY	GLY	ARG	GLY	GLY	GLY

	48 GF4 /J1.1 'CL	49 K- EV15 'CL	50 VKAPPA IV GERMLINE 'CL	51 PBL7IV 'CL	52 FK-001 'CL #	53 WALKER 'CL	54 OAUDI 'CL	55 VJ1 'CL	56 HK100 'CL	57 Va 'CL	58 V2 'CL	59 V14 'CL	60 V52 'CL	61 V55 'CL	62 K- EVJK11 'CL	63 PSEUDO VKAPPA 24'CL	# OF SEQUENCES	# OF AMINO ACIDS	
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-8	LEU	LEU	LEU		LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	SER	LEU	PHE	58	4	
-7	TRP	TRP	TRP		TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	59	3	
-6	LEU	ILE	ILE		ILE	ILE	LEU	ILE	LEU	LEU	LEU	LEU	LEU	PHE	LEU	ILE	59	5	
-5	PRO	SER	SER		SER	SER	ARG	ARG	SER	SER	PRO	PRO	PRO	PRO	PRO	LEU	59	5	
-4	GLU	ASP	GLY		GLY	GLY	GLY	ARG	GLY	GLY	GLY	GLY	GLY	ASP	GLY	ASP	GLU	63	5
-3	THR	THR	ALA		ALA	ALA	VAL	ALA	ALA	ALA	ALA	ALA	THR	THR	THR	VAL	63	5	
-2	SER	ARG	TYR		TYR	TYR	ARG	ARG	TYR	ARG	ARG	ARG	ARG	ARG	THR	SER	63	6	
-1	GLY	ALA	GLY		GLY	GLY	CYS	CYS	GLY	TYR	CYS	CYS	CYS	SER	GLY	GLY	63	6	


GET CHAINS SUBGROUP I
RESIDUES
1 2C12 'CL
2 1B11 'CL
3 1H1 'CL
4 2A12 'CL
5 BR
6 DEN
7* 3D6 'CL
8 HK102 'CL
9 EU
10 CAR
11 FA
12 HK 137 'CL
13 RAV
14 KUE
15* MF2-1/17
16 VD 'CL
17 VD' 'CL
18 GAL (I)
19 OU (IOC)
20* MEA
21 MIL (=7)
22 WE3
23 DAUD 'CL
24 HK 134 'CL
FR1
CDR1
FR2
CDR2
FR3
CDR3
FR4

HUMAN KAPPA LIGHT CHAINS SUBGROUP I (cont'd)

		25 Va 'CL	26 WALKER 'CL	27 HIL01 'CL	28 Va 'CL	29 VRI-CHEJ 'CL	30 AMYLOID BAN	31 Va 'CL	32 VJ1 'CL	33 DEE	34 WIL (-)	35 AMO	36 V108 'CL	37 VRI-II 'CL	38 REI	39 AU	40 ROY	41 SI	42 HOVH CAMP 'CL	43 AG	44 SCW	45 REV	46 KA	47 LAY	48 SEL	49 NI
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	1	ASP	ASP	ASP	ala	ASP	ASP	ala	val	ASX	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP
	2	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	val	ILE	ILE	ILE	ILE
	3	GLN	GLN	GLN	GLN	GLN	GLN	GLN	asp	GLX	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	4	leu	MET	MET	leu	MET	leu	MET	MET	MET	MET	MET	val	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	leu	MET
	5	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR
	6	GLN	GLU	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	7	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	8	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
	9	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
FR1	10	the	ser	ser	ser	ser	ser	ser	leu	ser	THR	ser	ser	ser	ser	ser	ser	pro	ser	ser	ser	ser	THR	ser	ser	ser
	11	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU
	12	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	13	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	val	ALA	ALA
	14	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	thr
	15	VAL	VAL	VAL	VAL	VAL	VAL	thr	thr	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL
	16	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY
	17	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	gly	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP
	18	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	SER	asp	asp	asp	asp	asp	asp	asp	asp
	19	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val
	20	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	ile	THR	THR	THR	THR	THR
	21	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	phe	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	leu
	22	THR	THR	THR	THR	THR	THR	THR	ser	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	leu
	23	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS
	24	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	gln	gln	gln	gln	lys	gln	gln	ARG	glu	gln	glu	gln
	25	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ser	ALA	val	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA
	26	SER	SER	asp	SER	SER	SER	SER	gly	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	27	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	his	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	27A	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	ser
	27B	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	val
CDR1	27C	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	leu
	27D	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	gln
	27E	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	ile
	27F	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	thr
	28	gly	SER	gly	gly	gly	SER	gly	gly	SER	SER	SER	gly	gly	asp	asp	asp	asp	asn	asp	asp	SER	thr	asn	asx	SER
	29	ILE	ILE	ILE	ILE	ILE	val	ILE	ILE	val	val	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ser	val	val	ILE	gly
	30	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	GLY	ser	ser	ile	ser	ser	asn	asp	asn	arg	val	leu	asn	ser	ser
	31	asx	ASN	ser	asx	ASN	ASN	asx	ser	lys	lys	gly	ASN	ASN	lys	asp	ile	ASN	lys	asp	lys	asp	ser	gln	lys	ser
	32	tyr	tyr	TRP	ala	asn	tyr	tyr	tyr	tyr	TRP	ser	gly	ser	lys	asp	ile	ser	tyr	TRP	lys	asp	ser	gln	lys	thr
	33	LEU	LEU	LEU	LEU	LEU	val	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	phe
	34	ALA	asn	ALA	ALA	asn	ALA	ALA	asn	ALA	asn	asn	asn	asn	asn	asn	asn	ile	asn	asn	asn	asn	asn	asn		ALA
	35	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP		TRP
	36	tyr	tyr	tyr	tyr	tyr	PHE	tyr	tyr	tyr	tyr	tyr	tyr	SER	tyr	tyr	SER	SER	tyr	tyr	tyr	tyr	tyr	tyr		tyr
	37	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	his	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	asp	GLN	GLN	GLN		GLN
	38	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN		GLN
	39	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	the	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS
	40	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
	41	GLY	GLY	glu	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	lys
FR2	42	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS
	43	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	the	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	pro	ALA
	44	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
	45	lys	lys	lys	leu	lys	lys	lys	glu	lys	ASN	ser	thr	lys	lys	lys	lys	lys	lys	lys	lys	lys	lys	lys	glu	lys
	46	leu	leu	ser	leu	leu	ser	leu	leu	VAL	leu	leu	leu	leu	leu	leu	leu	phe	leu	ile	leu	leu	leu	leu	leu	leu
	47	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE
	48	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE
	49	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR		TYR	phe	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	phe	TYR	TYR	TYR	TYR
	50	ala	ala	ala	ser	ala	asp	ala	ala	ala	glu	gly	ala	ala	glu	asp	asp	asp	asn	asp	gly	asp	ala	gly	asp	asp
	51	ASA	ASA	ASA	ASA	ASA	ASA	ASA	ASA	ASA	ASA	ASA	ASA	ASA	ASA	ASA	ASA	ASA	ASA	ASA	ASA	ASA	ASA	ASA	ASA	ASA
CDR2	52	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	53	thr	ser	ser	ser	ser	thr	thr	thr	ser	ile	ser	ser	ser	ASN	ASN	lys	ASN	ASN	ASN	chr	ASN	ser	thr	thr	ASN
	54	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	asp	LEU	LEU
	55	gln	gln	gln	gln	gln	gln	gln	gln	LYS	asp	arg	gln	gln	glu	glu	glu	glu	glu	glu	glu	glu	glu	glu	LYS	glu
	56	ser	ser	ser	ser	ser	ser	ser	ser	ser	ile	ser	ser	ser	his	his	ile	ser	ser	ser	ile	ser	thr	thr	thr	thr
	57	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY
	58	PRO	thr	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
	59	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	60	ARG	ARG	ARG	ARG	ARG	asn	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG
	61	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE
	62	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	63	GLY	GLY	GLY	GLY	asp	GLY	GLY	GLY	GLY	GLY	GLY	GLY	asp	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY
	64	SER	SER	SER	SER	SER	SER	SER	SER	SER	ser	SER	SER	SER	SER	gly	thr	SER	SER	SER	gly	gln	SER	SER	SER	SER
	65	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLN	GLY	ile	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	asp	GLY	GLY	GLY	GLY	GLY
	66	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	67	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLN
	68	THR	THR	THR	ala	THR	THR	THR	THR	THR	THR	THR	THR	ala	THR	ala	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR
	69	GLU	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	asp	his	asp	asp	asp	asp	asx	asp	his	asp	asp	asp
	70	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	tyr	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE
	71	THR	THR	THR	THR	ile	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	ala	THR	THR	THR	THR	THR	THR	THR	THR
FR3	72	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	phe	phe	phe	LEU	phe	phe	LEU	LEU	phe	phe	LEU	phe
	73	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	ser	THR	THR	THR	THR	THR
	74	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ile	ser	ile	ile	ile	ile	ile
	75	ser	ser	ser	arg	ser	ser	ser	ser	ser	ser	thr	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser	ser
	76	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	gly	SER	SER	SER	SER	gly
	77	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU
	78	GLN	GLN	GLN	GLN	GLN	GLN	GLN	leu	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	79	pro	PRO	pro	PRO	pro	pro	SER	SER	PRO	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro
	80	glu	glu	glu	glu	glu	glu	glu	glu	glu	ASP	glu	glu	glu	glu	glu	glu	glu	glu	glu	asp	glu	glu	ala	glu	glu
	81	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	asn	ASP	ASP	asp	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASN
	82	PHE	ser	PHE	PHE	PHE	PHE	PHE	PHE	PHE	ser	PHE	val	PHE	ile	ile	ile	PHE	ile	ile	ile	PHE	PHE	ile	PHE	PHE
	83	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	gly	ALA	ALA	ALA	ALA	ALA
	84	IHR	THR	IHR	THR	THR	THR	THR	THR	THR	asp	ala	THR	ala	THR	THR	THR	THR	THR	THR	ser	THR	THR	THR	val	THR
	85	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR
	86	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	phe	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR	TYR
	87	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS
	88	gln	gln	gln	gln	gln	gln	gln	gln	glx	gln	gln	leu	gln	gln	gln	gln	gln	leu	gln	gln	gln	gln	gln	gln	gln
	89	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln	gln
	90	leu	ser	tyr	phe	ser	tyr	tyr	tyr	ser	tyr	ser	asp	ser	tyr	tyr	phe	tyr	his	ser	tyr	tyr	tyr	tyr	tyr	tyr
	91	SER	tyr	SER	SER	ASP	SER	SER	SER	tyr	SER	SER	ASP	SER	ASP	ASP	ASP	tyr	ile	ASP	ASP	tyr	leu	asn	ASX	ASP
	92	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
CDR3	94	tyr	thr	tyr	tyr	thr	tyr	tyr	PHE	thr	tyr	ser	thr	thr	leu	len	len	len	arg	leu	val	asn	leu	trp	PHE	leu
	95	pro	leu	pro	pro	pro	pro	pro	pro	pro	---	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro	pro
	95A	---	---		---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	GLU	---	---	---	---
	95B	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	95C	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	95D	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	95E	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	95F	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	96	---	ILE	---	---	---	TYR	---	---	TYR	---	TRP		TYR	ARG	ARG	ILE	VAL	ARG	PRO	LEU	SER				
	97	---	THR	---	---	---	THR	---	---	THR	---	THR		THR	THR	THR	THR	THR	THR	THR	THR	THR				
	98	---	PHE	---	---	---	PHE	---	---	PHE	PHE	PHE		PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE	PHE				
	99	---	GLY	---	---	---	GLY	---	---	GLY	GLY	GLY		GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY				
	100	---	GLN	---	---	---	GLN	---	---	PRO	GLN	GLN		GLN	GLN	GLN	GLY	GLN	GLN	GLN	GLY	GLN				
	101	---	GLY	---	---	---	GLY	---	---	GLY	GLY	GLY		GLY	GLY	GLY	SER	GLY	GLY	GLY	GLY	GLY				
	102	---	THR	---	---	---	---	---	---	THR	THR	THR		THR	THR	THR	THR	THR	THR	THR	THR	THR				
FR4	103	---	ARG	---	---	---	LYS	---	---	LYS	LYS	LYS		LYS	LYS	LYS	LYS	LYS	LYS	ARG	THR	LYS	LYS	GLU	LYS	
	104	---	LEU	---	---	---		---	---	VAL	LEU	VAL		LEU	VAL	VAL	LEU	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	
	105	---	ILE	---	---	---		---	---	GLU	GLU	GLN		GLN	GLU	ASP	GLU	GLU	GLU	GLU	ASP	ASP	GLU	GLU	GLU	
	106	---	ILE	---	---	---	ILE	---	---	MET	ILE	ILE		ILE	ILE	PHE	ILE	ILE	ILE	ASN	ILE	LEU	VAL	VAL	SER	
	106A	---	---	---	---	---	---	---	---	---	---	---		---	---	---	---	---	---	---	---	---	---	---	---	
	107	---	LYS	---	---	---	LYS	---	---	THR	LYS	ARG		THR	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS	LYS
	108	---	---	---	---	---	ARG	---	---	ARG	ARG			ARG	ARG	ARG	ARG	ARG	ARG	GLY	ARG	ARG	ARG		ARG	
	109	---	---	---	---	---		---	---	THR	THR			THR	THR	THR	THR		IHR	THR		THR				THR

HUMAN KAPPA LIGHT CHAINS SUBGROUP I (cont'd)

		50 RI	51* Ku RSV19 VK #	52 HBJ 4	53 BJ 26 #	54 BJ 19 #	55 RFZ	56 HA	57 PAU	58 PSM	59 JBL #	60 NE	61 FRA	62 BJ 40	63 MF3- 1C/6	64 MF2, 1/13B	65 MF2, 18/2	66 GR'	67 GI 'CL #	68 NOM	69 KER	70 ESM IGG	71 ESM IGM	72* DAV	73* FIN	74 PW #	75 SHE	76 ADA
	0	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---		---	---	---	---	---	---	---	---
	1	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP		ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP
	2	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE		ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE
	3	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN		GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	4	MET	leu	MET	MET	leu	MET	MET	MET	MET	MET	leu	MET	MET	MET	MET	MET	MET		MET	MET	PZI	MET	MET	MET	leu	val
	5	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR		THR	THR	THR	THR	THR	THR	THR	THR
	6	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLN	GLN	GLN	GLN		GLX	GLN	GLN	GLN	GLN	GLN	GLN	GLN
	7	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	SER	SER	SER	SER	SER	SER
	8	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO		PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
	9	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	SER	SER	SER	SER	thr	SER
F	10	ser	ser	THR	ser	ser	ser	ser	THR	ser	ser	ser	THR	ser	ser	ser	ser	THR		ser	ser	ser	ser	ser	ser	ser	ser
FR	11	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	SER	LEU	LEU	LEU		LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU
1	12	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	SER	SER	SER	SER	SER	SER
	13	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA		ALA	ALA	ALA	ALA	thr	thr	ALA	val
	14	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	SER	SER	val	val	SER	phe
	15	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL		VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL
	16	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY		GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY
	17	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASX	ASP	ASP	ASP	ASP	ASP	ASP		ASP	ASP	ASP	ASP	ASP	ASP	ASX	ASP
	18	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg		arg	arg	arg	arg	arg	arg	arg	arg
	19	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val	val		val	ILE	val	val	val	val	val	val
	20	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR		THR	THR	THR	THR	THR	THR	THR	THR
	21	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE		ILE	ILE	ILE	ILE	ILE	val	phe	ILE
	22	THR	THR	THR	THR	THR	THR	THR	THR	THR	ala	THR	THR	THR	THR	THR	THR	THR		THR	THR	THR	THR	THR	arg	THR	THR
	23	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS		CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS
	24	gln	ARG	ARG	gln	gln	gln	gln	ARG	gln	ARG	glx	ARG		ARG	ARG	ARG	ARG		gln	gln	ARG	ARG	asp	asp	ARG	glx
	25	ALA	ser	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA		ALA	ALA	ALA	ALA		ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA
	26	asn	SER	SER	SER	SER	asn	SER	SER	SER	SER	SER	SER		SER	SER	SER	SER		arg	SER	SER	SER	SER	SER	SER	SER
	27	GLN	GLN	GLN	GLN	GLN	GLN	---	GLN	GLN	GLN	GLX	GLX		GLN	GLN	GLN	arg		his	GLN	GLN	GLN	GLN	GLN	GLN	GLX
	27A	---	thr	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---
	27B	---	leu	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---
C	27C	---	val	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---
D	27D	---	ala	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---
R	27E	---	thr	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---
1	27F	---	---	---	---	---	---	---	---	---	---	---	---		---	---	---	---		---	---	---	---	---	---	---	---
	28	val	asp	SER	SER	asp	val	---	SER	asp	SER	asn	asn		gly	gly	gly	SER		asp	asp	SER	SER	asn	asn	SER	asn
	29	ILE	gly	val	ILE	ILE	ILE	---	ILE	ILE	ILE	ILE	ILE		ILE	ILE	ILE	ILE		ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE
	30	ser	asn	asn	asn	asn	ser	arg	ser	arg	ser	ser	asn		asn	arg	arg	ser		ser	lys	ser	ser	ser	ser	GLY	ser
	31	lys	thr	ASN	lys	thr	lys	ASN	ser	ser	thr	ASX	ASX		ASN	ASN	ASN	ser		ASN		ser	ser	ser	ser		ASX	ASX
	32	ser	TYR	TRP	TYR	phe	ser	TYR	ser	TYR	phe	TYR	TYR		asp	asp	asp	TRP		TYR		TYR	TYR	TRP	TYR	TYR	TYR
	33	LEU	LEU	LEU	ala	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU		LEU		LEU	LEU	LEU	LEU	LEU	LEU
	34	asn	glu	ALA	asn	asn	asn	asn	ALA	asn	ser	asx	ALA		gly	gly	gly	ALA		asn		asx	asx	ile	ile	asx	asx
	35	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP	TRP		TRP	TRP	TRP	TRP		TRP		TRP	TRP	TRP	TRP	TRP	TRP
	36	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr	tyr		tyr	tyr	tyr	tyr		PHE		tyr	tyr	tyr	tyr	tyr	tyr
	37	GLN	GLN	GLN	GLN	glu	GLN	GLN	GLX	GLN	GLN	GLX	GLX		GLN	GLN	GLN	GLN		GLN		GLX	GLX	GLN	GLN	GLN	GLY
	38	GLN	GLN	glu	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLX	GLX		GLN	GLN	GLN	gly		GLN				GLN	GLN	GLN	tyr
	39	arg	LYS	LYS	#	#	arg	LYS	LYS	LYS	LYS	LYS	LYS		LYS	LYS	LYS	arg		LYS				tyr	tyr	LYS	LYS
	40	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	gln	PRO	PRO		PRO	PRO	PRO	PRO		PRO				PRO	PRO		PRO
F	41	GLY	GLY	GLY	#	#	GLY	GLY	GLY	GLY	GLY	GLY	GLY							GLY							
FR	42	gln	LYS	glx	LYS	LYS	gln	arg	glx	LYS	LYS	glx															
2	43	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA		ALA													
	44	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO		PRO													
	45	lys	lys	lys	lys	lys	lys	lys	lys	lys	lys	lys		lys													
	46	leu	leu	leu	leu	leu	leu	leu	leu	leu	leu	VAL															
	47	LEU	LEU	LEU		LEU	LEU	LEU	LEU	LEU	LEU	LEU		met													
	48	ILE	ILE	ILE		ILE	ILE	ILE	ILE	ILE	ILE			ILE													
	49	TYR	TYR	asx		TYR	TYR	TYR	TYR	TYR	TYR	TYR		gly													
	50	asp	arg	LYS		asp	asp	asp	glx	asp				asp													
C	51	ile	val	thr	asp	ALA	ALA	ALA		SER				ALA													
D	52	SER	SER	SER	SER	SER	ala	phe						SER													
R	53	ASN	ASN	asx	arg	ser	ASX	ASN						asx													
2	54	LEU	arg	LEU	LEU	LEU	LEU	LEU		LEU				LEU													
	55	glu	phe	glu	glu	glu	glu	glu		glu				asx													
	56	ser	ser	arg	thr	thr		ala						ser													
	57	GLY	GLY	GLY	GLY	GLY	GLY	GLY						GLY													
	58	val	val	asx	val	val		val						val					ILE								
	59	PRO	PRO	PRO	PRO	gln	PRO	PRO						val					PRO								
	60	SER	SER	SER	SER	SER		SER						SER					SER								
	61	ARG	ARG	ARG	lys	ARG	ARG	ARG						ARG					ARG								
	62	PHE	PHE	PHE	val	PHE	PHE							gln					PHE								
	63	thr	SER	ala	SER	leu	thr							SER					SER								
	64	GLY	GLY	GLY	GLY	GLY	GLY							GLY					GLY								
	65	SER	SER	SER	SER	SER								SER					SER								
	66	GLY	GLY	GLY	asx	GLY								GLY					GLY								
	67	SER	SER		phe									SER					SER								
	68	GLY	GLY		glu	asx								GLY					GLY								
	69	THR	THR		THR	THR								THR					THR								
	70	asp	asp		asp	lys								asp					GLU								
	71	PHE	PHE		val									pro					PHE								
F	72	phe	THR		THR									ser					THR								
FR	73	LEU	phe		val									LEU					LEU								
3	74	THR	THR		asn									gln					THR								
	75	ile	ile		gln									VAL					VAL								
	76	ser	ser		ser									ser					ILE								
	77	ser	ser		ser									arg					ASN								
	78	LEU	LEU		LEU														LEU								
	79	arg	GLN		GLN														GLN								
	80	pro	pro		pro														SER								
	81	gln	glu		glu														ASP								
	82	ASP	ASP		ASP														ASP								
	83	val	ile		ile														PHE								
	84	ALA	ALA		ALA														ALA								
	85	THR	THR		pro														THR								
	86	TYR	TYR		lys														TYR		TYR						
	87	phe	TYR																TYR		TYR						
	88	CYS	CYS																CYS		CYS						
	89	glu	phe		gln														HIS		gln						
	90	glu	gln		gln														HIS		gln						
	91	tyr	gly		arg	phe								gly					ASN		tyr						
	92	ala	ser		ASP	ASP								glu					ASP		asp						
	93	asn	ala		asp	asp								SER					thr		asp						
C	94	len	leu		leu	leu								asp					PHE		leu						
D	95	pro	pro		pro	pro								SER					SER		pro						
R	95A	---	---		---	---								---					---		---						
3	95B	---	---		---	---								---					---		---						
	95C	---	---		---	---								---					---		---						
	95D	---	---		---	---								---					---		---						
	95E	---	---		---	---								---					---		---						
	95F	---	---		---	---								---					---		---						
	96	LEU	ARG		TYR	TYR								ARG					TRP		PRO						
	97	THR	THR		THR	THR								THR					THR		THR						
	98	PHE	PHE		LEU	PHE								PHE					PHE		PHE						
	99	GLY	GLY		GLY	GLY								GLY					GLY		GLY						
	100	GLY	GLY		GLY	PRO								GLN					GLN		PRO						
	101	GLY	GLY		GLY	GLY								GLY					GLY		GLY						
	102	THR	THR		THR	THR								THR					THR		THR						
F	103	LYS	LYS		LYS	LYS								LYS					LYS		LYS						
FR	104	VAL	VAL		VAL	VAL								VAL					VAL		VAL						
4	105	GLU	GLU		GLU	GLU								GLU					LEU		ASP						
	106	ILE	ILE		MET	LEU								VAL					ILE		LEU						
	106A	---			---	---								---					---		---						
	107	LYS			LYS	LYS								LYS					LYS		LYS						
	108																		ARG								
	109																										

HUMAN KAPPA LIGHT CHAINS SUBGROUP I (cont'd)

		77 AMYLOID VIII-B	78 PAUL	79 RI #	80 WAT	81* LOW	82 AMYLOID ES305 #	83 OCO	84 BOL	85 AMYLOID X	86 SAC	87 BJ	88 GO	89 F- GUI #	90 S- GUI #	91 HF6- 21/28	92 DIE	93 CAR A	94 TEI	95 MON	96 ALE	97 JOH	98 POT	99 CON	100 TRA	101* R-G2b
	0	---	---	pca	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	1	ASP	ASP	ASP	ASP	ASP	ASP	ASX	ASP	ASP	ASP	ASX	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASX	ASP	ASP	ASP	ASP	ASP
	2	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE		val	ILE	ILE	val	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE
	3	GLN	GLN	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLN		GLN	GLN	GLN	val	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLN	GLN	GLN
	4	MET	MET	MET	MET	MET	leu	MET	ile	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET
	5	THR	THR	THR	THR	THR	THR	THR	met	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR
	6	GLN	GLN	GLN	GLN	GLN	GLN	GLX	GLX	GLN	GLN	GLN	GLX	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLN	GLN	GLN
	7	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	ala	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	8	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	ala	PRO		PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO
	9	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	SER	leu	SER	SER	SER	SER	SER	SER	SER	ala	SER	SER
F	10	ser	THR	THR	ser	ser	ser	ser	ser	ser	ser		phe	ser	ser	ser	ser	ser	ser	THR		ser	ser	ser	ser	ser
R	11	LEU	LEU	LEU	LEU	LEU	LEU	LEU	val	LEU	LEU		val	LEU	LEU	LEU	LEU	LEU	LEU	LEU		LEU	LEU	LEU	LEU	LEU
I	12	SER	SER	SER	SER	SER	SER	val	SER	SER	SER		SER	SER	SER	pro	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	13	ALA	ALA	ALA	ALA	ALA	ALA	met	ala	ALA	ALA		ALA	ALA	ALA	val	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA
	14	SER	SER	SER	SER	SER	SER	pro	SER	SER	SER		ALA	SER	SER	thr	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER
	15	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL		VAL	VAL	VAL	leu	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL	VAL
	16	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY		GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY	GLY
	17	ASP	ASP	ASP	ASP	ASP	ASP	ASX	ASX	ASP	ASP		ASX	ASX	ASX	gly	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP
	18	arg	arg	gly	arg	arg	leu	arg	arg	arg	---	arg	arg	arg	arg	pro	arg	arg	arg	arg	arg	arg	arg	arg	arg	arg
	19	val	val	val	val	val	val	val	val	val	---	val	val	val	val	ala	val	val	val	val	val	val	val	val	val	val
	20	THR	THR	THR	THR	THR	THR	THR	THR	ile	---	THR	THR	THR	THR	ser	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR
	21	ILE	ILE	ILE	ILE	ILE	?	ILE	ILE	ILE	---	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	leu	ILE	ILE	ILE	ILE	ILE
	22	THR	THR	ser	THR	THR	THR	THR	THR		---	THR	THR	THR	THR	ser	THR	THR	THR	THR	ser	THR	THR	THR	ILE	THR
	23	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	---	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS	CYS			
	24	gln	ARG	ARG	glx	ala	gln	glx	ARG	glx	---	gln	ARG		ARG	ARG					ARG					
	25	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	ALA	---	ALA	ALA	ALA	ALA	ser										
	26	SER	SER	SER	SER	SER	SER	SER	SER		---	SER	SER			SER										
	27	GLN	GLN	GLX	GLN	GLN	glu	GLX	GLX	GLX	---	GLN				GLN										
	27A	----	----	----	----	----	----	----	----	----	---	----				ser										
	27B	----	----	----	----	----	----	----	----	----	---	----				leu										
	27C	----	----	----	----	----	----	----	----	----	---	----				val										
C	27D	----	----	----	----	----	----	----	----	----	---	----				lys										
D	27E	----	----	----	----	----	----	----	----	----	---	----				ser										
R	27F	----	----	----	----	----	----	----	----	----	---	----				---										
1	28	asx	SER	SER	asp	asx	ala	asx	gly	asn	---	asp				asp										
	29	ILE	ILE	ILE	ILE	ILE	?	ILE	ILE	ILE	---	ILE				gly										
	30	GLY	ser	ser	ser	ser		ser			---	ser				ser										
	31		asp		tyr		ASN	ASX		pro	---	asn				ser										
	32	tyr	ser		val		tyr	tyr		tyr	---	lys				tyr										
	33	LEU			val		LEU			LEU	---					LEU										
	34				asn						---					glu										
	35	TRP			TRP		TRP				---					TRP										
	36						tyr			tyr	---					tyr										
	37										---					GLN										
	38										---					GLN										
	39										---					LYS										
	40										---					PRO										
F	41										---															
R	42										---															
2	43										---															
	44										---															
	45										---															
	46										---															
	47										---															
	48										---															
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	50										LYS															
	51										ser															
C	52										---															
D	53										---															
R	54										---															
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	87										---		TYR													
	88										CYS		CYS													
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	90										gln		gln													
	91										glx		tyr													
	92										ASX		gln													
	93										ala		asn													
C	94										ala		leu													
D	95										thr		pro													
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	102										THR															
F	103										LYS															
R	104										VAL															
4	105										ASX															
	106										VAL															
	106A										---															
	107										LYS															
	108										ARG															
	109										THR															

HUMAN KAPPA LIGHT CHAINS SUBGROUP I (cont'd)

	102* K-G2	103 BRA	104 LUX	105* HEI	106 PAP	107* R-G2s	108 CRA	109 AMYLOID LEP	110* WAG	111 MOE	112 HBJ 1	113 AMYLOID 547	114 WEB	115 HBJ 10	116 LOO	117 BEN	118 MAA	119 GR	120 MUR	121 GM131 'CL 8	122 AMYLOID 394	123* MAR	124 CL*
0																							
1	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP	ASP		ASP	ASP	ASP
2	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE	ILE		ILE	ILE	ILE
3	GLN	GLN	GLN	GLN	GLN		GLN	GLN	GLN	GLN	leu	GLX	GLN	GLN	GLN	GLN	GLN	GLN	GLN		GLN	GLN	GLN
4	MET	MET	leu	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET	MET2		MET	leu	MET	MET		MET	leu	---
5	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR	THR		THR	THR	---
6	GLN	GLX	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLN	GLX	GLN	GLN	GLN	GLN	GLN	GLN	GLN		GLN	GLN	---
7	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	---
8	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO	PRO		PRO	PRO	---
9	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	thr	SER	SER	SER	SER	SER	SER	SER	SER		SER	SER	---
10	SER	SER	phe	THR	SER	SER	SER	SER	SER	THR	SER	SER	ser	SER	THR	ser	THR	ser	ser		SER	SER	---
11	LEU	LEU	LEU	LEU	LEU	LEU	LEU	LEU	SER	LEU	LEU	LEU	val	LEU		LEU	LEU	LEU	LEU		LEU	LEU	---
12	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER				---
13	ALA	val	ALA	ALA	val	ALA	ALA	ALA	ALA	ALA	SER	ALA	ALA	ALA	ALA	ALA	ALA	val					---
14	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER	SER									---
15	VAL	VAL	VAL	VAL	VAL	VAL	leu	VAL	VAL	VAL	VAL	VAL	VAL		VAL								---
16	GLY	GLY	GLY	GLY	GLY	GLY	arg	GLY	GLY		GLY												---
17	ASP	gln	ASP	ASP	ASP	ASP	ASP	ASP	ASP		ASP												---
18	arg	arg	arg	arg	arg	arg	arg	arg															---
19	val	val	val	val	val	val	val	val															---
20	THR	THR	THR	THR	THR		THR	THR															---
21	ILE	ILE	ILE		ILE	ILE	ILE																---
22	THR	THR	THR		ala	THR	THR																---
23		CYS																					---
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79																			GLN				---
80																			PRO				---
81																			glu				---
82																			ASP				---
83																			PHE				---
84																			ALA				---
85																			THR				---
86																			TYR				---
87																			TYR				---
88																			CYS				---
89																			gln				---
90																			phe				---
91																			his				---
92																			ASP				ASP
93																			his				ile
94																			leu				PHE
95																			pro				pro
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95E																			---				---
95F																			---				---
96																			LEU				GLY
97																			THR				THR
98																			PHE				PHE
99																			GLY				GLY
100																			GLY				GLN
101																			GLY				GLY
102																			THR				THR
103																			THR				LYS
104																			VAL				VAL
105																			GLU				GLU
106																			MET				ILE
106A																			---				---
107																			LYS				LYS
108																			ARG				ARG
109																			THR				THR

Samsung Bioepis Exhibit No. 1002
Page 5162

HUMAN KAPPA LIGHT CHAINS SUBGROUP I (cont'd)

		125 AMYLOID	126 ABJ .6	127 AMYLOID MS #	128 BEN	# OF SEQUENCES	# OF AMINO ACIDS	OCCURRENCES OF MOST COMMON AMINO ACID	VARIABILITY
	0	---	---	---	---	1	1	1(PCA)	
	1	ASP	ASP	ASP	ASP	125	3,4	122(ASP),118(ASP)	3.1,4.2
	2	ILE	ILE	#	ILE	123	2	120(ILE)	2.
	3	glu	GLN	GLN	GLN	123	7	116(GLN),112(GLN)	7.4,7.7
	4	MET	MET	MET	MET	124	4	109(MET)	4.6
	5	pro	THR	THR		124	3	122(THR)	3.
	6	GLN	GLN			123	1,2	123(GLN),116(GLN)	1.,2.1
	7	SER				121	2	120(SER)	2.
	8	PRO				121	2	120(PRO)	2.
	9	SER				121	4	117(SER)	4.1
FR1	10	ser				120	5	89(SER)	6.7
	11	LEU				119	4	111(LEU)	4.5
	12					118	5	113(SER)	7.1
	13					117	4	107(ALA)	4.4
	14					113	6	106(SER)	6.4
	15					113	3	109(VAL)	3.1
	16					109	2	108(GLY)	2.
	17					109	3,4	106(ASP),98(ASP)	3.1,4.6
	18					106	6	97(ARG)	6.6
	19					107	3	101(VAL)	3.2
	20					106	4	102(THR)	4.2
	21					104	4	99(ILE)	4.2
	22					104	6	95(THR)	6.6
	23					96	1	96(CYS)	1.
	24					88	6	57(ARG)	9.3
	25					88	6	82(ALA)	6.4
	26					85	4	79(SER)	4.3
	27					84	4	79(GLN),66(GLN)	4.3,5.1
	27A					3	2	2(SER)	
	27B					3	2	2(LEU)	
	27C					3	2	2(VAL)	
CDR1	27D					3	3	1(+)	
	27E					2	2	1(+)	
	27F								
	28					84	7	29(SER)	20.
	29					83	5	72(ILE)	5.8
	30					81	11	41(SER)	22.
	31					79	12	27(ASN),24(SER)	35.,39.
	32					79	9	34(TYR)	21.
	33					77	4	73(LEU)	4.2
	34					73	7,8	30(ALA)	17.,19.
	35					76	1	76(TRP)	1.
	36					74	2	66(TYR)	2.2
	37					73	4	68(GLN),61(GLN)	4.3,4.8
	38					71	4	68(GLN),63(GLN)	4.2,4.5
	39					68	4	62(LYS)	4.8
	40					70	3	68(PRO)	3.1
	41					68	3	54(GLY)	3.2
	42					60	6	49(LYS)	7.3
FR2	43					61	4	57(ALA)	4.5
	44					61	1	61(PRO)	1.
	45					61	7,8	46(LYS)	9.3,11.
	46					60	7	42(LEU)	10.
	47					59	2	58(LEU)	2.
	48					57	2	56(ILE)	2.
	49					59	4	55(TYR)	4.3
	50					59	9	12(ALA)	20.
	51					59	5	50(ALA)	5.9
	52					58	6	53(SER)	6.6
CDR2	53					57	6,7	26(SER)	13.,15.
	54					58	2	56(LEU)	2.1
	55					58	8,9	23(GLU)	19.,21.
	56					56	8	34(SER)	13.
	57					57	1	57(GLY)	1.
	58					57	3	50(VAL)	3.5
	59					57	4	54(PRO)	4.2
	60					57	1	57(SER)	1.
	61					58	3	56(ARG)	3.1
	62					57	3	55(PHE)	3.1
	63					57	6	50(SER)	6.8
	64					57	2	55(GLY)	2.1
	65					56	5	51(SER)	5.5
	66					57	4	54(GLY)	4.2
	67					55	3	51(SER)	3.2
	68					55	3	52(GLY)	3.2
	69					55	2	51(THR)	2.2
	70					55	5,6	33(ASP),31(ASP)	8.5,11.
	71					54	4	49(PHE)	4.4
FR3	72					54	5	50(THR)	5.4
	73					54	3	43(LEU)	3.8
	74					54	4	51(THR)	4.2
	75					54	3	47(ILE)	3.4
	76					53	5	43(SER)	6.2
	77					54	6	40(SER)	8.1
	78					54	2	53(LEU)	2.
	79					54	3,4	52(GLN),48(GLN)	3.1,4.5
	80					54	2	46(PRO)	2.3
	81					54	3,5	37(GLU),34(GLU)	4.4,7.9
	82					54	2	53(ASP),50(ASP)	2.,2.2
	83					54	4	40(PRE)	5.4
	84					54	2	53(ALA)	2.
	85					54	6	48(THR)	6.7
	86					56	2	55(TYR)	2.
	87					55	2	52(TYR)	2.1
	88					56	1	56(CYS)	1.
	89					57	4,5	46(GLN),43(GLN)	5.,6.6
	90					57	2,3	51(GLN),46(GLN)	2.2,3.7
	91					58	11,12	20(TYR)	23.,25.
	92					60	9	20(ASN),19(+)	27.,28.
	93					60	9	29(SER)	19.
	94					60	12	15(LEU)	48.
CDR3	95					56	4	44(PRO)	5.1
	95A					1	1	1(GLU)	
	95B								
	95C								
	95D								
	95E								
	95F								
	96					44	12	10(TRP)	53.
	97					43	4	39(THR)	4.4
	98					46	3	44(PHE)	3.1
	99					46	1	46(GLY)	1.
	100					46	4,5	28(GLN),27(GLN)	6.6,8.5
	101					46	1	46(GLY)	1.
	102					46	2	45(THR)	2.
	103					46	4	38(LYS)	4.8
FR4	104					45	2	36(VAL)	2.5
	105					45	4,5	29(GLU),28(GLU)	6.2,8.
	106					46	7	30(ILE)	11.
	106A								
	107					45	3	42(LYS)	3.2
	108					33	2	31(ARG)	2.1
	109					22	1	22(THR)	1.

Samsung Bioepis Exhibit No. 1002
Page 5163

D9

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30. Dez. 2013

European Patent Office

80298 M U N I C H

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

Samsung Bioepis Exhibit No. 1002
Page 5165

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

Samsung Bioepis Exhibit No. 1002
Page 5166

(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.

Samsung Bioepis Exhibit No. 1002
Page 5167

(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)

Samsung Bioepis Exhibit No. 1002
Page 5168

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

Samsung Bioepis Exhibit No. 1002
Page 5169

> 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.

Samsung Bioepis Exhibit No. 1002
Page 5174

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.

Samsung Bioepis Exhibit No. 1002
Page 5175

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
Page 5178

L27 ANSWER 2 OF 2 REGISTRY COPYRIGHT 2009 ACS on STN
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 ***
*** USE 'SQD' OR 'SQIDE' FORMATS TO DISPLAY SEQUENCE ***
59 REFERENCES IN FILE CA (1907 TO DATE)
2 REFERENCES TO NON-SPECIFIC DERIVATIVES IN FILE CA
59 REFERENCES IN FILE CAPLUS (1907 TO DATE)

The European Agency for the Evaluation of Medicinal Products
Evaluation of Medicines for Human Use

London, 20 February 2003
CPMP/QWP/419/03

COMMITTEE FOR PROPRIETARY MEDICINAL PRODUCTS (CPMP)

<DRAFT>

NOTE FOR GUIDANCE ON EXCIPIENTS, ANTIOXIDANTS AND ANTIMICROBIAL PRESERVATIVES IN THE DOSSIER FOR APPLICATION FOR MARKETING AUTHORISATION OF A MEDICINAL PRODUCT

DISCUSSION IN THE QUALITY WORKING PARTY	April 2002, January 2003
TRANSMISSION TO CPMP	February 2003
RELEASE FOR CONSULTATION	February 2003
DEADLINE FOR COMMENTS	August 2003

Note:
This Note for Guidance replaces 3AQ9a Excipients in the Dossier for Application for Marketing Authorisation of a Medicinal Products and CPMP/CVMP/QWP/115/95 Note for Guidance on Inclusion of Antioxidants and Antimicrobial Preservatives in Medicinal Products

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AMG1052

NOTE FOR GUIDANCE ON EXCIPIENTS, ANTIOXIDANTS AND ANTIMICROBIAL PRESERVATIVES IN THE DOSSIER FOR APPLICATION FOR MARKETING AUTHORISATION OF A MEDICINAL PRODUCT

INTRODUCTION

This note for guidance is concerned with the application to excipients, antioxidants and antimicrobial preservatives of Module 3, sections P.1, P.2, P.4, P.5, P.8 of the Common Technical Document with a view to granting a marketing authorisation for a new medicinal product.

The data should be presented according to the standard format described in the CTD Module 3, part P.

Antioxidants and Antimicrobial Preservatives are substances which are used to extend the shelf-life of medicines by respectively retarding the oxidation of active substances and excipients, and by reducing microbial proliferation.

The properties of these substances are due to certain chemical groups which are usually aggressive towards living cells and which lead to certain risks when used in man.

If it is not absolutely necessary to add these substances in medicinal products they must be avoided.

The purpose of this note for guidance is to describe the information that needs to appear in application for marketing authorisations with regards to the addition of any antioxidants or antimicrobial preservatives.

For each antioxidants and antimicrobial preservative the application should contain :

- reason for inclusion
- proof of efficacy
- the method of control in finished product
- details of the labelling of the finished product
- safety information

Several guidelines should be also taken into account :

- ICH Topic Q 6 A : Specifications : Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products : Chemical Substances (3.3.2.2. Oral liquids : d) Antimicrobial preservative content, e) Antioxidants preservative content ; 3.3.2.3. Parenteral Drug Products : g) h) and Decision Tree 8 : Microbiological Attributes of Non-sterile drug products).
- Note for Guidance on Development Pharmaceutics : 3.3. Liquid and Semi-solid Formulations.
- 3BC7A : Excipients in the Label and Package leaflet of Medicinal Products for Human Use
- Note for guidance on maximum shelf-life for sterile products after first opening or following reconstitution.

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Samsung Bioepis Exhibit No. 1002
Page 5181

SECTION P.1 Description and Composition of the Drug Product

Excipients must be listed, specifying their common name, their quantity and the use and reference to any relevant standard. When the common name is not sufficient to indicate functional specifications, the brand name with commercial grade should be specified. In the case of excipients presented as a mixture of compounds, details as to the composition should be provided in qualitative and quantitative terms. However, for flavouring agents and aromatic substances, it is permitted to give the qualitative composition only.

Antimicrobial preservatives and antioxidants should be chemically defined (reference to existing European Pharmacopoeia monographs may be used) and designated by the Chemical Abstract Service Registry Number (RN-CAS).

The purpose for the inclusion of any antioxidant or microbial preservative should be stated (antioxidant for the benefit of active substance or excipient or both, or antimicrobial preservative).

SECTION P.2 Pharmaceutical development

SECTION P.2.1 Components of the Drug Product

The section P.2.1.2 should comprise an explanation of the choice of the excipient (and grade where necessary) and the level of excipient according to the note for guidance "Development pharmaceutics and process validation".

For antimicrobial preservatives and antioxidants, during the pharmaceutical development of the product the applicant should demonstrate:

- the necessity to add an antioxidant or preservative to the finished product at the chosen level
- the physical and chemical compatibility of the antioxidant and of the preservative with other constituents of the finished product, the container and the closures.

The used concentration must be justified in terms of efficacy and safety, such that the minimum concentration of preservative is used which gives the required level of efficacy. The appropriate test method for efficacy of antimicrobial preservation is that of the European Pharmacopoeia. Tbis should be used to determine whether the required level of activity is achieved.

In the case of antioxidants, these should only be used once it has been shown that their use cannot be avoided, even if the manufacturing process is optimised to minimise the potential for oxidation, for instance by manufacturing and filling products under an inert headspace gas.

The safety of the antioxidant or preservative should be supported by bibliographic and /or experimental data.

Some antioxidants or antimicrobial preservatives may be undesirable under certain circumstances :

- mercury containing preservatives : see the CPMP Position Paper on Thiomersal, Implementation of the Warning Statement Relating to Sensitisation (CPMP/2612/99) and EMEA Position Statement on recent developments concerning thiomersal in vaccines (EMEA/CPMP/1578/00). This kind of preservative should be strictly avoided except if no other possibility may be considered but in this case the choice should be fully justified.

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Samsung Bioepis Exhibit No. 1002
Page 5182

- benzyl alcohol : when used in parenteral products for children under the age of two years. Its degradation product and metabolite is the benzaldehyde, toxic for the CNS.
- Benzoic acid esters (Para hydroxy benzoate and their derivates etc) : when used in any dosage-form for parenteral use.
- Sulphites and Metabisulphites.

Parenteral infusions do not contain any added antimicrobial preservatives and no antimicrobial preservatives are added when the medicinal product is intended for administration by routes where for medical reasons an antimicrobial preservative is unacceptable, such as intercisternally or by any other route of administration which gives access to the cerebrospinal fluid or retro-ocularly.

SECTION P.4 Control of Excipients

Examples of different kinds of excipients are given in the annex.

1. Specifications (P.4.1), Analytical Procedures (P.4.2), Validation of Analytical Procedures (P.4.3) and Justification of Specifications (P.4.4) :

1.1 Excipients described in the European Pharmacopoeia or, if not described in the European Pharmacopoeia, pharmacopoeia of a Member State

The routine tests which are to be carried out on each batch of starting materials must be stated in the application for marketing authorisation. If tests other than those mentioned in the pharmacopoeia are used, proof must be supplied that the test methods used are suitable to establish that the starting materials meet the quality requirements of that pharmacopoeia. When the monograph covers a family of related products, the particular specifications chosen for the excipients must be submitted. In addition and when necessary, the test used to determine the quality of the excipient should be shown to be in relation to the function that it fulfils in the medicinal product.

Data on microbiological contamination of the excipients used in the manufacture of sterile products should always be given where membrane filtration is used to achieve sterility.

Antimicrobial preservatives and antioxidants are defined as excipients and as such should be controlled following the rules governing medicinal products in the European Union. These data should be provided in part P.4.

1.2 Excipients not described in the European Pharmacopoeia or in the pharmacopoeia of a Member State

An appropriate specification of the excipient must be established, based on the following types of tests:

- Physical characteristics
- Identification tests
- Purity tests, including limits for total or individual impurities, which should be named. Purity tests may be physical, chemical, biological and, if appropriate, immunological.

  Where sterile filtration is used in the manufacture of a parental medicinal product, data and routine tests on microbiological contamination of excipients should always be given.
- Other relevant tests including, e.g. the tests on parameters which may influence the performance of the dosage form.

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Samsung Bioepis Exhibit No. 1002
Page 5183

* Assay or limit tests if necessary.

When an excipient is not described in the European Pharmacopoeia or in the pharmacopoeia of a Member State or in an other compendium of established use (e.g. USNF Pharmacopoeia and Japanese Pharmacopoeia), validation data of the test methods used should be presented, where appropriate.

1.3 Justification of Specifications

Justifications of specifications takes into account the choice and use of an excipient which is used for a particular purpose: it will determine the properties which must be checked during the routine tests and which will be the subject of certain specifications in connection with the bioavailability of the product (see Note for Guidance "Specifications and control tests on the finished product").

Nevertheless, justification of specifications are not systematically required for well-known excipients. For example, they are not required for excipients which have been used in similar medicinal products for a long period of time and when their characteristics and properties have not changed significantly.

For solid and semi-solid dosage forms, the justification of specifications should, if necessary, provide information on the relevant characteristics of the excipient. Special tests are often necessary (e.g. to verify the capacity of the excipient to emulsify and disperse, or to measure the viscosity...).

Appropriate data are needed for excipients used in a new route of administration.

Justification of specifications on excipients already included in the European Pharmacopoeia, or if not included in the European Pharmacopoeia, in the pharmacopoeia of a Member State and other well-known excipients already used in a medicinal product.

For these excipients, justification of specifications will normally not be required. However, any particular specification concerning the characteristics, as defined in Section P.2.1.2, should be justified (e.g. sieve analysis, in relation to microcrystallinity).

Excipients of Human or Animal Origin (P.4.5)

Viral Safety and TSE Risk should be documented in accordance with the relevant directives.

Novel Excipients (P.4.6)

For novel excipients : a dossier should be established containing the same data as required for new active substances:

a) A strict definition of the excipient, its function and its conditions of use. If the excipient is complex or is made of a mixture of compounds, the composition must be specified in qualitative and quantitative terms.

b) For new excipients and for excipients presented as a mixture of compounds the following should be taken into consideration:

   i. Any bibliographical data on the chemistry and on the toxicology and the field in which the product is already used.

   ii. The Community provisions concerning additives in foodstuff: any criteria which are based on the toxicological data, with cross-references to these data.

   The quality specifications which have been laid down in the directives are satisfactory as long as the routine control tests used are validated.

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Samsung Bioepis Exhibit No. 1002
Page 5184

iii. The international specifications (FAO/WHO/JECFA), and other publications such as the Food Chemical Codex.

iv. For medicinal products for topical use, data on the starting material in cosmetic products (Directive 76/768/EEC).

v. Data concerning the toxicology of the new excipient should be presented according to the dosage form and the route of administration of the medicinal product (if applicable).

c) Documentation on chemistry of excipients is required for all new excipients, taking as its basis the Note for Guidance "Chemistry of Active Substances".

* The origin of the excipient, including the name and address of manufacturer.
* A general outline of the synthesis (manufacture and purification).
* Structure.
* Physical, chemical properties, identification and purity tests.
* Validated methods of analysis with a presentation of batch results.
* Miscellaneous information (microbiological tests, etc).
* Contamination, presence of foreign substances, residual solvents, etc.
* In the case of an excipient obtained from a mixture of several components, the quality of each component and the physico-chemical tests for the mixture should be described.

The routine test procedures and limits should be established on the basis of the documentation given in the dossier.

SECTION P.5 Control of Drug Product

Apart from those situations envisaged in the note for guidance "Specifications and control tests on the finished product" it is not usually necessary to carry out identity testing and an assay of the excipients in the finished product at release.

Nevertheless, the finished product release specifications should include an identification test and limits for any antioxidants and antimicrobial preservatives present in the formulation. The finished product specification against which the product is tested throughout its shelf-life should also include limits for the antimicrobial preservatives present.

Where antioxidants are used up during the manufacture of the product, the release limits should be justified by batch data. The adequacy of specified limits should be justified on the basis of controlled conditions and in-use stability testing to ensure that sufficient antioxidant remains to protect the product throughout its entire shelf-life and during the proposed in-use period.

The control of antioxidants and antimicrobial preservatives should comply with the requirements identified in the guideline : ICH Topic Q 6 A : Specifications : Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products : Chemical Substances .

The antimicrobial preservative and antioxidants must be routinely identified and quantified at release.

P.8 Stability

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Samsung Bioepis Exhibit No. 1002
Page 5185

The maintenance of the physico-chemical properties of the finished product depend on the properties and the stability of the excipients (see note for guidance "Specifications and control tests on the finished product").

For new excipients, stability data should be provided as required for new active substances.

The application should follow the current QWP/CPMP guidelines on the stability of new dosage forms and should ensure that antimicrobial preservative and antioxidants levels are quantified periodically throughout the shelf-life of the finished product. In addition the efficacy of the preservatives should be established using the European Pharmacopoeia test for efficacy of antimicrobial preservation. This should be performed on the finished product at the end of the shelf-life and at the lower preservative limit in the end of shelf-life specification. The former is necessary, even if no evidence of degradation of the antimicrobial preservative and of the antioxidant is observed on storage, as other chemical and physical changes in the finished product may influence the efficacy of the antimicrobial preservative and the antioxidant.

In the case of products presented in multidose containers, the efficacy of the antimicrobial preservative under simulated in-use conditions must be established. The tests should be performed under the same condition as it is expected to be used by the final user. It may also be appropriate to examine the efficacy of the antimicrobial preservative following storage of opened or used containers for the proposed in use shelf-life.

R LABELLING

- Labelling must in accordance with relevant EC directive 2001/83/EC, Note for Guidance : Excipients in the Label and Package leaflet of Medicinal Products for Human Use and CPMP Position Paper on Thiomersal, Implementation of the Warning Statement Relating to Sensitisation (CPMP/2612/99).

However, if a product is presented in a multidose container without a preservative because :

a) it is intended for single use only (e.g. cytotoxic),

b) the product is self-preserving,

c) the product is oils based,

the labelling and product literature should indicate the absence of a preservative. This would not only emphasise the increased risk associated with the use of such products, but also aid the physician to specifically identify a product without preservative.

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Samsung Bioepis Exhibit No. 1002
Page 5186

ANNEX

EXAMPLES OF REQUIREMENTS CONCERNING DIFFERENT KINDS OF EXCIPIENTS

1. Excipients, which are a single chemical entity, include, for example, organic and inorganic acids and their salts, sugars and alcohols.

   They may have undergone physical treatments, which gave them special technological characteristics (e.g. micronisation).

2. Chemically transformed excipients include excipients which have undergone a special chemical treatment in order to confer certain technological characteristics (e.g. modified starch). The name and quality of such excipients should be defined in such a way as to avoid confusion with an unmodified excipient.

3. Mixtures of chemically related components include, for example, polyol esters (mixture of mono, di and tri esters), hydrogenated glucose syrup, maltitolsyrup. For these products the dossier should specify the following characteristics of the excipient:

   * the nature and content of each component with a statement of its acceptable limits;
   * technological criteria (appropriate criteria to the performance of dosage form);
   * any additives which may be present.

4. Mixed excipients are ready-for-use preparations, for example for direct compression or film coating.

   * The qualitative and quantitative composition of the mixed excipient should be submitted, the specifications of the product as a whole and of each component must be stated.

5. Excipients of natural origin, so called "natural" products have often undergone some kind of chemical treatment. In general and if relevant for the quality control of the product, data should give an outline of the operations carried out to obtain and to purify the product, and any special characteristics: decomposition products, specific impurities, chemical substances used during the treatment with residual limits, methods of sterilisation or decontamination, with a description of the effect of these processes on the excipient (e.g. modification of the physical structure).

6. For biological excipients of animal or human origin, the risk of transmitting adventitious agents should be considered and appropriate documentation submitted (e.g.; method of preparation and control of tissues and body fluids used as starting material). In addition, the name of the manufacturer and site of manufacture should be specified.

7. Flavouring agents (flavours and aromatic substances) are either natural products and/or products obtained by chemical synthesis. Because of the complexity of their composition, it is only necessary to describe the general qualitative composition mentioning the main constituents with an appropriate process of identification to ensure the consistency of the composition (in particular, identification of the main constituents and if necessary carriers).

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Samsung Bioepis Exhibit No. 1002
Page 5187

Most constituents of artificial flavours have internationally accepted purity criteria in food use (FAO/WHO). Reference to these standards is acceptable for medicinal products.

8. Colouring matters: - Community legislation on colouring matters in medicinal products is applicable.

ANTIOXIDANTS

Antioxidants are used to reduce the oxidation of active substances and excipients in the finished product. Antioxidants should not be used to disguise poorly formulated products or inadequate packaging. The need to include an antioxidant should be explained and fully justified. Oxidative degradation can be accelerated by light and by the presence of mineral or metallic impurities, due to the formation of free radicals.

There are three types of antioxidants :

Type	Definition	Example
True antioxidants	These are thought to block chain reactions by reacting with free radicals	Butylated hydroxytoluene (BHT)
Reducing agents	These have a lower redox potential than the drug or excipient they are protecting	Ascorbic acid
Antioxidants synergists	These enhance the effects of antioxidants	Sodium edetate

The efficacy obtained for an antioxidants depends on its nature, the stage at which it is incorporated within the finished product, the nature of the container and the formulation.

The efficacy of antioxidants must be assessed in the finished product in conditions, which simulate actual use by measuring the extent of degradation in the finished product, with and without the antioxidant.

Antioxidants should only be included in a formulation if it has been proven hat their use cannot be avoided. This applies to cases where the manufacturing process is optimised to minimise the potential for oxidation.

ANTIMICROBIAL PRESERVATIVES

Antimicrobial Preservatives are used to prevent or inhibit the growth of micro-organisms which could present a risk of infection or degradation of the medicinal product. These micro-organisms may proliferate during normal conditions of use of the product by the patient, particularly in multidose preparations.

On no account should preservatives be used as an alternative to Good Manufacturing Practice (GMP).

Preparations at greatest risk of contamination are those which contain water such as solutions, suspensions and emulsions to be taken orally, solution for external use, creams and sterile preparations used repeatedly (e.g. injectable multidose preparations and eye-drops).

The level of efficacy will vary according to the chemical structure of the preservative, its concentration, the physical and chemical characteristics of the medicinal product (especially pH) and the type and level of initial microbial contamination. The design of the pack and the

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Samsung Bioepis Exhibit No. 1002
Page 5188

temperature at which the product is stored will also affect of activity of any antimicrobial preservatives present.

The antimicrobial efficacy of the preservative in the finished should be assessed during product development, particularly during stability studies and at the end of the proposed shelf-life, using Pharmacopoeia European test.

If products do not contain a preservative and do not have inherent preservative efficacy they must not be packaged in multidose presentations without a sound justification.

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Samsung Bioepis Exhibit No. 1002
Page 5189

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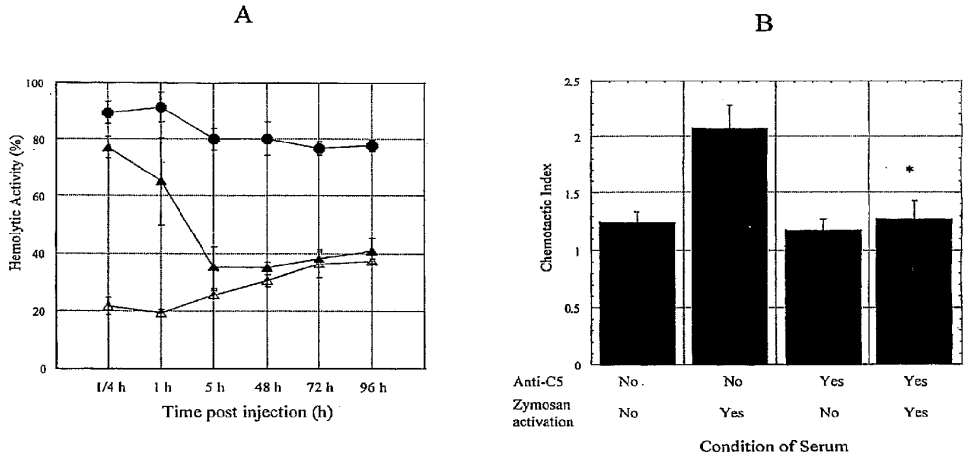
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(54) Title: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES



(57) Abstract: The present application relates to methods and compositions employing an antibody that inhibits activation of the complement system and can be used to prevent or treat a pulmonary disease or condition.

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Nebulization of Monoclonal Antibodies for Treating Pulmonary Diseases

Related Applications

This application claims priority to and benefit of the filing date of U.S. Patent Application Serial No. 11/127,438, filed May 11, 2005, which is a continuation-in-part application of U.S. Patent Application Serial No. 10/655,861, filed September 5, 2003, which claims priority to and benefit of the filing date of U.S. Provisional Application Serial No. 60/469,189, filed May 9, 2003 and U.S. Provisional Application Serial No. 60/408,571, filed September 6, 2002, all of which are hereby incorporated by reference in their entirety.

Background

Asthma, bronchitis and emphysema are known collectively as Chronic Obstructive Pulmonary Diseases. These diseases are characterized as generalized airways obstruction, especially of small airways, associated with varying degrees of symptoms of chronic bronchitis, asthma, and emphysema. These diseases may often coexist in an individual, and it may be difficult to determine the primary cause of an airway obstruction. Airway obstruction is defined as an increased resistance to airflow during forced expiration. Obstruction of large airways may also occur in these diseases, particularly in asthma. Currently available therapy for asthma remains problematic. Similarly, improved therapy is also desirable for treating or preventing other pulmonary diseases, for example lung cancers.

Effective delivery to a patient is a critical aspect of any successful drug therapy. Various routes of delivery exist, and each has its own advantages and disadvantages. Oral drug delivery of pills, capsules, elixirs, and the like is perhaps the most convenient method, but many drugs are degraded in the digestive tract before they can be absorbed. Subcutaneous injection is frequently an effective route for systemic drug delivery, including the delivery of proteins, but enjoys a low patient acceptance. Since injection of drugs one or more times a day can frequently be a source of poor patient compliance, a variety of alternative routes of administration have also been developed, including transdermal, intranasal, intrarectal, intravaginal, and pulmonary delivery. Thus, it is desirable to improve

Samsung Bioepis Exhibit No. 1002
Page 5191

drug delivery methods and compositions, particularly for antibody-based therapeutics in treating or preventing pulmonary diseases.

Summary

Accordingly, this application provides compositions and methods suitable for delivering antibody-based therapeutics. These antibody-based therapeutics can be particularly useful in preventing or treating pulmonary diseases or conditions such as for example asthma. Examples of antibodies useful in this application include anti-C5 antibody or antibodies that inhibit activation of the complement cascade, for example, the antibodies as described in U.S. Patent No. 6,355,245. In addition to antibodies, other therapeutics are also contemplated to be employed with the compositions and methods of the present application, for example, the chimeric complement inhibitor proteins as described in U.S. Patent No. 5,627,264. Certain preferred embodiments employ pexelizumab or eculizumab as the antibody therapeutic. In certain embodiments, the methods suitable for delivering antibody-based therapeutics for treating or preventing pulmonary diseases are not designed for systemic delivery of the therapeutics, and therefore, systemic effect of the therapeutics may not be observed using these methods.

A first aspect of the application provides a method for preventing or treating a pulmonary disease or condition in a subject comprising administering to the subject a therapeutically effective amount of an antibody that inhibits activation of the complement cascade or blocks the function of one or more subsequently activated components of the complement system. In certain embodiments, an additional active agent is also administered to the same subject. Administration of the antibody and the additional active agent may occur simultaneously or sequentially in either order. In certain embodiments, the antibody and the additional active agent can be administered to the subject via the same delivery method or route, for example, by inhalation. In alternative embodiments, the antibody and the additional active agent can be administered to the subject via different delivery methods or routes, for example, the antibody delivered by inhalation and the additional active agent by injection or by oral intake.

Samsung Bioepis Exhibit No. 1002
Page 5192

A second aspect of the application provides an aerosol composition comprising an antibody that inhibits activation of the complement cascade, wherein the composition is suitable for preventing or treating a pulmonary disease or condition in a subject. The antibody is formulated in a composition suitable for aerosolization. The antibody may be formulated in combination with an additional active agent, and the combination formulation is suitable for aerosolization. Alternatively, the antibody and an additional active agent may be formulated separately, such that they will be combined after aerosolization occurs or after being administered to a subject.

A third aspect of the application provides a nebulization composition comprising an antibody that inhibits activation of the complement cascade, wherein the composition is suitable for preventing or treating a pulmonary disease or condition in a subject. The antibody is formulated in a composition suitable for nebulization. Similarly, the antibody may be formulated in combination with an additional active agent, and the combination formulation is suitable for nebulization. Alternatively, the antibody and an additional active agent may be formulated separately, such that they will be combined after nebulization occurs or after being administered to a subject.

A further aspect of the application provides a biopharmaceutical package comprising an antibody that inhibits activation of the complement cascade and a nebulizer, wherein the package is suitable for preventing or treating a pulmonary disease or condition in a subject. The biopharmaceutical package may further comprise an active agent in addition to the antibody. The biopharmaceutical package may also comprise instructions for use.

Pulmonary diseases or conditions contemplated by the application include, but are not limited to, asthma, bronchial constriction, bronchitis, a chronic obstructive pulmonary disease (COPD), interstitial lung diseases, lung malignancies, α-1 anti-trypsin deficiency, emphysema, bronchiectasis, bronchiolitis obliterans, sarcoidosis, pulmonary fibrosis, and collagen vascular disorders.

The timing of administering a therapeutic to a subject can vary, for example, depending on the identity of the subject or the pulmonary disease or condition to be

Samsung Bioepis Exhibit No. 1002
Page 5193

treated or prevented, or both. For example, the administration may occur before the manifestation of the pulmonary condition (e.g., pre-asthmatic attack), during the manifestation of the pulmonary condition (e.g., during the asthmatic attack), or after the manifestation of the pulmonary condition (e.g., post-asthmatic attack).

An antibody of the present application can be specific to C5 such that it prevents the cleavage of C5 into C5a and C5b. The antibody can be specific to the C5 convertase. Alternatively, the antibody may be specific to a component of the complement system, for example, C5a, C5b, or C5b-9, and the antibody specific to the component preferably inhibits the component's function, for example, by blocking the component's binding to its respective receptor, or by blocking its function in activating the subsequent signaling or events in the complement cascade. Certain embodiments employ eculizumab or pexelizumab, or both. An antibody or antibody therapeutic of the present application can be a full length immunoglobulin, a monoclonal antibody, a chimeric antibody (e.g., a humanized antibody), a single chain antibody, a domain antibody, an Fab fragment, or an antibody having an Fab fragment and a mutated Fc portion. In certain embodiments, the mutated Fc portion does not activate complement, or the mutation(s) in the Fc portion decreases the Fc portion's ability to activate complement. An antibody of the present application may be produced or processed in bulk and packaged in an ampule made of a suitable material (e.g., glass or plastic) at different doses.

An additional active agent (or an active agent in addition to the antibody therapeutic) of the present application can be another antibody therapeutic (e.g., an anti-IgE antibody such as Xolair® or omalizumab, an anti-IL-4 antibody or an anti-IL-5 antibody), an anti-IgE inhibitor (e.g., Singulair® or montelukast sodium), a sympathomimetic (e.g., albuterol), an antibiotic (e.g., tobramycin), a deoxyribonuclease (e.g., pulmozyme), an anticholinergic drug (e.g., ipratropium bromide), a corticosteroid (e.g., dexamethasone), a β-adrenoreceptor agonist, a leukotriene inhibitor (e.g., zileuton), a 5 Lipoxygenase inhibitor, a PDE inhibitor, a CD23 antagonist, an IL-13 antagonist, a cytokine release inhibitor, a histamine H1 receptor antagonist, an anti-histamine, an anti-inflammatory agent (e.g. cromolyn sodium) or a histamine release inhibitor.

Samsung Bioepis Exhibit No. 1002
Page 5194

An example of formulation suitable for aerosolization or nebulization of an antibody is in physiologic osmolarity (e.g., between 280 and 320 mM) at a suitable pH (e.g., pH 6 to 8). A formulation of the present application may further comprise an excipient, for example polysorbate 80 which can be used at 0.0015 to 0.02%.

A nebulizer of the present application can be a jet air nebulizer (e.g., Pari LC Jet Plus or Hudson T Up-draft II), an ultrasonic nebulizer (e.g., MABISMist II), a vibrating mesh nebulizer (e.g., Micro air by Omron) and a shockwave nebulizer (EvitLabs Sonik LDI20).

Brief Description of the Drawings

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

Fig. 1 illustrates blocking the generation of C5a and C5b-9 by anti-C5 mAb, BB5.1. Pharmacodynamic profile (A) of inhibition of C5b-9 mediated hemolysis from animals given a single i.v. or i.p. injection of anti-C5 mAb (40 mg/kg) or control mAb (40 mg/kg). • represents data from 4 control animals that were given control mAb by either i.v. (n=2) or i.p. (n=2). ▲ represents i.p. anti-C5 mAb-treated animals (n=3). Δ represents i.v. anti-C5 mAb-treated animals (n=3). C5a-mediated neutrophil migration (B) was analyzed in an in vitro assay. Zymosan activated sera (20%) was used as the source of C5a. Anti-C5 mAb (100 μg/ml) was added to serum sample prior to zymosan activation as indicated (n=8). * indicates $p < 0.05$ when compared to zymosan activated serum samples. BALB/c mice were used in all figures except Figs. 4 C and D.

Fig. 2 shows airway response to OVA provocation and definition of three critical checkpoints. A: Representative DCP results indicating the longitudinal changes of sRaw. After aerosol challenge with 5% OVA for 10 min, the appearance of EAR was observed at 15 min, followed by the LAR at 5 h. B: Three critical points during the course of disease. Checkpoint 1: Anti-C5 mAb was given by i.p. injection on days 25, 29 and 31. Analysis of lower airway functions and quantification of airway inflammation were evaluated 5 h after aerosol challenge of 5% OVA on day 32. Checkpoint 2: Anti-C5 mAb was given by i.p. injection on day

Samsung Bioepis Exhibit No. 1002
Page 5195

33, after recovery from the first airway response on day 32. AHR was assessed by evaluating the changes of RL and Cdyn from baseline in response to aerosol Mch challenge on day 35. Checkpoint 3: C5 inhibition was given by either i.v. or aerosol administration of BB5.1 during EAR on day 35. Lower airway function was determined during the peak of LAR, 5 h after 5% OVA provocation.

Fig. 3 shows contribution of C5 at checkpoint 1, the initiation of airway inflammation. Mean serum OVA-specific antibody titers of 5-6 mice on day 32 were measured by ELISA (A). Cross hatched bars: OVA-specific IgG. Open bars: OVA-specific IgE. Also on day 32, animals were trachea cannulated for the measurement of RL (B) and Cdyn (C) 5 h after 5% aerosol OVA provocation, followed by histological analyses of lung (D). The mean histology score for control mAb-treated mice (F) is 2±0.28 compared to 0.64±0.21 for corticosteroid-treated animals (G) and 1.25±0.25 for anti-C5 mAb-treated animals (H) compared to total lack of inflammation, score =0, seen in sham-mice (E). * indicates $p<0.05$ when comparing treated animals (i.e. corticosteroid or anti-C5 mAb treatment) with control mAb treated animals, while ■ indicates $p<0.05$ when comparing corticosteroid treated mice with animals treated with antibody targeting C5 or C5aR in this and all following figures unless otherwise indicated. For clarity, all control mAb treated animals are labeled as control in all following figures. Arrow indicates eosinophil infiltration. Giemsa stain, magnification = 100X.

Fig. 4 shows contribution of C5 at checkpoint 2, the development of AHR. C5s BALB/cByJ mice (A&B) and C5d B10D2oSn mice (C&D) were immunized with OVA in identical manner (Fig. 2 B). Animals were randomized and given (i.p.) the indicated treatment on day 33. On day 35, aerosol Mch challenges were administered through a trachea cannula during measurement of RL (A&C) and Cdyn (B&D). Changes in RL and Cdyn are expressed as a percentage of baseline after each aerosol challenge. Subgroups of C5d mice with or without OVA immunization were reconstituted (i.v.) with 200μg of rmC5a 3 hours prior to Mch challenges. For C5s BALB/c mice, * indicates $p<0.05$ when comparing either steroid or anti-C5 mAb treated animals with control mAb treated animals. For C5d B10D2oSn mice, * indicates $p<0.05$ when comparing the mAb treated animals (n=6) given either control mAb (n=3) or anti-C5 mAb (n=3) with OVA immunized C5d B10D2oSn

Samsung Bioepis Exhibit No. 1002
Page 5196

mice reconstituted with rmC5a. There are no statistical differences between C5d animals treated with control mAb and anti-C5 mAb.

Fig. 5 shows contribution of C5 at checkpoint 3, during an on-going airway response. Animals that experienced previous airway response to allergen provocation on day 32 were given a second aerosol allergen challenge on day 35. During the peak of EAR, animals were randomized and given (i.v.) the indicated treatment. Animals were trachea cannulated for the measurement of RL (A) and Cdyn (B) 5 h after the OVA provocation. (n= 7-8 mice per group.)

Fig. 6 shows BALF WBC counts and differential analysis. BALF was obtained 5 h after aerosol allergen provocation at checkpoint 1 (A) or at checkpoint 3 (B&C). Total BALF WBC counts (A&B) and their differential analyses (C) were performed by a pathologist in a double blind fashion. For 6 (C), open bars: alveolar macrophages. Cross hatched bars: Eosinophils. Solid bars: Neutrophils. Hatched bars: Lymphocytes. (n= 6-8 mice per group.)

Fig. 7 shows BALF levels of inflammatory mediators. BALF was obtained 5 h after aerosol allergen provocation at checkpoint 1 (cross hatched bars) or checkpoint 3 (solid bars). BALF levels of IL-5 (A), IL-13 (B), histamine (C), eotaxin (D), RANTES (E), activated TGF-β (F), TNF-α (G) and proMMP9 (H) were measured by ELISA. (n= 6-8 samples per group.)

Fig. 8 shows contribution of intrapulmonary C5 activation at checkpoint 3. Results represent data pooled from 3 separate experiments with identical experimental procedures. Animals were aerosol challenged with 5% OVA and randomized. During EAR, animals were then given aerosol administration of one of the indicated treatments through a jet nebulizer. Sham-immunized mice were treated with aerosol PBS. All animals were trachea cannulated for the measurement of RL (A) and Cdyn (B) 5 h after the OVA provocation. BALF WBC counts (C) and proMMP9 (D) were analyzed in the majority of animals. The data from control mAb (n=15) and control sIgG treated mice (n=4) were pooled together. Except anti-C5 Fab (n=3), n=6-19 animals in all other cohorts.

Samsung Bioepis Exhibit No. 1002
Page 5197

Fig. 9 shows the result of combined therapy on the development of AHR in subjects with established airway inflammation. The results were obtained based on checkpoint 2 study as described herein. Treatment was delivered on day 32 by nebulization instead of i.p. Mch challenge occurred on day 35. For anti-C5, BB5.1 at 3 mg/ml was subjected to nebulization for 10 minutes. The steroid at 2 mg/ml was nebulized for 10 minutes. For the combination, the final concentration was 3 mg/ml for BB5.1 and 2 mg/ml for the steroid, which was subjected to nebulization for 10 minutes.

Fig. 10 shows exemplary formulations suitable for nebulization of an antibody.

Fig. 11 shows the result of Mch challenge two days after nebulization treatment.

Fig. 12 shows the particle size distribution.

Fig. 13 shows nebulization and delivery efficiencies.

Fig. 14 shows SDS-PAGE analysis of aerosolized eculizumab.

Fig. 15 shows SEC HPLC analysis of aerosolized eculizumab using two different nebulizers.

Fig. 16 shows that repeated long term aerosol administrations of anti-C5 monoclonal antibody do not induce airway inflammation. The mice here were subject to 10-week treatments: sham mice were treated with PBS for 10 min twice per week for 10 weeks; control mice were treated with HFN7.1 (an isotype-matched mouse antibody) for 10 min by nebulization, also twice a week for 10 weeks; the anti-C5 antibody (BB5.1) treated mice were treated with BB5.1 for 10 min by nebulization, also twice a week for 10 weeks.

Detailed Description of the Application

Overview

The present application abbreviates certain terms as follows:

AHR: airway hyper-responsiveness; BALF: bronchoalveolar lavage fluid; Cdyn - dynamic lung compliance; C5aR: C5a receptor; C5d: C5 deficient; C5s: C5

Samsung Bioepis Exhibit No. 1002
Page 5198

sufficient; DCP: double chamber plethysmograph; EAR: Early phase airway response; IC: immune complexes; LAR: Late phase airway response; Mch: methacholine; rmC5a: recombinant mouse C5a; RL: lung resistance; sRaw: Specific airway resistance; sIgG: serum IgG.

The Complement System

The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of cellular and viral pathogens. There are at least 25 complement proteins, which are found as a complex collection of plasma proteins and membrane cofactors. The plasma proteins make up about 10% of the globulins in vertebrate serum. Complement components achieve their immune defensive functions by interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions.

The complement cascade progresses via the classical pathway or the alternative pathway. These pathways share many components, and while they differ in their initial steps, they converge and share the same "terminal complement" components (C5 through C9) responsible for the activation and destruction of target cells.

The classical complement pathway is typically initiated by antibody recognition of and binding to an antigenic site on a target cell. The alternative pathway is usually antibody independent, and can be initiated by certain molecules on pathogen surfaces. Both pathways converge at the point where complement component C3 is cleaved by an active protease (which is different in each pathway) to yield C3a and C3b. Other pathways activating complement attack can act later in the sequence of events leading to various aspects of complement function.

C3a is an anaphylatoxin (see discussion below). C3b binds to bacterial and other cells, as well as to certain viruses and immune complexes, and tags them for removal from the circulation. (C3b in this role is known as opsonin.) The opsonic function of C3b is considered to be the most important anti-infective action of the complement system. Patients with genetic lesions that block C3b function are prone

Samsung Bioepis Exhibit No. 1002
Page 5199

to infection by a broad variety of pathogenic organisms, while patients with lesions later in the complement cascade sequence, i.e., patients with lesions that block C5 functions, are found to be more prone only to Neisseria infection, and then only somewhat more prone (Fearon, in Intensive Review of Internal Medicine, 2d ed. Fanta and Minaker, eds. Brigham and Women's and Beth Israel Hospitals, 1983).

C3b also forms a complex with other components unique to each pathway to form classical or alternative C5 convertase, which cleaves C5 into C5a and C5b. C3 is thus regarded as the central protein in the complement reaction sequence since it is essential to both the alternative and classical pathways (Wurzner, et al., Complement Inflamm. 8:328-340, 1991). This property of C3b is regulated by the serum protease Factor I, which acts on C3b to produce iC3b. While still functional as opsonin, iC3b cannot form an active C5 convertase.

C5 is a 190 kDa beta globulin found in normal serum at approximately 75 μg/ml (0.4 μM). C5 is glycosylated, with about 1.5-3 percent of its mass attributed to carbohydrate. Mature C5 is a heterodimer of a 999 amino acid 115 kDa alpha chain that is disulfide linked to a 656 amino acid 75 kDa beta chain. C5 is synthesized as a single chain precursor protein product of a single copy gene (Haviland et al. J. Immunol. 1991, 146:362-368). The cDNA sequence of the transcript of this gene predicts a secreted pro-C5 precursor of 1659 amino acids along with an 18 amino acid leader sequence.

The pro-C5 precursor is cleaved after amino acid 655 and 659, to yield the beta chain as an amino terminal fragment and the alpha chain as a carboxyl terminal fragment, with four amino acids deleted between the two. C5a is cleaved from the alpha chain of C5 by either alternative or classical C5 convertase as an amino terminal fragment comprising the first 74 amino acids of the alpha chain. Approximately 20 percent of the 11 kDa mass of C5a is attributed to carbohydrate. The cleavage site for convertase action is at or immediately adjacent to amino acid residue 733 of the 1659-amino acid pro-C5 precursor sequence with an 18-amino acid leader sequence (SEQ ID NO:2 as described in U.S. Patent No. 6,355,245). A compound that would bind at or adjacent to this cleavage site would have the

Samsung Bioepis Exhibit No. 1002
Page 5200

potential to block access of the C5 convertase enzymes to the cleavage site and thereby act as a complement inhibitor.

C5 can also be activated by means other than C5 convertase activity. Limited trypsin digestion (Minta and Man, J. Immunol. 1977, 119:1597-1602; Wetsel and Kolb, J. Immunol. 1982, 128:2209-2216) and acid treatment (Yamamoto and Gewurz, J. Immunol. 1978, 120:2008; Damerau et al., Molec. Immunol. 1989, 26:1133-1142) can also cleave C5 and produce active C5b.

C5a is another anaphylatoxin. C5b combines with C6, C7, and C8 to form the C5b-8 complex at the surface of the target cell. Upon binding of several C9 molecules, the membrane attack complex (MAC, C5b-9, terminal complement complex--TCC) is formed. When sufficient numbers of MACs insert into target cell membranes the openings they create (MAC pores) mediate rapid osmotic lysis of the target cells. Lower, non-lytic concentrations of MACs can produce other effects. In particular, membrane insertion of small numbers of the C5b-9 complexes into endothelial cells and platelets can cause deleterious cell activation. In some cases activation may precede cell lysis.

As mentioned above, C3a and C5a are anaphylatoxins. These activated complement components can trigger mast cell degranulation, which releases histamine and other mediators of inflammation, resulting in smooth muscle contraction, increased vascular permeability, leukocyte activation, and other inflammatory phenomena including cellular proliferation resulting in hypercellularity. C5a also functions as a chemotactic peptide that serves to attract pro-inflammatory granulocytes to the site of complement activation.

Pulmonary Diseases or Conditions, and the Complement System

Certain pulmonary diseases or conditions such as asthma can be characterized by a combination of chronic airway inflammation, airway obstruction and airway hyper-responsiveness (AHR) to various stimuli. It is thought to be mediated primarily by adaptive immune responses including allergen-specific $CD4^+$ T cells, Th2 cytokines, and allergen specific IgE leading to pulmonary inflammation and AHR. Complement and its activated components, which form a central core of innate immune defense against bacterial, viral and fungal invasions (Nagy et al., J.

Samsung Bioepis Exhibit No. 1002
Page 5201

Allergy. Clin. Immunol. (2003) 112:729-734; Kasamatsu et al., Arerugi (1993) 42:1616-1622; Bjornson et al., Am. Rev. Respir. Dis. (1991) 143:1062-1066), can be activated through the classical pathway, alternative pathway and the lectin pathway (Lachmann, Res. Immunol. (1996) 147:69-70). All three activation pathways converge at complement component C5 prior to the generation of C5a and C5b-9, both of which induce potent biological responses including tissue injury, inflammation, anaphylatoxic responses, and cell lysis at very low concentrations (Takafuji et al., Int. Arch. Allergy Immunol. (1994) 104 Suppl. 1:27-29). In addition, C5 can be activated after allergen exposure (Nagata et al., J. Allergy Clin. Immunol. (1987) 80:24-32). Recent data from animal models of allergic asthma suggest that activated complement components, such as C5a, provide a critical link between innate and adaptive immunity (Karp et al., Nat. Immunol. (2000) 1:221-226). However, controversies remain in the art regarding the involvement of C5 and its activated components in the pathogenesis of asthma (Karp et al., *supra*; Gerard et al., Curr. Opin. Immunol. (2002) 14:705-708; Abe et al., J. Immunol. (2001) 167:4651-4660; Lukacs et al., Am. J. Physiol. Lung Cell. Mol. Physiol. (2001) 280(3):L512-L518).

Several experimental models for bronchial asthma have indicated that C5 and its activated components are involved in the development of airway inflammation and bronchoconstriction (or bronchial constriction). Inhibition studies with various complement inhibitors markedly reduced AHR or airway inflammation in rodents (Abe et al., *supra*; Lukacs et al., *supra*). The potential involvement of C5 activation was also extended to clinical observations that the severity of clinical symptoms was correlated with the extent of C5 activation (Gonczi et al., Allergy (1997) 52:1110-1114). On the other hand, studies have shown that C5 deficiency (C5d) leads to increased susceptibility to allergen-induced AHR in mice, and this finding is supported by evidence of decreased production of IL-12, a key Th1 cytokine reported to modulate the pathogenesis of asthma (Karp et al., *supra*). The key question at the center of the debate is whether C5 and its activated components are pro-inflammatory or anti-inflammatory during the sensitization phase and the effector phase of the pathogenesis as suggested respectively from studies of C5d animals (Karp et al., *supra*) and experiments with intervention during the course of

Samsung Bioepis Exhibit No. 1002
Page 5202

disease (Abe et al., *supra*; Lukacs et al., *supra*). In addition, another key issue is whether intrapulmonary-activated complement components play a significant role in the pathogenesis and overcome the potential anti-inflammatory effect of activated C5 components on the adaptive immune system (Karp et al., *supra*).

The present application relates to the study of the contribution of C5 and its activated components at three critical points during the course of disease, which outlines the key mechanisms of this essential component of the innate immune system in the pathogenesis of asthma. Comprehensive analyses of lower airway function and quantification of multiple parameters of airway inflammation have been conducted. As described herein, the study shows that C5 contributes to the initiation of airway inflammation, demonstrates the critical contribution of C5 in the development of AHR in animals with established airway inflammation and the significant role of activated C5 components in sustaining an on-going airway response to allergen challenge.

The pathophysiological hallmark necessary for the development of asthma-like symptoms is airway inflammation. Individuals with a genetic predisposition may have modulated adaptive immune responses to environmental exposures, including activation of allergen-specific $CD4^+$ T cells, polarization of Th2 cytokines, such as IL-4 and IL-5, which are critically involved in the production of allergen-specific IgE and the recruitment of eosinophils (Cieslewicz et al., J. Clin. Invest. (1999) 104:301-308). More recently, IL-13 was identified as a key player in the pathogenesis, and reported to be both necessary and sufficient to induce the airway response in animals (Wills-Karp et al., Science (1998) 282:2258-2261). As shown by the study described herein, the broad spectrum of anti-inflammatory activities of corticosteroid, including modulating the Th2 cytokine profile (Figs. 7 A and B) and blocking the recruitment of inflammatory cells into lower airways (Fig. 3 D), correlates with its potent antiasthmatic activity. Consistent with its anti-inflammatory activities reported earlier (Wang et al., Proc. Natl. Acad. Sci.U.S.A (1996) 93:8563-8568), C5 inhibition at checkpoint 1 resulted in significantly less airway inflammation without significant impact on the adaptive immune system's responses to allergen exposures. Further, C5d animals developed a similar degree of airway inflammation as that developed by C5s animals. One possible explanation is

Samsung Bioepis Exhibit No. 1002
Page 5203

that airways are directly exposed to allergens and infections. These exposures may activate other complement components, such as C3a, which has very similar proinflammatory properties (Takafuji et al., Int. Arch. Allergy Immunol. (1994) 104 Suppl. 1:27-29; Nagata et al., J. Allergy Clin. Immunol. (1987) 80:24-32), and was known to contribute to the pathogenesis (Gerard et al., Curr. Opin. Immunol. (2002) 14:705-708). The data provided in the present application indicate that activated C5 components are critically involved but are not necessary for the initiation of airway inflammation, which requires the activation of adaptive immune responses to aerosol exposures of allergen (Cieslewicz et al., *supra*). However, at checkpoints 2 and 3 when airway inflammation was already well established, the data described herein indicate that C5 inhibition has similar or better efficacy than corticosteroid in ameliorating airway inflammation and improving lower airway functions (see, e.g., Figs. 4-8).

The second hallmark of asthma, the development of AHR in response to non-specific stimuli, was selected as another critical checkpoint. By pre-screening several strains of C5s and C5d mice, the genetic influences on the function of muscarinic acetylcholine receptor responsible for the intrinsic AHR (De Sanctis et al., Am. J. Respir. Crit. Care Med. (1997) 156:S82-S88; Levitt et al., FASEB J. (1988) 2:2605-2608.) are eliminated from the study described herein. Using animals that do not have intrinsic AHR, C5 inhibition is shown to be sufficient to prevent the development of AHR to aerosol Mch challenges in C5s BALB/c mice with established airway inflammation (Figs. 4 A and B) while reconstitution of C5d B10D2oSn mice with rmC5a restored AHR, also in the presence of established airway inflammation
(Figs. 4 C and D). These data suggest that C5, most likely C5a, serves as a direct link between the innate immune responses and the key components of the airways responsible for AHR. C5aR was found on airway smooth muscle cells and airway epithelia (Drouin et al., J. Immunol. (2001) 166:2025-2032). Although the direct interaction of activated C5 components, in particular C5a, with receptors on airway smooth muscles and epithelium was demonstrated previously (Irvin et al., Am. Rev. Respir. Dis. (1986) 134:777-783; Larsen, Annu. Rev. Immunol. (1985) 3:59-85.), the data provided herein indicate that the maintenance of AHR depends upon

Samsung Bioepis Exhibit No. 1002
Page 5204

complex interactions among airway epithelia, airway smooth muscle cells, inflammatory cells and their inflammatory mediators as suggested by minimal impact on the functions of lower airways during Mch challenges after systemic reconstitution (i.v.) of sham B10D2.oSn mice with rmC5a (Figs. 4 C and D).

The on-going airway responses after aerosol allergen provocation, serve as the third critical checkpoint in the study described herein. The development of EAR and LAR together with massive production and release of multiple inflammatory mediators provide a good opportunity to examine the complex interaction of activated C5 components with other components of innate and adaptive immune responses and their role in pathogenesis. The data described herein demonstrate that C5 inhibition by either anti-C5 monoclonal antibody (mAb) or its Fab fragment achieved similar in vivo efficacy in ameliorating intrapulmonary inflammatory activities as well as in improving functions of lower airways.

The data here also suggest that in the presence of airway inflammation, activated C5 components function as the key regulators of the downstream inflammatory cascade. This regulatory effect is demonstrated by its ability to influence the migration of inflammatory cells into airway lumen as well as the activation and the release of multiple harmful mediators corresponding to the significant changes of lower airway functions at checkpoint 3. C5a is probably the most potent activator of inflammatory cells (Takafuji et al., *supra*) and C5aR has been identified on circulating leukocytes, mast cells, macrophages, and endothelial cells (Chenoweth et al., Proc. Natl. Acad. Sci. U.S.A. (1978) 75:3943-3947). The disassociation of significant improvement of lower airway functions with significant presence of intrapulmonary inflammatory activities after anti-C5aR sIgG treatment is consistent with the hypothesis that the direct engagement of C5a with its receptors expressed on airway smooth muscle cells and epithelia is, in part, responsible for airway constriction (Irvin et al., *supra*). The data also indicate that the chemotactic activity and cell activation properties of C5b-9 (Czermak et al., Am. J. Pathol. (1999) 154:1513-1524) are responsible for the significant intrapulmonary inflammatory activities in the absence of C5aR engagement. In the presence of established airway inflammation and IC, C5b-9 can sufficiently regulate the downstream inflammatory cascade by triggering releases of multiple mediators such

Samsung Bioepis Exhibit No. 1002
Page 5205

as proMMP9 independently of the engagement of C5a with its receptors expressed on inflammatory cells (Fig. 8 D). The presence of an elevated level of C5b-9 together with the presence of multiple inflammatory mediators contributed to migration of inflammatory cells into the bronchial lumen in animals treated with anti-C5aR sIgG (Fig. 8 C).

Both the adaptive and innate immune systems contributed in the complex process of the production and release of multiple inflammatory mediators, including leukotrienes, prostaglandins, histamine, alkaline proteins, cytokines, chemokines, and enzymes, which are responsible for bronchospasm, altered vascular permeability, adherence and migration of inflammatory cells, mucosal edema and excessive mucous secretion and ultimately lead to three unique but interdependent phenomena: airway inflammation, airway obstruction and AHR. Since there are many redundant biological mechanisms, under the right circumstances, such as in genetically deficient animals, these redundant mechanisms may compensate and contribute in a more significant fashion. The complex relationship and interactions of these components are responsible for airway inflammation, obstruction and AHR, as well as other pulmonary diseases or conditions.

Lung parenchymal cells possess the ability to produce C5 and cleave C5 into activated fragments (Desai et al, J. Exp. Pathol. (1984) 1:201-216). The activation of C5 as part of innate immune responses may well be an innocuous by-product of a self-defensive response, due in part to direct enzymatic cleavage of C5 (Maruo et al, J. Allergy Clin. Immunol. (1997) 100:253-260) after exposure to allergens (Nagata et al., *supra*), house dust (Maruo et al., *supra*), smoke (Robbins et al., Am. J. Physiol. (1991) 260:L254-L259.) or airway infections (Nagy et al., J. Allergy. Clin. Immunol. (2003) 112:729-734; Kasamatsu et al., Arerugi (1993) 42:1616-1622; Bjornson et al., Am. Rev. Respir. Dis. (1991) 143:1062-1066). The formation of intrapulmonary IC after allergen exposure may also activate complement cascade (Larsen, *supra*). This hypothesis proposes that activated C5 components are primarily located at the epithelial side of the airway and, in non-asthmatic individuals, the intrapulmonary activation of complement is well regulated (Varsano et al., Thorax (2000) 55:364-369). Consistent with this hypothesis, our data demonstrated that intrapulmonary C5 inhibition achieved similar efficacy as

Samsung Bioepis Exhibit No. 1002
Page 5206

systemic C5 inhibition at checkpoint 3 (Fig. 8) and at checkpoint 2. These data further suggest that intrapulmonary activated C5 components are major pro-inflammatory rather than anti-inflammatory forces.

The study also shows the effect of C5 inhibition on the inflammatory mediators produced or released by neutrophils or eosinophils (Fig. 7). These inflammatory mediators, in particular TGF-β, RANTES and proMMP9, may cause irreversible damage to airways by inducing bronchial smooth muscle hypertrophy and deposition of collagen under the basal membranes of respiratory epithelium, which leads to the remodel and repair of lower airways, the focal point for those individuals with a poor long term prognosis (Boulet et al., Am. J. Respir. Crit. Care Med. (2000) 162:1308-1313). Accordingly, C5 inhibition, which utilizes a unique and different anti-inflammatory mechanism, may complement the potent anti-inflammatory effect of corticosteroid and other active agents, and have an added benefit for long term prognosis.

This study clearly demonstrates that the complex functional changes of airways seen in individuals with asthma coexist with the complex inflammatory processes. On one hand, activated C5 components, such as C5a through its binding with airway C5aR, serve as the direct link between the innate immune system and AHR. On the other hand, both C5a and C5b-9 regulate the downstream inflammatory cascade through their chemotactic and cell activation activities in the presence of IC and established airway inflammation. Blocking the generation of C5a and C5b-9 not only improves the functioning of lower airways but also ameliorates intrapulmonary inflammatory activities. In contrast, C5aR antagonist significantly improves functions of lower airways without a dramatic impact on intrapulmonary inflammatory activities.

Aerosol or Nebulization Compositions

In one respect, this application is directed to pulmonary drug delivery compositions and/or devices for delivering an antibody that inhibits the activation of the complement cascade. The pulmonary drug delivery compositions are useful for treating a pulmonary disease or condition. For example, aerosol compositions are provided for the delivery of an antibody or an antibody combined with an additional

Samsung Bioepis Exhibit No. 1002
Page 5207

active agent to the respiratory tract. The respiratory tract includes the upper airways, including the oropharynx and larynx, followed by the lower airways, which include the trachea followed by bifurcations into the bronchi and bronchioli. The upper and lower airways are called the conductive airways. The terminal bronchioli then divide into respiratory bronchioli which then lead to the ultimate respiratory zone, the alveoli, or deep lung.

Pulmonary drug delivery may be achieved by inhalation, and administration by inhalation herein may be oral and/or nasal. Examples of pharmaceutical devices for pulmonary delivery include metered dose inhalers (MDIs), dry powder inhalers (DPIs), and nebulizers. Exemplary delivery systems by inhalation which can be adapted for delivery of the subject antibody and/or active agent are described in, for example, U.S. Patent Nos. 5,756,353; 5,858,784; and PCT applications WO98/31346; WO98/10796; WO00/27359; WO01/54664; WO02/060412. Other aerosol formulations that may be used for delivering the antibody and/or active agent are described in U.S. Patent Nos. 6,294,153; 6,344,194; 6,071,497, and PCT applications WO02/066078; WO02/053190; WO01/60420; WO00/66206.

Pressurized metered dose inhalers (pMDIs) are the most commonly used inhaler worldwide. The aerosol is created when a valve is opened (usually by pressing down on the propellant canister), allowing liquid propellant to spray out of a canister. Typically, a drug or therapeutic is contained in small particles (usually a few microns in diameter) suspended in the liquid propellant, but in some formulations the drug or therapeutic may be dissolved in the propellant. The propellant evaporates rapidly as the aerosol leaves the device, resulting in small drug or therapeutic particles that are inhaled. Propellants typically used in such pMDIs include but are not limited to hydrofluoroalkanes (HFAs). A surfactant may also be used, for example, to formulate the drug or therapeutic, with pMDIs. Other solvents or excipients may also be employed with pMDIs, such as ethanol, ascorbic acid, sodium metabisulfate, glycerin, chlorobutanol, and cetylpyridium chloride. Such pMDIs may further include add-on devices such as, for example, spacers, holding chambers and other modifications.

Samsung Bioepis Exhibit No. 1002
Page 5208

Nebulizers produce a mist of drug-containing liquid droplets for inhalation. They are usually classified into two types: ultrasonic nebulizers and jet nebulizers. A new type of nebulizer is also available, which does not require ultrasound or air pressure to function. Single breath atomizers have also been developed (e.g., Respimat®), which is used to deliver a drug in a single inhalation and may be preferred because of less contamination. Jet nebulizers are more common and use a source of pressurized air to blast a stream of air through a drug-containing water reservoir, producing droplets in a complex process involving a viscosity-induced surface instability that leads to nonlinear phenomena in which surface tension and droplet breakup on baffles play a role. Ultrasonic nebulizers produce droplets by mechanical vibration of a plate or mesh. In either type of nebulizer, the drug is usually contained in solution in the liquid in the nebulizer and so the droplets being produced contain drug in solution. However, for some formulations (e.g., Pulmicort) the drug is contained in small particles suspended in the water, which are then contained as particles suspended inside the droplets being produced. Certain excipients are usually included in formulations suitable for nebulization, such as sodium chloride (e.g., to maintain isotonicity), mineral acids and bases (e.g., to maintain or adjust pH), nitrogen headspace sparging, benzalkonium chloride, calcium chloride, sodium citrate, disodium edtate, and polysorbate 80.

The third type of inhaler is the dry powder inhaler (DPI). In DPIs, the aerosol is usually a powder, contained within the device until it is inhaled. The therapeutic or drug is manufactured in powder form as small powder particles (usually a few millionths of a meter, or micrometers, in diameter). In many DPIs, the drug or therapeutic is mixed with much larger sugar particles (e.g., lactose monohydrate), that are typically 50-100 micrometers in diameter. The increased aerodynamic forces on the lactose/drug agglomerates improve entrainment of the drug particles upon inhalation, in addition to allowing easier filling of small individual powder doses. Upon inhalation, the powder is broken up into its constituent particles with the aid of turbulence and/or mechanical devices such as screens or spinning surfaces on which particle agglomerates impact, releasing the small, individual drug powder particles into the air to be inhaled into the lung. The

Samsung Bioepis Exhibit No. 1002
Page 5209

sugar particles are usually intended to be left behind in the device and/or in the mouth-throat.

One aspect of the application provides an aerosol composition comprising an antibody that inhibits activation of the complement cascade, wherein the composition is suitable for preventing or treating a pulmonary disease or condition in a subject. An aerosol antibody composition can be a composition comprising aerosolized antibody or a composition comprising an antibody in a formulation suitable for aerosolization. The antibody may be formulated in combination with an additional active agent, and the combination formulation is suitable for aerosolization. Alternatively, the antibody and an additional active agent may be formulated separately, such that they will be combined after aerosolization occurs or after being administered to a subject.

Another aspect of the application provides a nebulization composition comprising an antibody that inhibits activation of the complement cascade, wherein the composition is suitable for preventing or treating a pulmonary disease or condition in a subject. A nebulization antibody composition can be a composition comprising a nebulized antibody or a composition comprising an antibody in a formulation suitable for nebulization. Similarly, the antibody may be formulated in combination with an additional active agent, and the combination formulation is suitable for nebulization. Alternatively, the antibody and an additional active agent may be formulated separately, such that they will be combined after nebulization occurs or after being administered to a subject.

A further aspect of the application provides a biopharmaceutical package comprising an antibody that inhibits activation of the complement cascade and a nebulizer, wherein the package is suitable for preventing or treating a pulmonary disease or condition in a subject. The biopharmaceutical package may further comprise an active agent in addition to the antibody. The biopharmaceutical package may also comprise instructions for use.

An antibody of the present application can be specific to C5 such that it prevents the cleavage of C5 into C5a and C5b. The antibody can be specific to the C5 convertase. Alternatively, the antibody may be specific to a component of the

Samsung Bioepis Exhibit No. 1002
Page 5210

complement system, for example, C5a, C5b, or C5b-9, and the antibody specific to the component preferably inhibits the component's function, for example, by blocking the component's binding to its respective receptor, or by blocking its function in activating subsequent signaling or events in the complement cascade. Certain embodiments employ eculizumab or pexelizumab, or both. An antibody or antibody therapeutic of the present application can be a full length immunoglobulin, a chimeric antibody (e.g., a humanized antibody), a single chain antibody, a domain antibody (e.g., a domain antibody as developed by Domantis, defined as the smallest functional binding units of antibodies, corresponding to the variable regions of either the heavy (VH) or light (VL) chains of human antibodies), an Fab fragment, or an antibody having an Fab fragment and a mutated Fc portion. In certain embodiments, the mutated Fc portion does not activate complement, or the mutation(s) in the Fc portion decreases the Fc portion's ability to activate complement. An antibody of the present application may be produced or processed in bulk and packaged in an ampule made of a suitable material (e.g., glass or plastic) at different doses. The antibody may be stable in a formulation at a concentration ranging from 1 mg/ml to 200 mg/ml.

An additional active agent (or an active agent in addition to the antibody therapeutic) of the present application can be another antibody therapeutic (e.g., an anti-complement or anti-C5 antibody, an anti-IgE antibody such as Xolair® or omalizumab, an anti-IL-4 antibody or an anti-IL-5 antibody), an anti-IgE inhibitor (e.g., Singulair® or montelukast sodium), a sympathomimetic (e.g., albuterol), an antibiotic (e.g., tobramycin), a deoxyribonuclease (e.g., pulmozyme), an anticholinergic drug (e.g., ipratropium bromide), a corticosteroid (e.g., dexamethasone), a β-adrenoreceptor agonist, a leukotriene inhibitor (e.g., zileuton), a 5 Lipoxygenase inhibitor, a PDE inhibitor, a CD23 antagonist, an IL-13 antagonist, a cytokine release inhibitor, a histamine H1 receptor antagonist, an anti-histamine, an anti-inflammatory agent (e.g. cromolyn sodium) or a histamine release inhibitor. As used herein, an active agent may also be referred to as a therapeutic or a drug.

An example of formulation suitable for aerosolization or nebulization of an antibody is in physiologic osmolarity (e.g., between 280 and 320 mM) at a suitable

Samsung Bioepis Exhibit No. 1002
Page 5211

pH (e.g., pH 6 to 8). A formulation of the present application may further comprise an excipient, for example polysorbate 80 which can be used at 0.0015 to 0.02%.

U.S. Patent No. 5,474,759 discloses aerosol formulations that are substantially free of chlorofluorocarbons, and having particular utility in medicinal applications. The formulations contain a propellant (such as 1,1,1,2,3,3,3,-heptafluoropropane), a medium-chain fatty acid propylene glycol diester, a medium-chain triglyceride, optionally a surfactant, and optionally auxiliary agents such as antioxidants, preservatives, buffers, sweeteners and taste masking agents.

Other pharmaceutically acceptable carriers may also be used in a formulation of the present application. The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. The phrase "pharmaceutically acceptable carrier" as used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject antagonists from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18)

Samsung Bioepis Exhibit No. 1002
Page 5212

Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

Methods of preparing these formulations or compositions include the step of bringing into association a compound of the present application with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association a compound of the present application with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

A nebulizer of the present application can be a jet air nebulizer (e.g., Pari LC Jet Plus or Hudson T Up-draft II), an ultrasonic nebulizer (e.g., MABISMist II), a vibrating mesh nebulizer (e.g., Micro air by Omron) and a shockwave nebulizer (EvitLabs Sonik LDI20).

"Aerosol composition" means an antibody and/or an active agent described herein in a form or formulation that is suitable for pulmonary delivery. The aerosol composition may be in the dry powder form, it may be a solution, suspension or slurry to be nebulized, or it may be in admixture with a suitable low boiling point, highly volatile propellant. It is to be understood that more than one antibody and optionally other active agents or ingredients may be incorporated into the aerosolized formulation or aerosol composition and that the use of the term "antibody" or "active agent" in no way excludes the use of two or more such antibodies or other agents or ingredients.

In certain preferred embodiments, an antibody or active agent retains more than 50% of its activity after nebulization, preferably more than 70%. In certain preferred embodiments, an antibody or active agent retains more than 50% of its purity after nebulization, preferably more than 70%.

Active agent formulations suitable for use in the present application include dry powders, solutions, suspensions or slurries for nebulization and particles suspended or dissolved within a propellant. Dry powders suitable for use in the present application include amorphous active agents, crystalline active agents and mixtures of both amorphous and crystalline active agents. The dry powder active agents have a particle size selected to permit penetration into the alveoli of the lungs,

Samsung Bioepis Exhibit No. 1002
Page 5213

that is, preferably 10 μm mass median diameter (MMD), preferably less than 7.5 μm, and most preferably less than 5 μm, and usually being in the range of 0.1 μm to 5 μm in diameter. The delivered dose efficiency (DDE) of these powders is >30%, usually >40%, preferably >50 and often >60% and the aerosol particle size distribution is about 1.0-5.0 μm mass median aerodynamic diameter (MMAD), usually 1.5-4.5 μm MMAD and preferably 1.5-4.0 μm MMAD. These dry powder active agents have a moisture content below about 10% by weight, usually below about 5% by weight, and preferably below about 3% by weight. Such active agent powders are described in WO 95/24183 and WO 96/32149, which are incorporated by reference herein.

Dry powder active agent formulations are preferably prepared by spray drying under conditions which result in a substantially amorphous powder. Bulk active agent, usually in crystalline form, is dissolved in a physiologically acceptable aqueous buffer, typically a citrate buffer having a pH range from about 2 to 9. The active agent is dissolved at a concentration from 0.01% by weight to 1% by weight, usually from 0.1% to 0.2%. The solutions may then be spray dried in a conventional spray drier available from commercial suppliers such as Niro A/S (Denmark), Buchi (Switzerland) and the like, resulting in a substantially amorphous powder. These amorphous powders may also be prepared by lyophilization, vacuum drying, or evaporative drying of a suitable active agent solution under conditions to produce the amorphous structure. The amorphous active agent formulation so produced can be ground or milled to produce particles within the desired size range. Dry powder active agents may also be in a crystalline form. The crystalline dry powders may be prepared by grinding or jet milling the bulk crystalline active agent.

The active agent powders of the present application may optionally be combined with pharmaceutical carriers or excipients which are suitable for respiratory and pulmonary administration. Such carriers may serve simply as bulking agents when it is desired to reduce the active agent concentration in the powder which is being delivered to a patient, but may also serve to improve the dispersability of the powder within a powder dispersion device in order to provide more efficient and reproducible delivery of the active agent and to improve handling

Samsung Bioepis Exhibit No. 1002
Page 5214

characteristics of the active agent such as flowability and consistency to facilitate manufacturing and powder filling. Such excipients include but are not limited to (a) carbohydrates, e.g., monosaccharides such as fructose, galactose, glucose, D-mannose, sorbose, and the like; disaccharides, such as lactose, trehalose, cellobiose, and the like; cyclodextrins, such as 2-hydroxypropyl-.beta.-cyclodextrin; and polysaccharides, such as raffinose, maltodextrins, dextrans, and the like; (b) amino acids, such as glycine, arginine, aspartic acid, glutamic acid, cysteine, lysine, and the like; (c) organic salts prepared from organic acids and bases, such as sodium citrate, sodium ascorbate, magnesium gluconate. sodium gluconate, tromethamin hydrochloride, and the like; (d) peptides and proteins such as aspartame, human serum albumin, gelatin, and the like; and (e) alditols, such as mannitol, xylitol, and the like. A preferred group of carriers includes lactose, trehalose, raffinose, maltodextrins, glycine, sodium citrate, human serum albumin and mannitol.

The dry powder active agent formulations may be delivered using Inhale Therapeutic Systems' dry powder inhaler as described in WO 96/09085 which is incorporated herein by reference, but adapted to control the flow rate at a desirable level or within a suitable range. The dry powders may also be delivered using a metered dose inhaler as described by Laube et al. in U.S. Patent No. 5,320,094, which is incorporated by reference herein.

Nebulized solutions may be prepared by aerosolizing commercially available active agent formulation solutions. These solutions may be delivered by a jet nebulizer such as the Raindrop, produced by Puritan Bennett, the use of which is described by Laube et al., *supra*. Other methods for delivery of solutions, suspensions of slurries are described by Rubsamen et al, U.S. Patent No. 5,672,581. A device that uses a vibrating, piezoelectric member is described in Ivri et al., U.S. Patent No. 5,586,550, which is incorporated by reference herein.

Propellant systems may include an active agent dissolved in a propellant or particles suspended in a propellant. Both of these types of formulations are described in Rubsamen et al., U.S. Patent No. 5,672,581, which is incorporated herein by reference.

Samsung Bioepis Exhibit No. 1002
Page 5215

In certain embodiments, an aerosol or nebulization antibody composition can be combined with one or more other aerosol or nebulization treatments, such as sympathomimetics (e.g., albuterol), antibiotics (e.g., tobramycin), deoxyribonucleases (e.g., pulmozyme), anticholinergic drugs (e.g., ipratropium bromide), or corticosteroids.

In certain embodiments, an aerosol or nebulization antibody composition can be combined with one or more other therapies (concurrently or sequentially) administered via nebulization, inhalation, intravenous or oral routes, such as sympathomimetics (e.g., albuterol), anticholinergic drugs (e.g., ipratropium bromide), inflammation inhibitors (e.g., cromolyn sodium), leukotriene inhibitors (e.g., zileuton), anti-IgE inhibitors (e.g., Singulair®) or corticosteroids.

As described herein, an antibody or therapeutic may be formulated as microparticles. Microparticles having a diameter of between 0.5 and 10 microns can penetrate the lungs, passing through most of the natural barriers. A diameter of less than ten microns is generally required to bypass the throat; a diameter of 0.5 microns or greater is usually required to avoid being exhaled.

In certain embodiments, the subject antibody or therapeutic is formulated in a supramolecular complex, which may have a diameter of between 0.5 and 10 microns, which can be aggregated into particles having a diameter of between 0.5 and 10 microns.

In other embodiments, the subject antibodies or therapeutics are provided in liposomes or supramolecular complexes appropriately formulated for pulmonary delivery.

(i). Polymers for forming Microparticles

In addition to the supramolecular complexes described above, a number of other polymers can be used to form the microparticles. As used herein, the term "microparticles" includes microspheres (uniform spheres), microcapsules (having a core and an outer layer of polymer), and particles of irregular shape.

Polymers are preferably biodegradable within the time period over which release of the antibody or therapeutic is desired or relatively soon thereafter,

Samsung Bioepis Exhibit No. 1002
Page 5216

generally in the range of one year, more typically a few months, even more typically a few days to a few weeks. Biodegradation can refer to either a breakup of the microparticle, that is, dissociation of the polymers forming the microparticles and/or of the polymers themselves. This can occur as a result of change in pH from the carrier in which the particles are administered to the pH at the site of release, as in the case of the diketopiperazines, hydrolysis, as in the case of poly(hydroxy acids), by diffusion of an ion such as calcium out of the microparticle, as in the case of microparticles formed by ionic bonding of a polymer such as alginate, and by enzymatic action, as in the case of many of the polysaccharides and proteins. In some cases linear release may be most useful, although in others a pulse release or "bulk release" may provided more effective results.

Representative synthetic materials are: diketopiperazines, poly(hydroxy acids) such as poly(lactic acid), poly(glycolic acid) and copolymers thereof, polyanhydrides, polyesters such as polyorthoesters, polyamides, polycarbonates, polyalkylenes such as polyethylene, polypropylene, poly(ethylene glycol), poly(ethylene oxide), poly(ethylene terephthalate), poly vinyl compounds such as polyvinyl alcohols, polyvinyl ethers, polyvinyl esters, polyvinyl halides, polyvinylpyrrolidone, polyvinylacetate, and poly vinyl chloride, polystyrene, polysiloxanes, polymers of acrylic and methacrylic acids including poly(methyl methacrylate), poly(ethyl methacrylate), poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate), polyurethanes and co-polymers thereof, celluloses including alkyl cellulose, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, nitro celluloses, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxy-propyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxylethyl cellulose, cellullose triacetate, and cellulose sulphate sodium salt, poly(butic acid), poly(valeric acid), and poly(lactide-co-caprolactone).

Natural polymers include alginate and other polysaccharides including dextran and cellulose, collagen, albumin and other hydrophilic proteins, zein and

Samsung Bioepis Exhibit No. 1002
Page 5217

other prolamines and hydrophobic proteins, copolymers and mixtures thereof. As used herein, chemical derivatives thereof refer to substitutions, additions of chemical groups, for example, alkyl, alkylene, hydroxylations, oxidations, and other modifications in the art.

Bioadhesive polymers include bioerodible hydrogels described by H. S. Sawhney, C. P. Pathak and J .A. Hubell in Macromolecules, 1993, 26, 581-587, polyhyaluronic acids, casein, gelatin, glutin, polyanhydrides, polyacrylic acid, alginate, chitosan, and polyacrylates.

To further illustrate, the matrices can be formed of the polymers by solvent evaporation, spray drying, solvent extraction and other methods known to those skilled in the art. Methods developed for making microspheres for drug delivery are described in the literature, for example, as described by Mathiowitz and Langer, J. Controlled Release 5,13-22 (1987); Mathiowitz, et al., Reactive Polymers 6, 275-283 (1987); and Mathiowitz, et al., J. Appl. Polymer Sci. 35, 755-774 (1988). The selection of the method depends on the polymer selection, the size, external morphology, and crystallinity that is desired, as described, for example, by Mathiowitz, et al., Scanning Microscopy 4,329-340 (1990); Mathiowitz, et al., J. Appl. Polymer Sci. 45, 125-134 (1992); and Benita, et al., J. Pharm. Sci. 73, 1721-1724 (1984).

In solvent evaporation, described for example, in Mathiowitz, et al., (1990), Benita, and U.S. Pat. No. 4,272,398 to Jaffe, the polymer is dissolved in a volatile organic solvent. The antibody and/or therapeutic, either in soluble form or dispersed as fine particles, is added to the polymer solution, and the mixture is suspended in an aqueous phase that contains a surface active agent such as poly(vinyl alcohol). The resulting emulsion is stirred until most of the organic solvent evaporates, leaving solid microspheres.

In general, the polymer can be dissolved in methylene chloride. Several different polymer concentrations can be used, for example, between 0.05 and 0.20 g/ml. After loading the solution with drug, the solution is suspended in 200 ml of vigorously stirring distilled water containing 1% (w/v) poly(vinyl alcohol) (Sigma Chemical Co., St. Louis, Mo.). After four hours of stirring, the organic solvent will

Samsung Bioepis Exhibit No. 1002
Page 5218

have evaporated from the polymer, and the resulting microspheres will be washed with water and dried overnight in a lyophilizer.

Microspheres with different sizes (1-1000 microns, though less than 10 microns for aerosol applications) and morphologies can be obtained by this method which is useful for relatively stable polymers such as polyesters and polystyrene. However, labile polymers such as polyanhydrides may degrade due to exposure to water. For these polymers, hot melt encapsulation and solvent removal may be preferred.

In hot melt encapsulation, the polymer is first melted and then mixed with the solid particles of antibodies or therapeutics, preferably sieved to appropriate size. The mixture is suspended in a non-miscible solvent such as silicon oil and, with continuous stirring, heated to
5 °C above the melting point of the polymer. Once the emulsion is stabilized, it is cooled until the polymer particles solidify. The resulting microspheres are washed by decantation with petroleum ether to give a free-flowing powder. Microspheres with diameters between one and 1000 microns can be obtained with this method. The external surface of spheres prepared with this technique are usually smooth and dense. This procedure is useful with water labile polymers, but is limited to use with polymers with molecular weights between 1000 and 50000.

In spray drying, the polymer is dissolved in an organic solvent such as methylene chloride (0.04 g/ml). A known amount of antibody and/or therapeutic is suspended (if insoluble) or co-dissolved (if soluble) in the polymer solution. The solution or the dispersion is then spray-dried. Microspheres ranging in diameter between one and ten microns can be obtained with a morphology which depends on the selection of polymer.]

Hydrogel microspheres made of gel-type polymers such as alginate or polyphosphazines or other dicarboxylic polymers can be prepared by dissolving the polymer in an aqueous solution, suspending the material to be incorporated into the mixture, and extruding the polymer mixture through a microdroplet forming device, equipped with a nitrogen gas jet. The resulting microspheres fall into a slowly stirring, ionic hardening bath, as described, for example, by Salib, et al.,

Samsung Bioepis Exhibit No. 1002
Page 5219

Pharmazeutische Industrie 40-111A, 1230 (1978). The advantage of this system is the ability to further modify the surface of the microspheres by coating them with polycationic polymers such as polylysine, after fabrication, for example, as described by Lim, et al., J. Pharm. Sci. 70, 351-354 (1981). For example, in the case of alginate, a hydrogel can be formed by ionically crosslinking the alginate with calcium ions, then crosslinking the outer surface of the microparticle with a polycation such as polylysine, after fabrication. The microsphere particle size will be controlled using various size extruders, polymer flow rates and gas flow rates.

Chitosan microspheres can be prepared by dissolving the polymer in acidic solution and crosslinking with tripolyphosphate. For example, carboxymethylcellulose (CMC) microsphere are prepared by dissolving the polymer in an acid solution and precipitating the microspheres with lead ions. Alginate/polyethyleneimine (PEI) can be prepared to reduce the amount of carboxyl groups on the alginate microcapsules.

(ii). Pharmaceutical Compositions

The microparticles can be suspended in any appropriate pharmaceutical carrier, such as saline, for administration to a patient. In the most preferred embodiment, the microparticles will be stored in dry or lyophilized form until immediately before administration. They can then be suspended in sufficient solution, for example an aqueous solution for administration as an aerosol, or administered as a dry powder.

(iii). Targeted Administration

The microparticles can be delivered to specific cells or organ, especially phagocytic cells and organs. Where systemic delivery of the drug or therapeutic is desirable, endocytosis of the microparticles by macrophages in the lungs can be used to target the microparticles to the spleen, bone marrow, liver, lymph nodes, and other parts of the body.

The microparticles can also be targeted by attachment of ligands, such as those described above, which specifically or non-specifically bind to particular targets, for example, particular targets within the pulmonary system or associated with a pulmonary disease or condition (e.g., a target specific for cancerous cells in

Samsung Bioepis Exhibit No. 1002
Page 5220

the lung). Examples of such ligands also include antibodies and fragments including the variable regions, lectins, and hormones or other organic molecules having receptors on the surfaces of the target cells.

(iv). Storage of the Microparticles

In certain embodiments, the microparticles are stored lyophilized. The dosage is determined by the amount of encapsulated antibodies and/or therapeutics, the rate of release within the pulmonary system, and the pharmacokinetics of the antibodies and/or therapeutics.

(v). Delivery of Microparticles

The microparticles can be delivered using a variety of methods, ranging from administration directly into the nasal passages so that some of the particles reach the pulmonary system, to the use of a powder instillation device, to the use of a catheter or tube reaching into the pulmonary tract. As described above, dry powder inhalers are commercially available, although those using hydrocarbon propellants are no longer used and those relying on the intake of a breath by a patient can result in a variable dose. Examples of suitable propellants include hydrofluoroalkane propellants, such as 1,1,1,2-tetrafluoroethane (CF3CH2F) (HFA-134a) and 1,1,1,2,3,3,3-heptafluoro-n-propane (CF3CHFCF3) (HFA-227), perfluoroethane, monochloroifluoromethane, 1,1 difluoroethane, and combinations thereof.

Therapeutic Methods

A first aspect of the application provides a method for preventing or treating a pulmonary disease or condition in a subject comprising administering to the subject a therapeutically effective amount of an antibody that inhibits activation of the complement cascade. In certain embodiments, an additional active agent is also administered to the same subject. Administration of the antibody and the additional active agent may occur simultaneously or sequentially in either order.

The term "preventing" is art-recognized, and when used in relation to a condition, such as a local recurrence (e.g., pain), a disease such as cancer, a syndrome complex such as heart failure or any other medical condition, is well understood in the art, and includes administration of a composition which reduces the frequency of, or delays the onset of, symptoms of a medical condition (e.g.,

Samsung Bioepis Exhibit No. 1002
Page 5221

asthma) in a subject relative to a subject which does not receive the composition. Thus, prevention of cancer includes, for example, reducing the number of detectable cancerous growths in a population of patients receiving a prophylactic treatment relative to an untreated control population, and/or delaying the appearance of detectable cancerous growths in a treated population versus an untreated control population, e.g., by a statistically and/or clinically significant amount. Prevention of an infection includes, for example, reducing the number of diagnoses of the infection in a treated population versus an untreated control population, and/or delaying the onset of symptoms of the infection in a treated population versus an untreated control population. Prevention of pain includes, for example, reducing the magnitude of, or alternatively delaying, pain sensations experienced by subjects in a treated population versus an untreated control population.

The term "treating" includes prophylactic and/or therapeutic treatments. The term "prophylactic or therapeutic" treatment is art-recognized and includes administration to the host of one or more of the subject compositions. If it is administered prior to clinical manifestation of the unwanted condition (e.g., disease or other unwanted state of the host animal) then the treatment is prophylactic, (i.e., it protects the host against developing the unwanted condition), whereas if it is administered after manifestation of the unwanted condition, the treatment is therapeutic, (i.e., it is intended to diminish, ameliorate, or stabilize the existing unwanted condition or side effects thereof).

Pulmonary diseases or conditions contemplated by the application include, but are not limited to, asthma, bronchial constriction, bronchitis, a chronic obstructive pulmonary disease (COPD), interstitial lung diseases, lung malignancies, α-1 anti-trypsin deficiency, emphysema, bronchiectasis, bronchiolitis obliterans, sarcoidosis, pulmonary fibrosis, and collagen vascular disorders.

The subject or patient receiving this treatment is any animal in need, including primates, in particular humans, and other mammals such as equines, cattle, swine and sheep, and poultry and pets in general.

The phrase "therapeutically effective amount" as used herein means that amount of a compound, material, or composition comprising a compound of the

Samsung Bioepis Exhibit No. 1002
Page 5222

present invention which is effective for producing some desired therapeutic effect by inhibiting C5 or activation of the complement cascade in at least a sub-population of cells or an organ in an animal and thereby blocking the biological consequences of that function in the treated cells or an organ, at a reasonable benefit/risk ratio applicable to any medical treatment.

The timing of administering a therapeutic to a subject can vary, for example, depending on the identity of the subject or the pulmonary disease or condition to be treated or prevented, or both. For example, the administration may occur before the manifestation of the pulmonary condition (e.g., pre-asthmatic attack or pre-bronchial constriction), during the manifestation of the pulmonary condition (e.g., during the asthmatic attack or bronchial constriction), or after the manifestation of the pulmonary condition (e.g., post-asthmatic attack or post-bronchial constriction).

The phrase "inhibits the activation of complement cascade" refers to any inhibitory effect or action on any component of the complement system as described above conferred by an antibody or active agent of the present application. The inhibitory effect may be manifested by decrease in or lack of a response typical to activation of complement. For example, typical cellular and other biological responses to activation of complement include, but are not limited to, release of histamine, smooth muscle contraction, increased vascular permeability, leukocyte activation, chemotaxis (e.g., movement of granulocytes to the site of complement activation), and other inflammatory phenomena including cellular proliferation. In certain embodiments, the inhibitory effect is specific to the C5 component. In certain embodiments, the inhibitory effect is specific to C5 convertase, for example, blocking C5 convertase's function to convert C5 into C5a and C5b-9. In certain embodiments, the inhibitory effect is specific to C5a. In certain embodiments, the inhibitory effect is specific to C5b or C5b-9.

Examples

The following examples are offered by way of illustration and not by way of limitation.

Samsung Bioepis Exhibit No. 1002
Page 5223

Materials and Methods

Animals: Male C5s BALB/cByJ, C57BL/6J mice and C5d B10D2oSn/J, AKR/J and SWR/J mice were purchased from the Jackson Laboratory and housed in a pathogen free facility. In addition to serum C5b-9 mediated hemolysis, the C5 genotype was further confirmed by PCR performed on tail DNA using a pair of primers (5'-CAC GAT AAT GGG AGT CAT CTG GG-3' (SEQ ID NO:1) and 5'-AAG TTG GAG TGT GGT CTT TGG GCC-3' (SEQ ID NO:2) that amplify a 280-bp DNA fragment from both C5s and C5d DNA. This fragment encodes a HindIII site that is selectively destroyed by a mutation in the C5 gene, such that HindIII (New England BioLabs,MA) digestion selectively cleaves the C5s but not the C5d PCR products into 150 and 130-bp fragments. All animal protocols were reviewed by Institutional Committee and were in accordance with NIH guidelines.

Reagents: Anti-mouse C5 mAb (BB5.1) (Wang et al., Proc. Natl. Acad. Sci. U.S.A (1996) 93:8563-8568) and an isotype matched irrelevant control mAb (HFN7.1), which is specific to human fibronectin, were purified from ascites. BB5.1 Fab, which was purified with a kit (Pierce Biotechnology), had retained similar activity of BB5.1 and was more than 97% in purity. The anti-C5aR sIgG, which was shown to prevent mortality in experimental sepsis (Riedemann et al., J. Clin. Invest. (2002) 110:101-108), was able to prevent zymosan induced neutrophil migration. Both anti-C5aR sIgG and the control sIgG were purified from sera harvested from C5aR deficient mice (Gerard et al., Curr. Opin. Immunol. (2002) 14:705-708) that were repeatedly immunized with either PBS or peptide spanning the N-terminus of the mouse C5aR and one extra cysteine (Riedemann et al., *supra*) emulsified with CFA. Antibodies from either ascites or serum samples were purified by protein A affinity chromatography (Amersham). Corticosteroid (dexamethasone) was purchased from Sigma. Recombinant mouse C5a, which was free of endotoxin contamination and had potent chemotactic activity in neutrophil migration assay, was cloned and purified as previously reported (Riedemann et al., *supra*).

Samsung Bioepis Exhibit No. 1002
Page 5224

Induction of Airway Inflammation and Severe Airway Response

BALB/c and B10D2oSn Mice 10-12 wks of age were sensitized on day 1 and day 14 with i.p. injection of 20 µg OVA (Grade V; Sigma) emulsified in 2 mg aluminum hydroxide (Alum Inject; Pierce Biotechnology). Mice were immunized with 1% OVA for 10 min via airway on days 28, 29 and 30 with a jet nebulizer (Harvard Apparatus) connected to a single housing chamber (Buxco Electronics, Inc.). On day 32, all sensitized animals were aerosol challenged with 5% OVA for 10 min. In checkpoint 3 experiments, BALB/c mice were aerosol challenged with 5% OVA again on day 35. Sham C5s or C5d mice were immunized with Alum only and aerosol challenged with PBS instead of OVA.

Non-Invasive Measurement of sRaw

sRaw, which measures the resistance of both upper and lower airways, was measured by a DCP in conscious animals with spontaneous breathing (Buxco). The DCP was used to monitor the longitudinal changes of sRaw prior to and after each 5% OVA aerosol challenge. In checkpoint 3 experiments, a DCP was used to ensure the appearance of EAR prior to randomizing animals into different treatment groups.

Invasive Measurement of Lower Airway Functions

Changes in RL and Cdyn were measured by using Buxco Biosystem software and a Buxco whole body plethysmograph. Mice were anesthetized (Avertin, 160 mg/kg) and tracheas cannulated. Spontaneous breathing was blocked by pancuronium bromide (0.3 mg/kg i.p.). Respirations were maintained by a Harvard Apparatus Inspira ventilator, which calculated a tidal volume and respiratory rate according to body weight. Measurements of RL and Cdyn were performed during the peak of LAR, 5 h after aerosol challenge with 5% OVA. Real-time changes of RL and Cdyn were recorded by Buxco software and reported as the mean value of five min of recording. AHR was evaluated based on changes of RL and Cdyn during aerosol Mch challenges. Changes in RL and Cdyn were expressed as a percentage of baseline after each aerosol challenge. A Buxco aerosol control and sonicating nebulizer unit was attached to the ventilator for aerosol delivery of Mch through tracheal cannulation. PBS or Mch (1.6 mg/ml) was delivered at the

Samsung Bioepis Exhibit No. 1002
Page 5225

rate of 10 puffs or 20 puffs per 10 seconds, with each puff of aerosol delivery lasting 15 ms.

C5 Inhibition

(1) For C5 inhibition at checkpoint 1, anti-C5 mAb (BB5.1) or a control mAb (HFN7.1) was administered i.p. at 40 mg/kg on days 25, 29, and 31. Dexamethasone (2 mg/kg) was used as a positive control. (2) Animals that experienced airway response to 5% OVA provocation on day 32 were randomized for the following studies. For C5 inhibition at checkpoint 2, animals were given one i.p. injection of either dexamethasone (2 mg/kg), anti-C5 mAb (40 mg/ml) or control mAb (40 mg/kg) on day 33. On day 35, animals were anesthetized and tracheas cannulated for measurement of RL and Cdyn during aerosol Mch challenges. (3) At checkpoint 3, anti-C5 mAb (40 mg/kg), control mAb (40 mg/kg), or dexamethasone (2 mg/kg) was administered by i.v. injection 20 min after aerosol challenging animals with 5% OVA on day 35. For intrapulmonary C5 inhibition at this critical point, animals were given aerosol administration of either anti-C5 mAb (3 mg/ml), anti-C5 Fab (3 mg/ml), control mAb (3 mg/ml), anti-C5aR sIgG (3 mg/ml), control sIgG (2 mg/ml), or corticosteroid (2 mg/ml) for 10-30 min by using a jet nebulizer connected to a single chamber housing the animal. Sham-immunized mice were given sham treatment with the same volume of PBS solution administered through the same route. The results from three intrapulmonary C5 inhibition experiments, which were conducted with identical protocols, were pooled together.

Analysis of Bronchoalveolar Lavage Fluid (BALF)

Approximately 5.5 h after allergen provocation, after the measurement of RL and Cdyn, BALF was harvested by instilling 1 ml PBS through the tracheal cannula, followed by gentle aspiration. The BALF was resuspended in 400 μl PBS. The total numbers of WBC in BALF were counted by using a hemocytometer or an automatic cell counter as in the aerosol experiments (Cell-Dyn 3700 Abbott). Cytospin slides were prepared, fixed and stained using Diff-Quik (VWR International, Inc.). The WBC differential was determined by a certified pathologist after counting a total of 300 WBC per slide on a 100x microscopic lens. The BALF levels of proMMP9, activated TGF-β, RANTES, eotaxin and IL-13 (R&D Systems,

Samsung Bioepis Exhibit No. 1002
Page 5226

Inc.), IL-5 (Amersham Pharmacia Biotech, Inc.), and histamine (Immunotech, Inc.) were measured by ELISA according to the manufacturer's instructions.

Analysis of Serum OVA Specific Antibodies

Serum samples were harvested 5 h after 5% allergen provocation on day 32 for OVA specific IgG and IgE by ELISA.

Lung Histology

Lung was inflated with 10% buffered formalin (1 ml) through tracheal cannulation and fixed in 10% formalin at least 24 h. Lung samples were stained with Giemsa. Double blind histological analysis was performed to quantify airway inflammation according to the following criteria. 0: No detectable airway inflammation; 1: Less than 25% bronchials and surrounding vasculature were found with either perivascular or peribronchial inflammatory cell infiltration; 2: Approximately 25-50% of bronchials and surrounding vasculature were affected; 3: Approximately 50-75% bronchials and surrounding vasculature were affected; 4: >75% of bronchials and surrounding vasculature were affected.

C5b-9 Mediated Hemolytic Assay and in Vitro C5a Mediated Neutrophil Migration Assay

Serum harvested at the indicated time was used as the source of C5b-9, which mediated the hemolysis of chicken RBC as previously reported (Wang et al., *supra*). Mouse sera (Sigma) were incubated with either BB5.1 (100 µg/ml) or the same volume of PBS prior to activation with zymosan (1 mg/ml; Sigma). Diluted sera (20%) were used as the source of C5a for neutrophil chemotaxis experiments.

Statistical Analysis

The data were expressed as the means ± S.E.M. Student's one-tailed t-test assuming

equal variance was used (MS Windows). A P value less than 0.05 was considered significant.

Samsung Bioepis Exhibit No. 1002
Page 5227

Blocking the generation of C5a and C5b-9 by anti-C5 mAb, BB5.1

Functional inhibition of complement component C5 by anti-C5 mAb was determined by the pharmacodynamic profile of blocking serum C5b-9 mediated hemolytic activity (Fig. 1 A). In addition, C5a mediated neutrophil migration was determined in an in vitro assay (Fig. 1 B). The pharmacodynamics of C5 inhibition by a single i.v. injection of anti-C5 mAb, BB5.1, is very different from the pharmacodynamics of a single i.p. injection during the first 5 h after administration. More than 80% of C5b-9 mediated hemolysis was inhibited within 1 h of i.v. administration of BB5.1, compared to less than 30% of inhibition by i.p. injection. Administration of anti-C5 mAb continued blocking more than 60% of hemolysis between 5 h and 48 h after i.v. injection while approximately 56% of hemolysis was blocked during this period of time after i.p. injection. Serum samples harvested from control mAb treated mice had more than 80% of normal hemolytic activity during the entire period of the study (Fig. 1 A). Hemolytic activity gradually returned to the normal range one wk after a single injection of anti-C5 mAb.

An in vitro neutrophil migration assay was employed to test the ability of anti-C5 mAb on blocking the generation of C5a (Fig. 1 B). Inhibition of serum samples with anti-C5 mAb prior to zymosan activation significantly blocked human neutrophil chemotaxis which was mediated by the presence of C5a after zymosan activation, as seen in zymosan activated control sera (Fig.1, B). Since anti-C5 mAb, BB5.1, blocked the generation of both C5a and C5b-9 and did not bind to C5a directly, this anti-C5 mAb is probably specific to blocking cleavage of C5 by C5 convertase.

Development of Severe Airway Response in OVA Sensitized Animals and Definition of Three Critical Points

OVA-sensitized animals immediately developed a severe airway response after aerosol challenge with 5% OVA (Fig. 2 A). A typical airway response to aerosol provocation with allergen consisted of an early-phase airway response (EAR), which is known to be mediated by the release of histamine by mast cells, and a late-phase airway response (LAR), which is mediated by the combination of effects of infiltration of inflammatory cells, edema and bronchial constriction

Samsung Bioepis Exhibit No. 1002
Page 5228

(Larsen, Annu. Rev. Immunol. (1985) 3:59-85). The airway response after 5% OVA provocation was monitored by a non-invasive double chamber plethysmograph (DCP) in each mouse to measure the longitudinal changes of specific airway response (sRaw), which indicates the appearance of EAR and LAR (Fig. 2 A). EAR was typically a brief elevation of sRaw 15 min after 5% OVA provocation and LAR was typically observed 5 h later, consistent with an earlier report (Cieslewicz et al., J. Clin. Invest. (1999)104:301-308). Both EAR and LAR have magnitudes between 5 to 30 fold over the baseline. The severe airway response during the peak of LAR was generally evident as animals showed obvious labored breathing. Since inhibition of C5 can be achieved by the administration of anti-C5 mAb, we sought to dissect the critical involvement of C5 and its activated components at three critical points

(Fig. 2 B) during critical stages of the disease. The three critical checkpoints are: 1) initiation of airway inflammation, one of the two hallmarks of asthma's pathogenesis; 2) development of AHR to nonspecific stimuli, another hallmark of asthma; and 3) sustainment of an on-going airway response after allergen provocation. Both checkpoint 2 and checkpoint 3 are designed to evaluate the contribution of C5 in subjects that had experienced severe airway response previously (Fig. 2 B) and had established airway inflammation. Checkpoint 3 studies were conducted during an on-going airway response after allergen provocation by administering treatment via either an i.v. or aerosol route during the EAR. Functions of lower airways and quantification of airway inflammation were evaluated during the peak of LAR, 5 h after 5% OVA provocation.

Checkpoint 1: Involvement of C5 in the initiation of airway inflammation

C5 inhibition at checkpoint 1, at the time of repeat aerosol sensitization of 1% OVA, had no significant impact on serum levels of OVA-specific IgE or IgG on day 32 (Fig. 3 A). Corticosteroid treated mice also had elevated levels of OVA-specific IgE and IgG, that may have been generated long before the corticosteroid or anti-C5 mAb was administered on day 25. Sham treated mice had a negligible level of OVA specific IgG or IgE. As a result of repeated administration (i.p.) of anti-C5 mAb, approximately 80% of C5b-9 mediated hemolysis was inhibited on day 32 (21.1%±4) as compared to the normal hemolytic activity seen in control mAb and

Samsung Bioepis Exhibit No. 1002
Page 5229

corticosteroid treated mice (92.7%±6.8). Two key functions of lower airways were examined by tracheal cannulation at 5 h after 5% OVA provocation. The increase in lung resistance (RL) is commonly associated with significant obstruction of lower airways (Fig. 3 B) and a decrease in the dynamic lung compliance (Cdyn) is associated with the loss of lung elasticity, an important characteristic of the return to normal volume after pressure changes seen in a normal lung (Fig. 3 C). The significantly increased RL and reduced Cdyn seen in control mAb-treated mice indicated an increased airway obstruction (Figs. 3 B and C). Sham-mice provided normal ranges of RL and Cdyn at 5 h after exposure to aerosol PBS solution.

Correlating to its potent anti-inflammatory and anti-asthmatic activities, corticosteroid treatment at checkpoint 1 significantly blocked the increase of RL and prevented the loss of Cdyn. Similarly, C5 inhibition at checkpoint 1 markedly reduced the increase of RL 5 h after allergen provocation with an RL of 1.66 cm H2O/ml/second ±0.2, compared to an RL of 2.17 cm H_2O/ml/second ±0.33 seen in control mAb treated animals (Fig. 3 B). C5 inhibition also slightly reduced the loss of Cdyn (Fig. 3 C). Double blind histological analysis (Fig. 3 D) of lung tissue confirmed data from the function analysis and demonstrated that control mAb-treated BALB/c mice had severe perivascular and peribronchial infiltration of inflammatory cells (Fig. 3 F). A significant reduction of histology score was observed in corticosteroid treated mice (Fig. 3 G) while less impressive reduction was observed in anti-C5 mAb-treated mice (Fig. 3 H). Similar to severe allergic asthma in humans, eosinophils were the predominant inflammatory cells (more than 50%) in the perivascular and peribronchial lesions along the airways (Figs. 3 F-H).

Checkpoint 2: Contribution of C5 in the development of AHR in animals with established airway inflammation

In addition to airway inflammation, AHR to non-specific stimuli is another hallmark of asthma. AHR was evaluated as changes of RL and Cdyn expressed as a percentage of baseline in response to increasing doses of aerosol methacholine (Mch) challenges (Figs. 4 A and B). Control mAb-treated BALB/c mice had significant increases of RL and significant losses of Cdyn during the course of aerosol Mch challenges, while sham-mice responded only modestly to aerosol Mch

Samsung Bioepis Exhibit No. 1002
Page 5230

challenges. Two of eight among control mAb-treated mice died during high doses of aerosol Mch challenge due to severe respiratory stress. In contrast, C5 inhibition prevented dramatic increases of RL and the reduction of Cdyn during the course of aerosol Mch challenges, similar to those animals treated with corticosteroid (Figs. 4 A and B). The loss of Cdyn and increase of RL during the course of aerosol Mch challenges compared to sham-mice were probably due to the presence of established airway inflammation in these animals. There were no noticeable differences in the degree of airway inflammation among the three treated cohorts. Anti-C5 mAb treated mice had approximately 45% of normal hemolytic activity on day 35.

In order to compare our results of C5 inhibition at checkpoint 2 with data from a previous report (Karp et al., Nat. Immunol. (2000) 1:221-226), the presence or absence of native intrinsic AHR was examined in some strains of normal C5d mice as previously reported (De Sanctis et al., Am. J. Respir. Crit. Care Med. (1997) 156:S82-S88; Levitt et al., FASEB J. (1998) 2:2605-2608). Non-immunized C5d AKR/J mice have dramatic airway responses to increasing doses of aerosol Mch challenges with significant increases of RL over baseline (84.2%±16.7 with 20 puffs Mch) and a significant reduction of Cdyn under baseline (40% ±5.4 with 20 puffs of Mch) whereas C5d SWR/J and B10D2oSn mice have minimal changes of RL and Cdyn, indistinguishable from C5 sufficient (C5s) BALB/c mice and C57BL/6 mice, with no more than a maximal change of 15% of either RL or Cdyn away from baseline (n=3 for each strain, data not shown). Next, C5d B10D2oSn mice, which do not have intrinsic AHR, were immunized and challenged with aerosol Mch in the identical manner as C5s BALB/c mice in Figs. 4 A and B. Subgroups of sensitized C5d animals were also given either control or anti-C5 mAb treatment on day 33. C5d B10D2oSn mice developed a similar degree of airway inflammation as C5s mice, with an average histology score of 1.91 ± 0.37 (n=6) compared to a histology score of 2.25 ± 0.25 in C5s BALB/c mice (n=4). No noticeable histological difference was observed between anti-C5 mAb and control mAb treated C5d mice. In contrast to a previous report (Karp et al., *supra*), increasing doses of aerosol Mch challenges did not induce significant changes of lower airway functions in OVA immunized B10D2.oSn mice (Figs. 4 C and D). Treatment with either anti-C5 mAb or control mAb on day 33 had no impact on the development of AHR. The data

Samsung Bioepis Exhibit No. 1002
Page 5231

from both mAb treated cohorts were pooled together as an mAb treated cohort (Figs. 4 C and D). Furthermore, reconstitution of OVA immunized B10D2.oSn mice with recombinant mouse C5a (rmC5a) completely restored AHR in response to aerosol Mch challenge as indicated by the significant increase of RL and reduction of Cdyn (Figs. 4 C and D).

Reconstitution of rmC5a was not sufficient for the development of AHR since sham-B10D2.oSn mice reconstituted with rmC5a did not have significant changes of lower airway functions during Mch challenge (Figs. 4 C and D). Analysis of serum samples harvested from each mouse after Mch challenges confirmed the C5 deficient status of B10D2.oSn mice with average hemolytic activity of 8.1% ± 0.9 compared to 108%±3.8 of C5s BALB/c mice. Furthermore, random tail samples (n=2) from each lot of C5s and C5d animals were analyzed to confirm the C5 genotype status.

Checkpoint 3: Contribution of C5 during an on-going airway response

The role of C5 in sustaining an on-going airway response was also examined. Intervention was given during the peak of EAR, which was monitored by DCP at 15 min after aerosol exposure to 5% OVA on day 35. All control mAb-treated BALB/c mice developed severe LAR with significant increases of RL and reductions of Cdyn 5 h after allergen provocation (Figs. 5 A and B).

Administration of treatment through i.v. injection during EAR was selected to achieve rapid systemic C5 inhibition, which completely blocked the development of LAR with minimal increase of RL 5 h after 5% OVA provocation (Fig. 5 A) and prevented much of the loss of lung elasticity indicated by the minimal reduction of Cdyn (Fig. 5 B). Interestingly, corticosteroid treatment did not eliminate the development of LAR with significant elevation of RL and reduction of Cdyn at 5 h.

Although no immediate impacts (within 5 h) on lower airway functions were observed after i.v. corticosteroid treatment (Fig. 5 A), this treatment significantly modulated the intrapulmonary IL-13 (Fig. 7 B) and significantly improved lower airway functions 24 h later. Double blind histologic analysis of lung tissue showed comparable levels of perivascular and peribronchial infiltration of inflammatory

Samsung Bioepis Exhibit No. 1002
Page 5232

cells from the animals 5 h after i.v. intervention with either corticosteroid, anti-C5 mAb or control mAb, similar to the histological sample shown in Fig. 3 F.

Effect of C5 Inhibition on the Migration of Inflammatory Cells

The migration of inflammatory cells from airway tissue inflammation into bronchial lumen as enumerated was also examined by bronchial alveolar lavage fluid (BALF) WBC analysis. There was a significant reduction of total BALF inflammatory cells in anti-C5 mAb-treated BALB/c mice compared to control mAb-treated mice at checkpoint 1 (Fig. 6 A). The blockade on the migration of inflammatory cells by C5 inhibition exceeded the degree of improved lower airway function (Figs. 3 B and C) and reduced tissue inflammation (Fig. 3 D). Similarly, when BALF was harvested at checkpoint 3 (Fig. 6 B), C5 inhibition again had significant blockade on the migration of inflammatory cells into bronchial lumen, with significantly lower BALF WBC counts in anti-C5 treated animals than in animals treated with either corticosteroid or control mAb. This result is in dramatic contrast to the prominent presence of perivascular and peribronchial infiltration of inflammatory cells in lung tissue harvested before allergen provocation on day 35. The significant blockade on the migration of inflammatory cells by C5 inhibition correlated with the significantly enhanced efficacy of C5 inhibition over corticosteroid therapy at checkpoint 3 (Figs. 5 A and B).

Sham mice provided the baseline level of BALF WBC counts, which are primarily normal alveolar macrophages (82.3%±10.3) in WBC differential analysis (Fig. 6 C). Eosinophils constituted more than 45% of BALF inflammatory cells from all OVA immunized animals, regardless of the therapeutic intervention. There was a significant reduction in the percentage of neutrophils recovered in BALF from the animals treated at checkpoint 3 with either corticosteroid (4.5%±2.8) or anti-C5 mAb (6.3%±3.3) versus control mAb treated mice (18.3%±4.4). No significant reductions occurred in the percentages of eosinophils or lymphocytes in BALF samples from the animals given intervention at checkpoint 3 (Fig. 6 C).

Samsung Bioepis Exhibit No. 1002
Page 5233

Effect of C5 Inhibition on Intrapulmonary Th1/Th2 Cytokine Profile and Inflammatory Mediators

BALF harvested from control mAb treated mice had markedly increased levels of IL-5 and IL-13 (Figs. 7 A and B) as compared to sham mice. Corticosteroid treatment markedly reduced the BALF levels of IL-5 and IL-13, however, this did not translate into an immediate improvement of lower airway function (Figs. 5 A and B) during the 5 h period of treatment. The dramatic improvement of lower airway functions (Figs. 5 A and B) seen after C5 inhibition at checkpoint 3 did not correlate with any reduction of BALF level of IL-5 and IL-13. The statistical difference of the BALF level of IL-13 between anti-C5- and corticosteroid-treated mice equals a p value of 0.051 C5 inhibition or corticosteroid therapy at checkpoint 3 did not influence the BALF level of histamine (Fig. 7 C). This was expected since histamine had probably already been released into the airway lumen upon the engagement of IgE receptors of mast cells by aerosol allergen provocation and was responsible for the appearance of EAR when therapeutic intervention was given. Eosinophils, the most prominent inflammatory cells along lower airways (Figs. 3 F-H), are probably responsible for the production of eotaxin, RANTES and TGF-β (Figs. 7 D-F) in asthmatic individuals. Control mAb-treated animals had significantly elevated BALF levels of eotaxin (Fig. 7 D), RANTES (Fig. 7 E), or activated TGF-β (Fig. 7 F), compared to negligible or non-detectable levels of these mediators in sham-mice. In contrast, C5 inhibition had a marked impact on BALF levels of eotaxin, RANTES, and activated TGF-β when given either at checkpoint 1 (Figs. , D and E) or checkpoint 3 (Fig. 7 F). Corticosteroid treatment only reduced the BALF level of TGF-β when given at checkpoint 3 but had no obvious impact on the production and release of eotaxin or RANTES when given at checkpoint 1.

Neutrophils are commonly believed to be responsible for the production of bronchial TNF-α and proMMP9. C5 inhibition significantly reduced BALF levels of TNF-α (Fig. 7 G) when given at checkpoint 1, significantly different from control mAb-treated mice. The bronchial level of proMMP9 was dramatically reduced and was significantly different from control mAb-treated and corticosteroid-treated mice when C5 inhibition was given at checkpoint 3 (Fig. 7 H).

Samsung Bioepis Exhibit No. 1002
Page 5234

Checkpoint 3: Contribution of Intrapulmonary Activation Of C5 During An On-Going Airway Response

The massive migration of inflammatory cells from airway tissue inflammation into bronchial lumen probably results from powerful chemotactic forces from the epithelial mucosa of the airway. Thus, C5 may be activated intrapulmonarily during an airway response to allergen provocation. The functions of lower airways and parameters of inflammation were evaluated after blocking the intrapulmonary activation of C5. The potential impact at checkpoint 3 by inhibitory receptors, such as FcγRIIB (Katz, Curr. Opin. Immunol. (2002) 14:698-704; Ravetch et al., Annu. Rev. Immunol. (2001) 19:275-90), were also evaluated, as the result of interaction between intrapulmonary C5 and anti-C5 mAb and the subsequent formation of immune complexes (IC).

Effect of intrapulmonary C5 inhibition with anti-C5 mAb was directly compared to anti-C5 Fab in order to achieve this goal. Furthermore, the efficacy of an anti-C5aR serum IgG (sIgG) was evaluated in order to dissect the role of intrapulmonary C5a versus C5b-9 at this checkpoint. As demonstrated in Figs. 8 A and B, control mAb-treated animals developed severe LAR with significant increases of RL and significant reductions of Cdyn, in significant contrast to the normal RL and Cdyn seen in sham-mice. Aerosol administration of corticosteroid blocked the development of LAR as evidenced by the reduction of RL with corresponding preservation of Cdyn. Blocking intrapulmonary C5 activation with aerosol administration of either anti-C5 mAb or anti-C5 Fab during the peak of EAR also prevented the development of LAR with minimal increase of RL and minimal reduction of Cdyn (Figs. 8 A and B). RL and Cdyn of both anti-C5 treated cohorts were significantly different from control mAb treated animals. Blocking the binding of intrapulmonary C5a to its receptors with an anti-C5aR sIgG also prevented the development of LAR with minimal increase of RL and minimal reduction of Cdyn similar to animals treated with anti-C5 mAb. There was no statistical difference between the three cohorts treated with either anti-C5 mAb, anti-C5 Fab, or anti-C5aR sIgG (Figs. 8 A and B).

Samsung Bioepis Exhibit No. 1002
Page 5235

When the parameters of intrapulmonary inflammatory activities were examined, such as the migration of inflammatory cells (Fig. 8 C) and the BALF level of proMMP9 (Fig. 8 D), significant differences were found between blocking intrapulmonary activation of C5 versus blocking the binding of C5a to its C5aR. Aerosol administration of either anti-C5 mAb or its Fab fragment during EAR significantly blocked both the migration of inflammatory cells into bronchial airway lumen and the elevation of BALF level of proMMP9, whereas blocking the engagement of C5aR had no significant impact on these two key inflammatory parameters similar to the animals treated with control mAb (Figs. 8 C and D). Consistent with its ability to block the development of LAR (Figs. 8 A and B), aerosol administration of corticosteroid also ameliorated intrapulmonary inflammatory activities with marked reduction of the migration of inflammatory cells and the production of proMMP9 (Figs. 8 C and D) indicating the importance of inflammatory activities at the epithelial side of the bronchial lumen. Aerosol administration of anti-C5 mAb, anti-C5 Fab or anti-C5aR sIgG did not have any impact on serum C5b-9 mediated hemolytic activity.

Combination Therapy:

As demonstrated in Fig. 9, animals treated with a non-specific control antibody had significant increases of lung resistance during the course of Mch challenges. In this study, two animals challenged with high doses of Mch died due to severe respiratory stress. In contrast, both the steroid and anti-C5 treated animals have only moderate increases of lung resistance, higher than normal sham immunized animals but significantly lower than control placebo treated mice. Combined treatment with both anti-C5 antibody and steroid significantly further reduced the increases of lung resistance during the course of Mch challenges. Based on this result, both steroid and C5 inhibitor can prevent the development of airway hyper-responsiveness to non-specific aerosol stimuli in asthmatic individuals with established airway inflammation.

Nebulization Formulations

Examples of formulations suitable for nebulization of an antibody are shown in Fig. 10. Fig. 11 further demonstrates the effectiveness of nebulization treatment.

Samsung Bioepis Exhibit No. 1002
Page 5236

The results shown in Fig. 11 were obtained following a study that employed the same protocol as the Checkpoint 2 study described herein, except that the treatment here was delivered by nebulization on day 32 instead of i.p. For anti-C5 antibody, 3 mg/ml of BB5.1 was subject to 10 min nebulization, and 2 mg/ml steroid was also subject to 10 min nebulization. For the combination therapy, the final concentrations subjected to 10 min nebulization were: 3 mg/ml for BB5.1 and 2 mg/ml for the steroid.

Fig. 12 further shows that eculizumab in Formulation 1 (Fig. 10) at 30 mg/ml can be effectively and efficiently nebulized by a conventional nebulizer such as the Pari LC Jet Plus or a specialty nebulizer such as Sonik LDI. Fig. 12 shows that a majority of the particles after nebulization were less than 5 μM, which is suitable for deep lung delivery.

As shown in Fig. 13, eculizumab in Formulation 1 (Fig. 10) can be effectively and efficiently delivered by a conventional nebulizer, comparable to the aerosol characteristics of an existing pulmonary drug delivered by inhalation, Pulmozyme®, also using a conventional nebulizer. Eculizumab in Formulation 1 (Fig. 10) can also be effectively and efficiently delivered by a specialty nebulizer, for example, Sonik LDI.

Using SDS-PAGE and HPLC analyses, Fig. 14 and Fig. 15 demonstrate that eculizumab can be effectively and efficiently delivered by nebulization. Eculizumab in a suitable formulation was nebulized using either the Pari-Jet Air nebulizer or the Sonik LDI nebulizer. The nebulized antibody, in a spray form, was collected from the mouth piece of the nebulizer (e.g., F1D2F), and different sizes of mouth pieces (e.g., F1D2C1-C5) were used. The SDS-PAGE and HPLC analyses demonstrated the integrity of the nebulized antibody through various sizes of the spray mouth piece, as shown by the purity of the samples analyzed.

References

All publications and patents mentioned herein, are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

Samsung Bioepis Exhibit No. 1002
Page 5237

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the application described herein. Such equivalents are intended to be encompassed by the following claims.

Samsung Bioepis Exhibit No. 1002
Page 5238

We claim:

1. A method for preventing or treating a pulmonary condition in a subject comprising administering to the subject a therapeutically effective amount of an antibody that inhibits activation of the complement cascade and an additional active agent.

2. The method of claim 1, wherein the pulmonary condition is selected from the group consisting of: asthma, bronchitis, a chronic obstructive pulmonary disease (COPD) interstitial lung diseases, lung malignancies, α-1 anti-trypsin deficiency, emphysema, bronchiectasis, bronchiolitis obliterans, sarcoidosis, pulmonary fibrosis, and collagen vascular disorders.

3. The method of claim 1, wherein the subject is a human.

4. The method of claim 1, wherein the antibody is specific to C5.

5. The method of claim 1, wherein the antibody is specific to C5 convertase.

6. The method of claim 1, wherein the antibody is specific to C5a or C5b-9.

7. The method of claim 1, wherein the antibody is eculizumab or pexelizumab.

8. The method of claim 1, wherein the antibody is selected from the group consisting of: a full length immunoglobulin, a single chain antibody, a domain antibody, a Fab fragment, and an antibody having a Fab fragment and a mutated Fc portion.

9. The method of claim 7, wherein the mutated Fc portion does not activate complement.

10. The method of claim 1, wherein the antibody is administered prior to manifestation of the pulmonary condition, during manifestation of the pulmonary condition, or after manifestation of the pulmonary condition.

11. The method of claim 1, wherein the additional active agent is a corticosteroid.

12. The method of claim 1, wherein the corticosteroid is dexamethasone.

13. The method of claim 1, wherein the antibody and the additional active agent may be administered to the subject by the same delivery method or route.

Samsung Bioepis Exhibit No. 1002
Page 5239

14. The method of claim 1, wherein the antibody and the additional active agent may be administered to the subject by different delivery methods or routes.

15. An aerosol composition comprising an antibody that inhibits activation of the complement cascade, wherein the composition is suitable for preventing or treating a pulmonary condition in a subject.

16. The aerosol composition of claim 15, wherein the antibody is formulated in a composition suitable for aerosolization.

17. The aerosol composition of claim 15 further comprising an additional active agent.

18. A biopharmaceutical package comprising: a) an antibody that inhibits activation of the complement cascade; and b) a nebulizer, wherein the package is suitable for preventing or treating a pulmonary condition in a subject.

19. The biopharmaceutical package of claim 18, wherein the nebulizer is selected from the group consisting of a jet air nebulizer, an ultrasonic nebulizer, a vibrating mesh nebulizer and a shockwave nebulizer.

20. The biopharmaceutical package of claim 19 further comprising instructions for use.

21. The biopharmaceutical package of claim 19 further comprising an additional active agent.

22. A pulmonary drug delivery kit comprising: a) an inhaler; and b) an antibody formulated for inhalation delivery.

23. The kit of claim 22, wherein said inhaler is a metered dose inhaler.

24. The kit of claim 23, whereon the metered dose inhaler is a pressurized metered dose inhaler.

25. The kit of any one of claims 22 or 23, wherein said inhaler further comprises a holding chamber.

26. The kit of claim 22, wherein said inhaler is a nebulizer.

27. The kit of claim 26, wherein said nebulizer is an ultrasonic nebulizer.

Samsung Bioepis Exhibit No. 1002
Page 5240

28. The kit of claim 26, wherein said nebulizer is a jet air nebulizer.

29. The kit of claim 22, wherein said inhaler is a dry powder inhaler.

30. A pulmonary drug delivery kit comprising a) an anti C5 antibody composition comprising 1 mg/ml to 200 mg/ml; and b) a label indicating administration by nebulization.

Samsung Bioepis Exhibit No. 1002
Page 5241

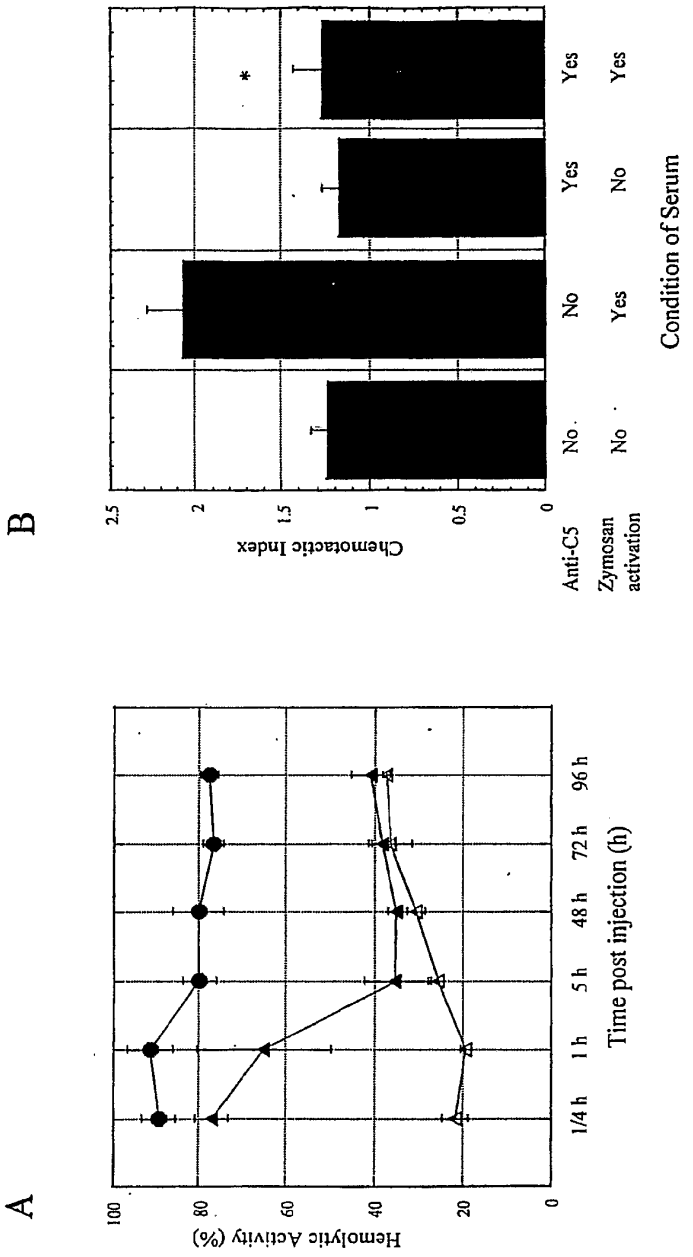

A
Hemolytic Activity (%)
100
80
60
40
20
0
1/4 h
1 h
5 h
48 h
72 h
96 h
Time post injection (h)
B
Chemotactic Index
2.5
2
1.5
1
0.5
0
*
Anti-C5
Zymosan activation
No
No
Yes
No
Yes
No
Yes
Yes
Condition of Serum

Fig. 1

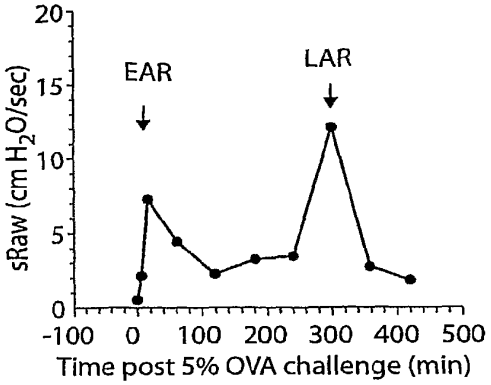


Fig. 2A

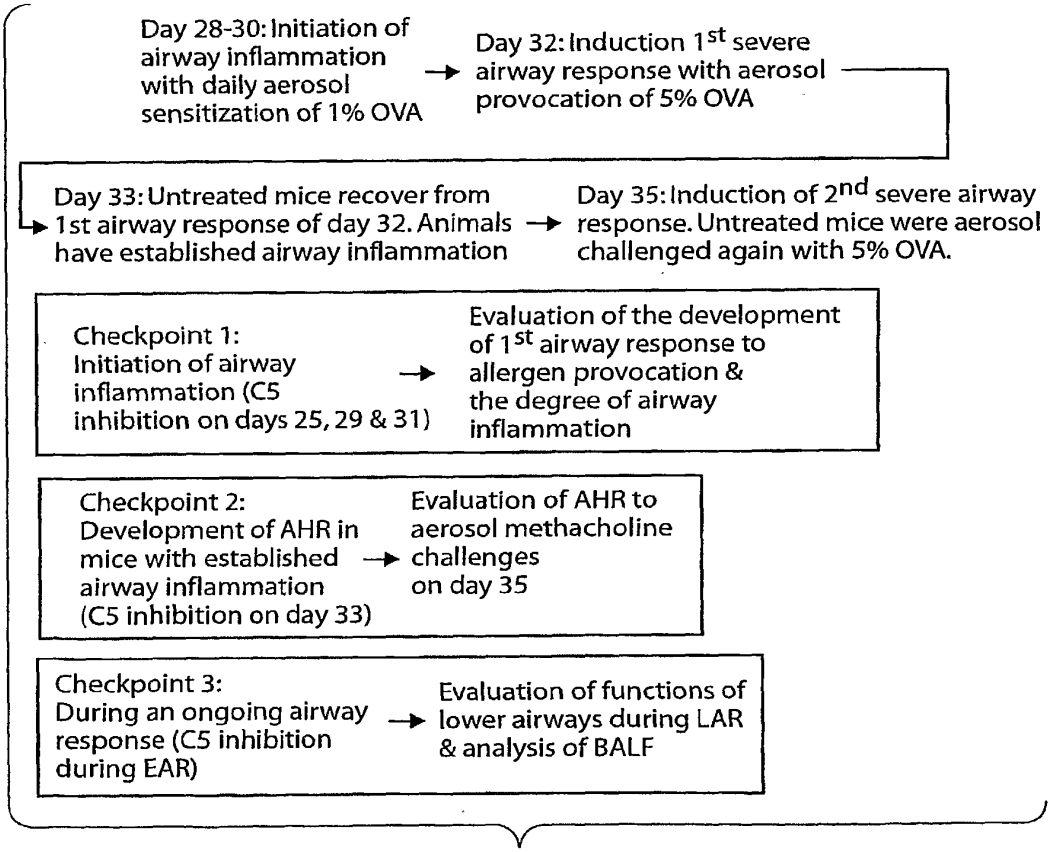


Fig. 2B

Samsung Bioepis Exhibit No. 1002
Page 5243

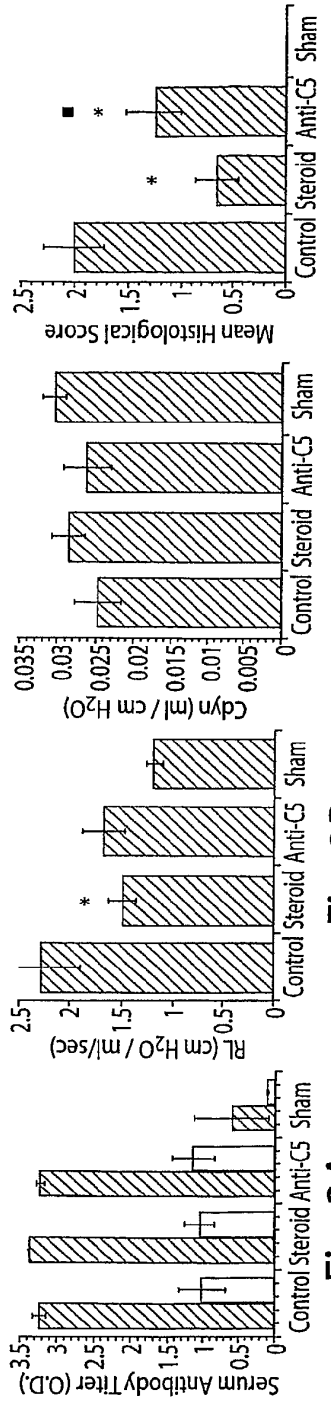


Fig. 3A

Fig. 3B

Fig. 3C

Fig. 3D



Fig. 3E

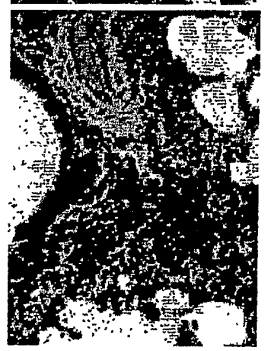


Fig. 3F



Fig. 3G



Fig. 3H

Samsung Bioepis Exhibit No. 1002
Page 5244

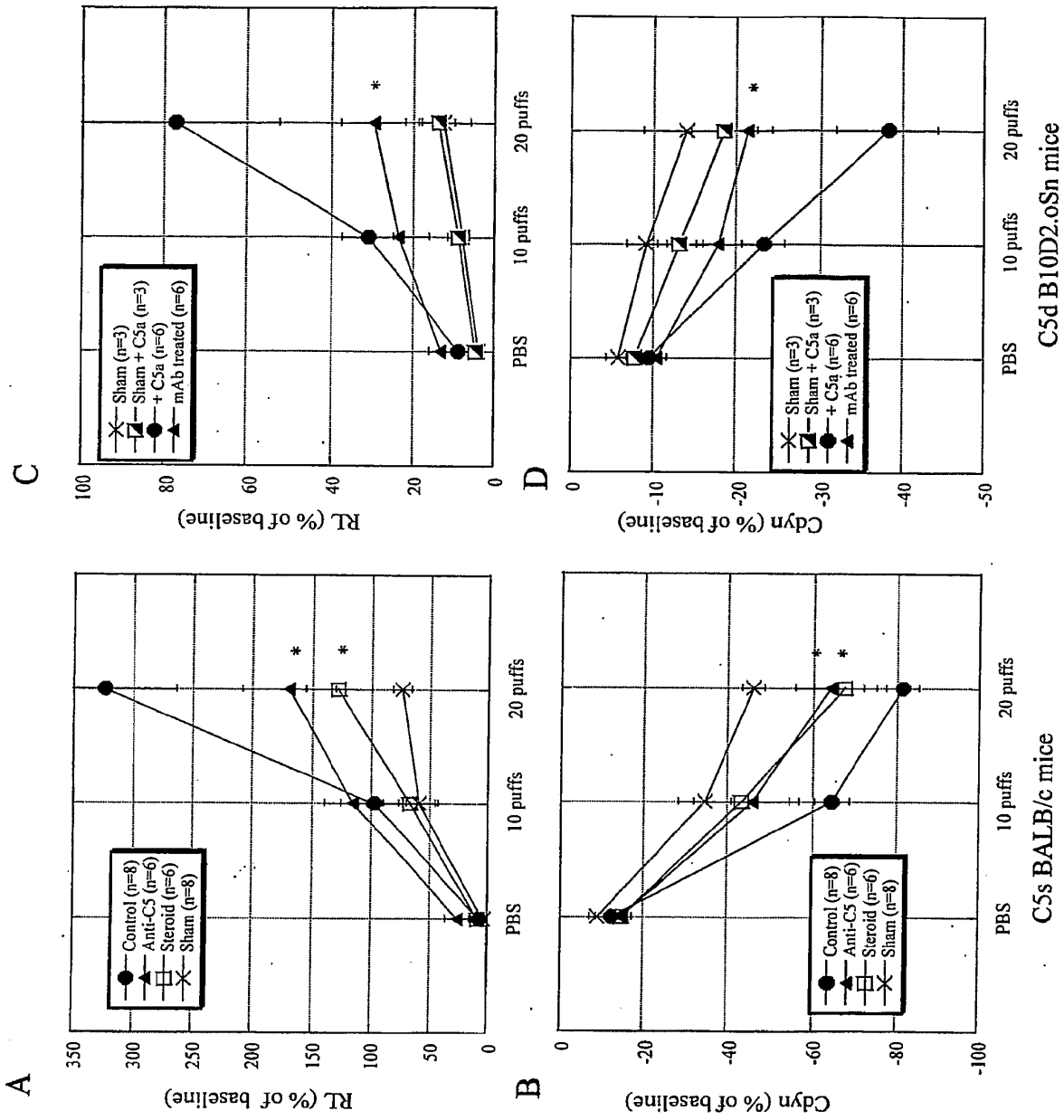

A
B
C
D
RL (% of baseline)
Cdyn (% of baseline)
PBS
10 puffs
20 puffs
Control (n=8)
Anti-C5 (n=6)
Steroid (n=6)
Sham (n=8)
Sham (n=3)
Sham + C5a (n=3)
+ C5a (n=6)
mAb treated (n=6)
C5s BALB/c mice
C5d B10D2.oSn mice

Fig. 4

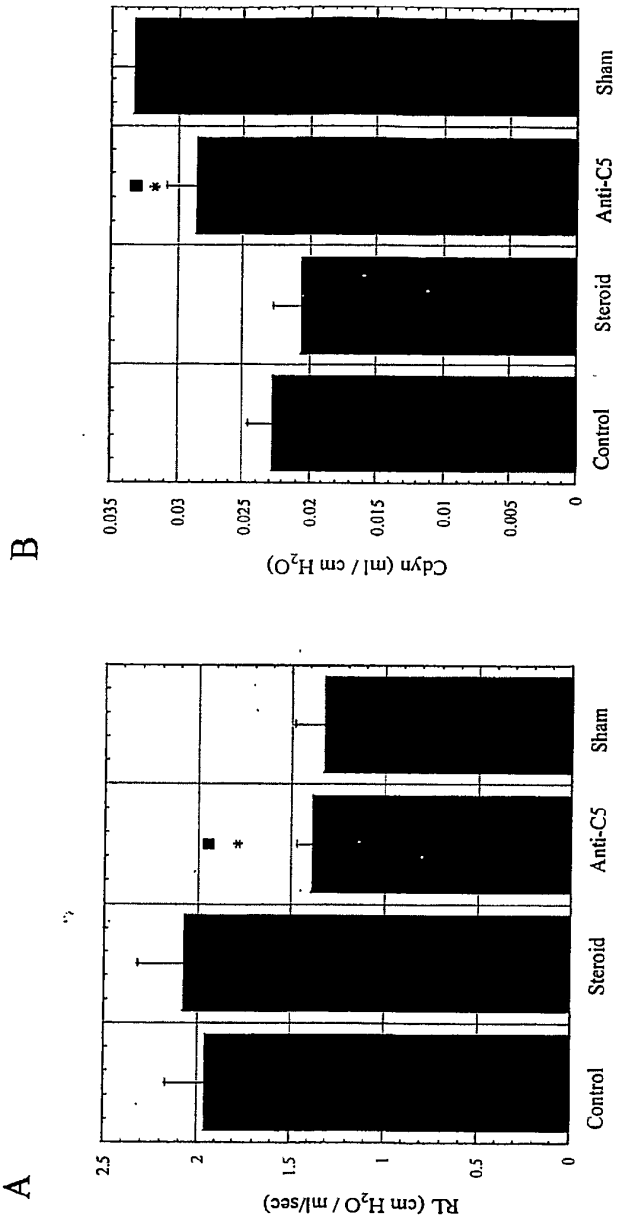

A
RL (cm H2O / ml/sec)
2.5
2
1.5
1
0.5
0
Control
Steroid
Anti-C5
Sham
*
B
Cdyn (ml / cm H2O)
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
Control
Steroid
Anti-C5
Sham
*

Fig. 5

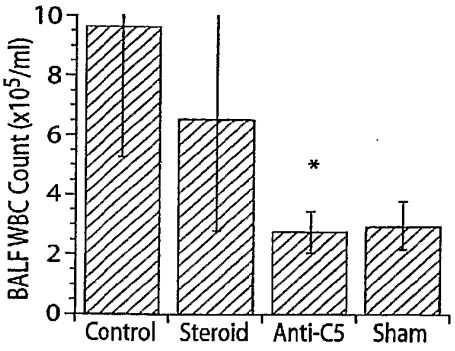


Fig. 6A

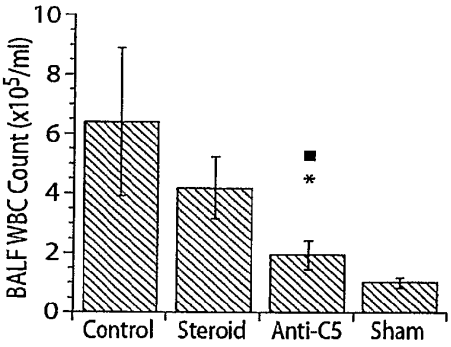


Fig. 6B

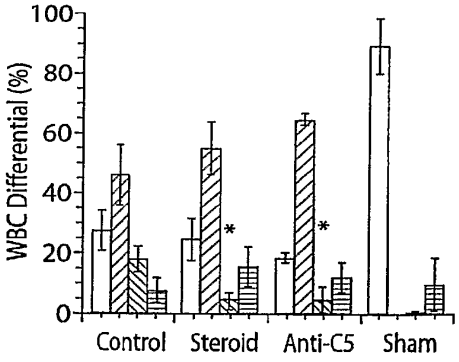


Fig. 6C

Samsung Bioepis Exhibit No. 1002
Page 5247

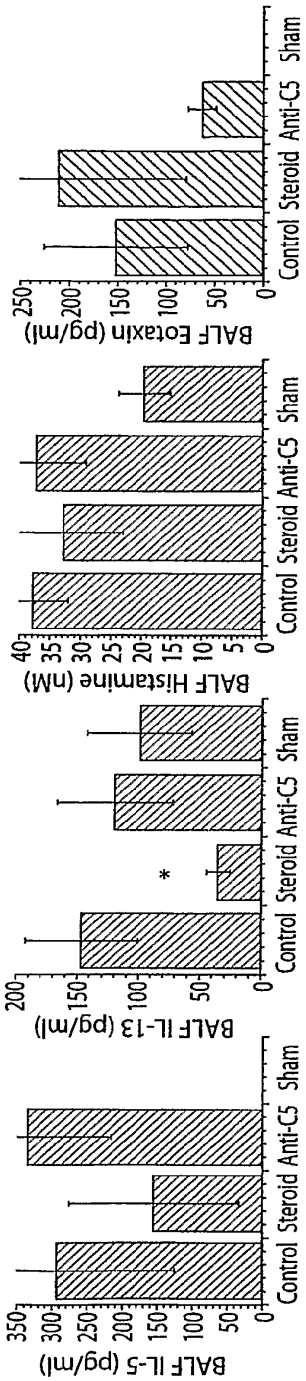


Fig. 7A

Fig. 7B

Fig. 7C

Fig. 7D

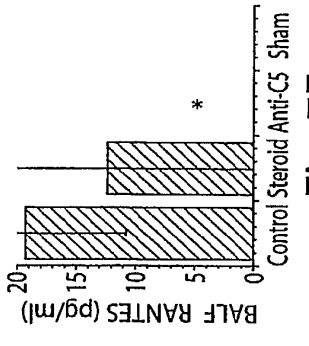


Fig. 7E

Fig. 7F

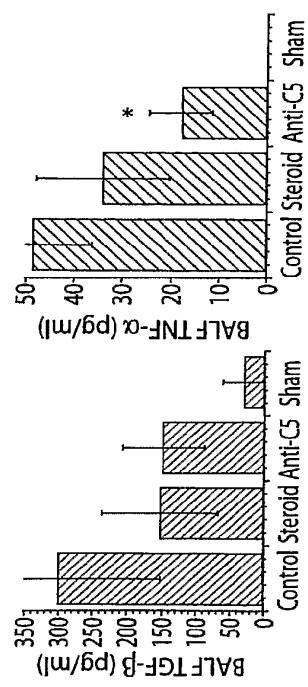


Fig. 7G

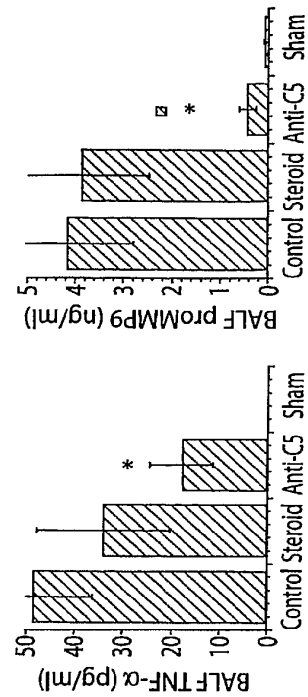


Fig. 7H

Samsung Bioepis Exhibit No. 1002
Page 5248

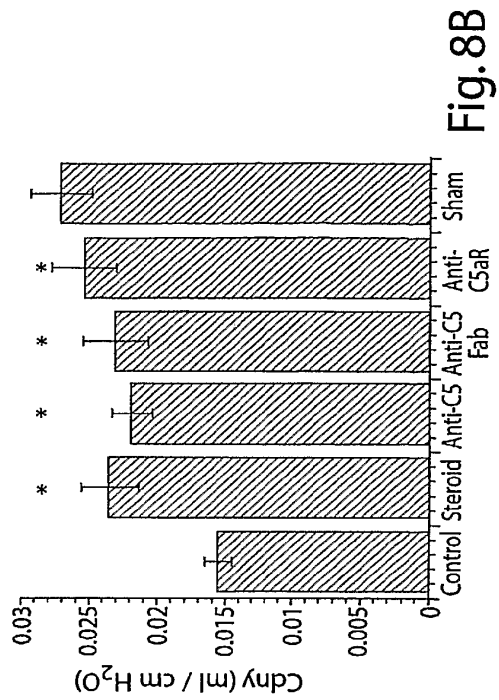


Fig. 8B

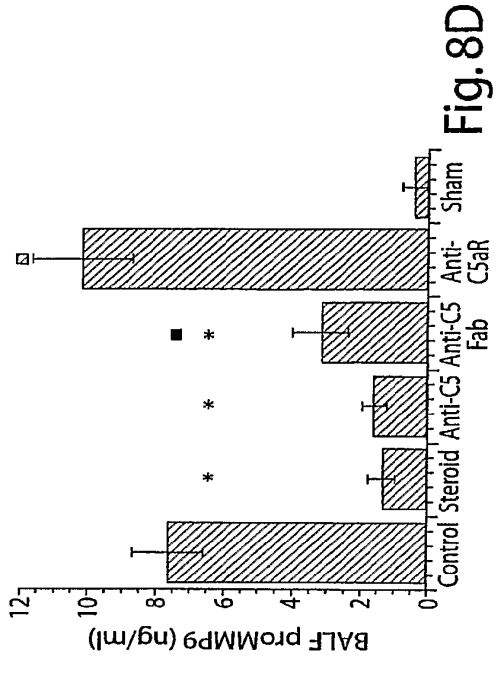


Fig. 8D

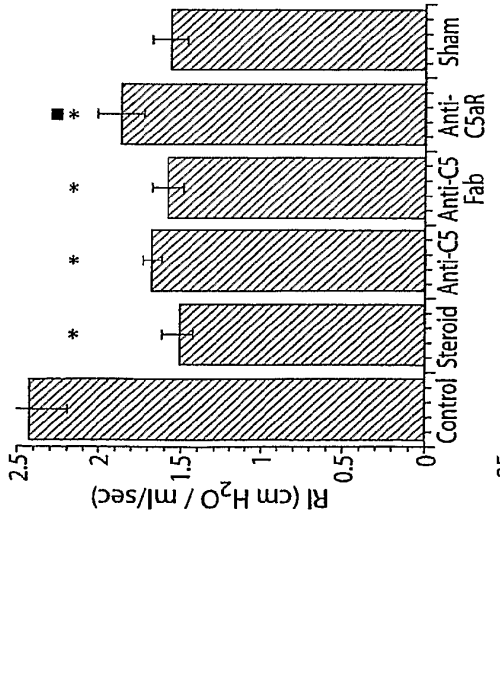


Fig. 8A

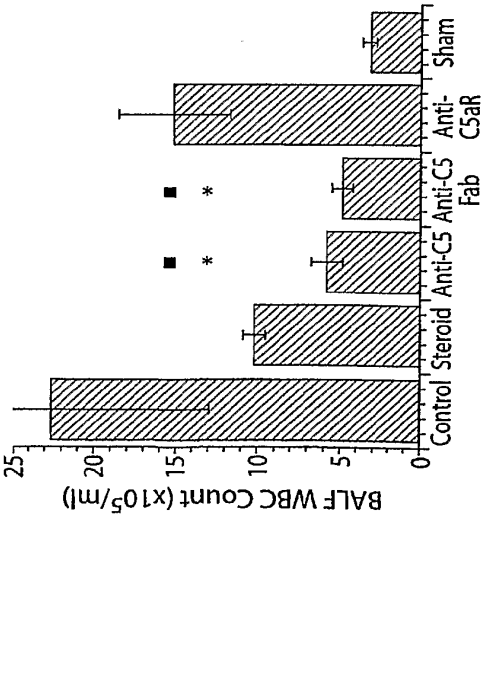


Fig. 8C

Samsung Bioepis Exhibit No. 1002
Page 5249

Fig. 9
2 days after aerosol Treatment

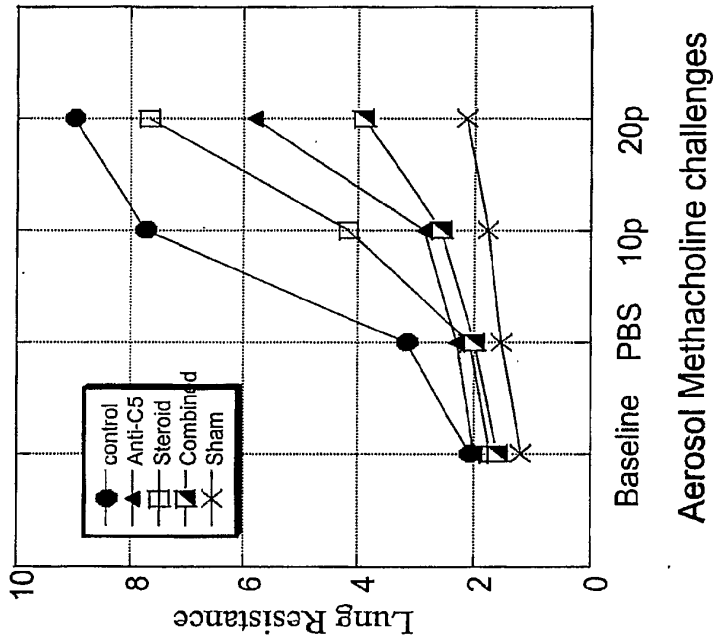


Fig. 10 Example of Suitable Formulations

Formulation 1

- Mab 1- 30 mg/ml
- 10 mM sodium phosphate
- 150 mM NaCl
- pH 6-8
- 0.001 to 0.02% Tween 80 v/v
- physiologic osmolality

Formulation 2

- 20mM Histidine
- 50mM Glycine
- 3% (w/v) Sorbital
- 1.5% (w/v) Mannitol
- pH 6-8
- 0.001 to 0.02% Tween 80
- 40mg/ml-200mg/ml
- physiologic osmolality

Fig. 11 Mch challenge two days after nebulization treatment

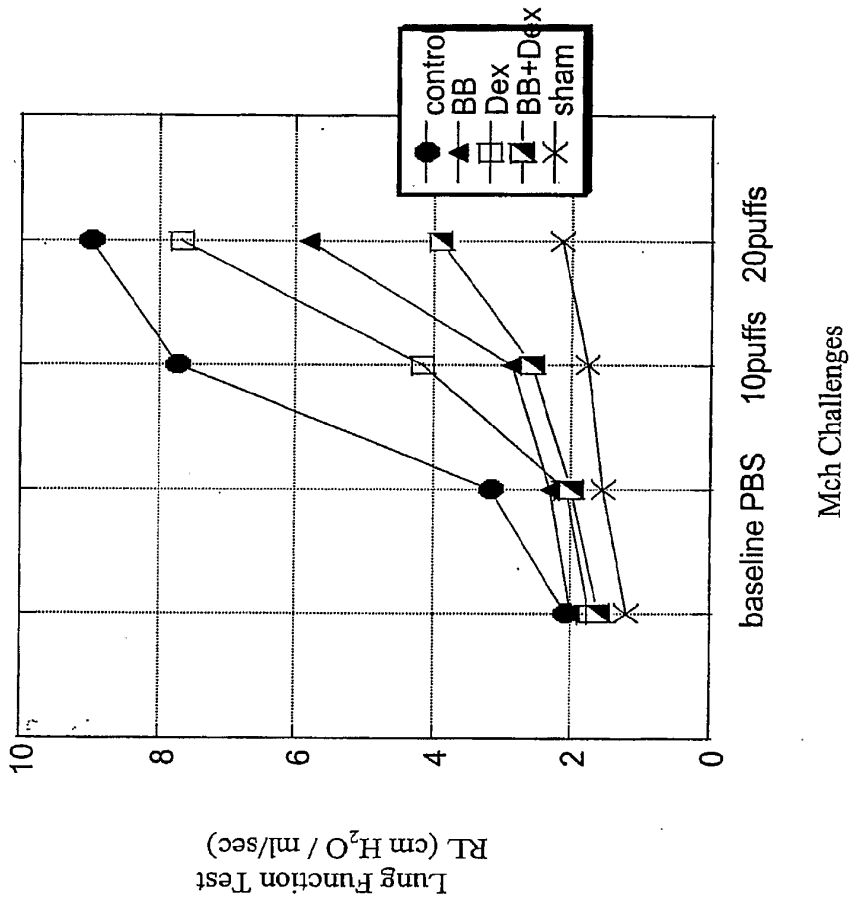


Fig. 12 Particle Size Distribution

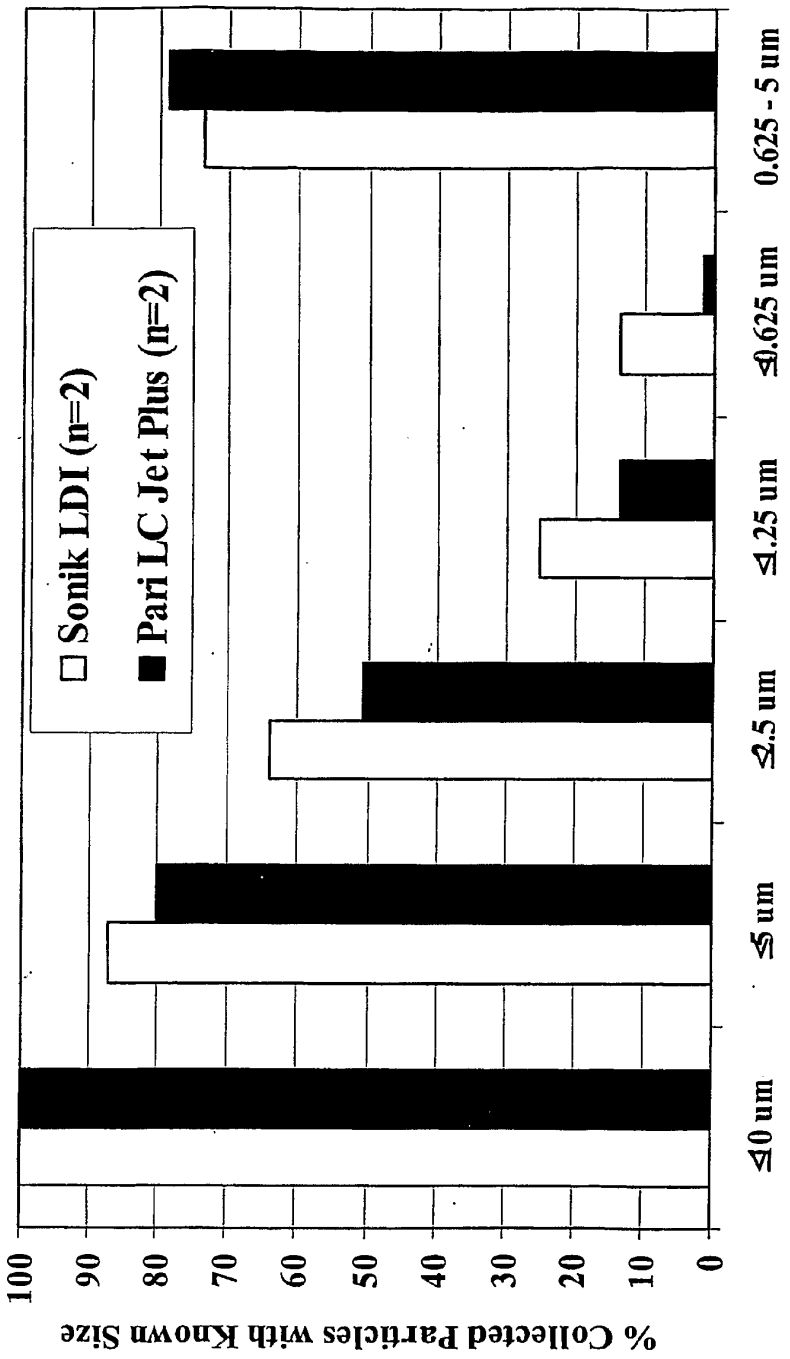


Fig. 13 Nebulization and Delivery Efficiency

	Sonik LDI Eculizumab	Pari LC Jet Plus Eculizumab	Pari LC Jet Plus Pulmozyme
Respirable fraction (%)	44.91 – 59.63	71.88 – 74.21	47.8 (1 – 5 µm)
Nebulizer efficiency (%)	11.24 – 19.91	42.51 – 52.46	51.4
Delivery (%)	6.70 – 8.94	31.55 – 37.71	24.6 (1 – 5 µm)

Respirable fraction: the fraction of the droplets delivered to the exit from the mouth piece in the range of 0.625 – 5 µm

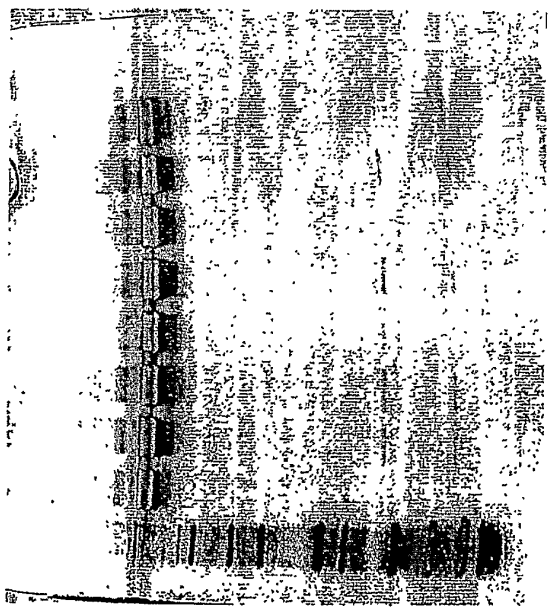
Nebulizer efficiency: the fraction of the dose initially placed in the nebulizer that reaches the exit from the mouthpiece

Delivery: the fraction of the initial dose that reaches the exit from the mouthpiece in the size range of 0.625 – 5 µm

Samsung Bioepis Exhibit No. 1002
Page 5254

Eculizumab Aerosolization

SDS-PAGE Analysis
4-20% TG, 1.5mm, 10 Well, Non Reduced
Reference NDG #1383 page 38-42
Pari-Jet Air



Lane	Sample	load (ug)	Purity(%)	% Peak Response
	MW marker	NA	NA	N/A
1	Ecu Ref Std	1.5	99.3	N/A
2	TR_CTRL-1 (30mg/mL)	1.5	99.1	102.7
3	16FEB05,F1D2C1	1.5	97.7	99.8
4	16FEB05,F1D2C2	1.5	98.5	98.6
5	16FEB05,F1D2C3	1.5	96.8	99.3
6	16FEB05,F1D2C4	1.5	94.3	94.1
7	16FEB05,F1D2C5	1.5	88.9	92.3
8	16FEB05,F1D2F	1.5	N/A	N/A
9	Blank	N/A	N/A	N/A

Fig. 14

Samsung Bioepis Exhibit No. 1002
Page 5255

Fig. 15 Eculizumab Aerosolization

SEC HPLC Purity Summaries

	15 Feb 05		16Feb 05	
	Sonik LDI	Pari Jet Air	Sonik LDI	Pari Jet Air
F1P1-W1	99.1	98.4	99.0	98.4
F1P2-W1	99.2	99.2	98.2	98.5
F1P3-W1	99.1	99.2	99.1	99.1
F1P4-W1	95.7	NA	93.8	n/a
F1C1-W1	[95.6]	97.5	94.2	97.7
F1C2-W1	[96.1]	98.2	95.8	98.5
F1C3-W1	[94.9]	96.9	[96.3]	96.8
F1C4-W1	[93.0]	94.0	95.2	94.3
F1C5-W1	[90.8]	89.3	[92.7]	88.9
[Peak responses < 90%]				

Samsung Bioepis Exhibit No. 1002
Page 5256

Fig. 16 Repeat Long Term Aerosol Administrations of Anti-C5
mAb Do Not Induce Airway Inflammation (2x per week for 10wk
Sham Treated (PBS)
Control mAb
Ant-C5 (BB5.1)

NOVARTIS

Simulect®

(basiliximab)

For Injection

Rx only

30154901

SAMPLE

WARNING

Only physicians experienced in immunosuppression therapy and management of organ transplantation patients should prescribe Simulect® (basiliximab). The physician responsible for Simulect® administration should have complete information requisite for the follow-up of the patient. Patients receiving the drug should be managed in facilities equipped and staffed with adequate laboratory and supportive medical resources.

DESCRIPTION

Simulect® (basiliximab) is a chimeric (murine/human) monoclonal antibody ($IgG_{1\kappa}$), produced by recombinant DNA technology, that functions as an immunosuppressive agent, specifically binding to and blocking the interleukin-2 receptor α-chain (IL-2Rα, also known as CD25 antigen) on the surface of activated T-lymphocytes. Based on the amino acid sequence, the calculated molecular weight of the protein is 144 kilodaltons. It is a glycoprotein obtained from fermentation of an established mouse myeloma cell line genetically engineered to express plasmids containing the human heavy and light chain constant region genes and mouse heavy and light chain variable region genes encoding the RFT5 antibody that binds selectively to the IL-2Rα.

The active ingredient, basiliximab, is water soluble. The drug product, Simulect®, is a sterile lyophilisate which is available in 6 mL colorless glass vials. Each vial contains 20 mg basiliximab, 7.21 mg monobasic potassium phosphate, 0.99 mg disodium hydrogen phosphate (anhydrous), 1.61 mg sodium chloride, 20 mg sucrose, 80 mg mannitol and 40 mg glycine, to be reconstituted in 5 mL of Sterile Water for Injection, USP. No preservatives are added.

CLINICAL PHARMACOLOGY

General

Mechanism of action: Basiliximab functions as an IL-2 receptor antagonist by binding with high affinity ($K_a = 1 \times 10^{10}\ M^{-1}$) to the alpha chain of the high affinity IL-2 receptor complex and inhibiting IL-2 binding. Basiliximab is specifically targeted against IL-2Rα, which is selectively expressed on the surface of activated T-lymphocytes. This specific high affinity binding of Simulect® (basiliximab) to IL-2Rα competitively inhibits IL-2-mediated activation of lymphocytes, a critical pathway in the cellular immune response involved in allograft rejection.

While in the circulation, Simulect® impairs the response of the immune system to antigenic challenges. Whether the ability to respond to repeated or ongoing challenges with those antigens returns to normal after Simulect® is cleared is unknown. *(See PRECAUTIONS)*

Samsung Bioepis Exhibit No. 1002
Page 5258

Pharmacokinetics

Adults: Single-dose and multiple-dose pharmacokinetic studies have been conducted in patients undergoing first kidney transplantation. Cumulative doses ranged from 15 mg up to 150 mg. Peak mean ± SD serum concentration following intravenous infusion of 20 mg over 30 minutes is 7.1 ± 5.1 mg/L. There is a dose-proportional increase in C_{max} and AUC up to the highest tested single dose of 60 mg. The volume of distribution at steady state is 8.6 ± 4.1 L. The extent and degree of distribution to various body compartments have not been fully studied. The terminal half-life is 7.2 ± 3.2 days. Total body clearance is 41 ± 19 mL/h. No clinically relevant influence of body weight or gender on distribution volume or clearance has been observed in adult patients. Elimination half-life was not influenced by age (20-69 years), gender or race. *(See DOSAGE AND ADMINISTRATION)*

Pediatric: The pharmacokinetics of Simulect® were assessed in 12 pediatric renal transplantation patients, children (2-11 years of age, n=8) and adolescents (12-15 years of age, n=4). These data indicate that in children, the volume of distribution at steady state was 5.2 ± 2.8 L, half-life was 11.5 ± 6.3 days and clearance was 17 ± 6 mL/h. Distribution volume and clearance are reduced by about 50% compared to adult renal transplantation patients. Disposition parameters were not influenced to a clinically relevant extent by age, body weight (9-37 kg) or body surface area (0.44-1.20 m^2) in this age group. In adolescents, the volume of distribution at steady state was 10.1 ± 7.6 L, half-life was 7.2 ± 3.6 days and clearance was 45 ± 25 mL/h. Disposition in adolescents was similar to that in adult renal transplantation patients. *(See DOSAGE AND ADMINISTRATION)*

Pharmacodynamics

Complete and consistent binding to IL-2Rα in adults is maintained as long as serum Simulect® levels exceed 0.2 µg/mL. As concentrations fall below this threshold, the IL-2Rα sites are no longer fully bound and the number of T-cells expressing unbound IL-2Rα returns to pretherapy values within 1-2 weeks. The relationship between serum concentration and receptor saturation was assessed in two pediatric patients (2 and 12 years of age) and was similar to that characterized in adult renal transplantation patients. *In vitro* studies using human tissues indicate that Simulect® binds only to lymphocytes.

At the recommended dosing regimen, the mean ± SD duration of basiliximab saturation of IL-2Rα was 36 ± 14 days *(See DOSAGE AND ADMINISTRATION)*. The duration of clinically significant IL-2 receptor blockade after the recommended course of Simulect® is not known. No significant changes to circulating lymphocyte numbers or cell phenotypes were observed by flow cytometry. Cytokine release syndrome has not been reported after Simulect® administration.

Clinical Studies

The safety and efficacy of Simulect® for the prophylaxis of acute organ rejection in adults following first cadaveric- or living-donor renal transplantation were assessed in two randomized, double-blind, placebo-controlled, multicenter trials. These studies compared two 20 mg doses of Simulect® with placebo when each was administered intravenously as part of a standard immunosuppressive regimen comprised of cyclosporine for microemulsion and corticosteroids, administered starting on Day 0, to prevent acute renal allograft rejection. The first dose of Simulect® or placebo was administered within 2 hours prior to transplantation surgery (Day 0) and the second dose administered on Day 4 post-transplantation. The regimen of Simulect® was chosen to provide 30-45 days of IL-2Rα saturation. 729 patients were enrolled in the two studies, of which 363 Simulect®-treated patients and 358 placebo-treated patients underwent transplantation. One study was conducted at 21 sites in Europe and Canada (EU/CAN Study); the second was conducted at 21 sites in the USA (US Study). Patients 18-75 years of age undergoing first cadaveric (EU/CAN and US Studies) or living-donor (US only) renal transplantation, with ≥1 HLA mismatch, were enrolled.

Samsung Bioepis Exhibit No. 1002
Page 5259

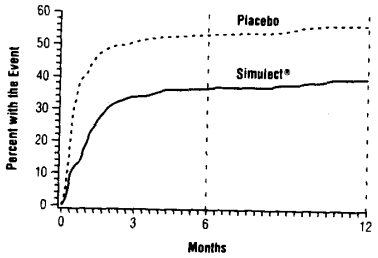
The primary efficacy endpoint in both studies was the incidence of death, graft loss or an episode of acute rejection during the first 6 months post-transplantation. Secondary efficacy endpoints included the primary efficacy variable measured during the first 12 months post-transplantation, the incidence of biopsy-confirmed acute rejection during the first 6 and 12 months post-transplantation, and patient survival and graft survival, each measured at 12 months post-transplantation. Table 1 summarizes the results of these studies. Figure 1 displays the Kaplan-Meier estimates of the percentage of patients by treatment group experiencing the primary efficacy endpoint during the first 12 months post-transplantation for the US study. Patients in both studies receiving Simulect® experienced a significantly lower incidence of biopsy-confirmed rejection episodes at both 6 and 12 months post-transplantation. There was no difference in the rate of delayed graft function, patient survival, or graft survival between Simulect®-treated patients and placebo-treated patients in either study.

There was no evidence that the clinical benefit of Simulect® was limited to specific subpopulations based on age, gender, race, donor type (cadaveric or living-donor allograft) or history of diabetes mellitus.

Table 1
Efficacy Parameters (Percentage of Patients)

	EU/CAN Study			US Study		
	Placebo (N=185)	Simulect® (N=190)	p-value	Placebo (N=173)	Simulect® (N=173)	p-value
Primary endpoint						
Death, graft loss or acute rejection episode (0-6 months)	57%	42%	0.003	55%	38%	0.002
Secondary endpoints						
Death, graft loss or acute rejection episode (0-12 months)	60%	46%	0.007	58%	41%	0.001
Biopsy-confirmed rejection episode (0-6 months)	44%	30%	0.007	46%	33%	0.015
Biopsy-confirmed rejection episode (0-12 months)	46%	32%	0.005	49%	35%	0.009
Patient survival (12 months)	97%	95%	0.29	96%	97%	0.56
Patients with functioning graft (12 months)	87%	88%	0.70	93%	95%	0.50

Figure 1
Kaplan-Meier Estimate of the Percentage of Subjects with Death, Graft Loss or First Rejection Episode
Month: 0–12



Samsung Bioepis Exhibit No. 1002
Page 5260

INDICATIONS AND USAGE
Simulect® (basiliximab) is indicated for the prophylaxis of acute organ rejection in patients receiving renal transplantation when used as part of an immunosuppressive regimen that includes cyclosporine and corticosteroids.

CONTRAINDICATIONS
Simulect® (basiliximab) is contraindicated in patients with known hypersensitivity to basiliximab or any other component of the formulation. See composition of Simulect® under *DESCRIPTION.*

WARNINGS: *See Boxed WARNING.*
General
Simulect® (basiliximab) should be administered under qualified medical supervision. Patients should be informed of the potential benefits of therapy and the risks associated with administration of immunosuppressive therapy. Anaphylactoid reactions following the administration of Simulect® have not been observed but can occur following the administration of proteins. Medications for the treatment of severe hypersensitivity reactions should be available for immediate use.

While neither the incidence of lymphoproliferative disorders nor opportunistic infections was higher in Simulect®-treated patients than in placebo-treated patients, patients on immunosuppressive therapy are at increased risk for developing these complications and should be monitored accordingly.

PRECAUTIONS
General
It is not known whether Simulect® (basiliximab) use will have a long-term effect on the ability of the immune system to respond to antigens first encountered during Simulect®-induced immunosuppression.

Re-administration of Simulect® after an initial course of therapy has not been studied in humans. The potential risks of such re-administration, specifically those associated with immunosuppression and/or the occurrence of anaphylaxis/anaphylactoid reactions, are not known.

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Samsung Bioepis Exhibit No. 1002
Page 5261

Immunogenicity
Of renal transplantation patients treated with Simulect® (basiliximab) and tested for anti-idiotype antibodies, 1/246 developed an anti-idiotype antibody response, with no deleterious clinical effect upon the patient. In the US Study, the incidence of human anti-murine antibody (HAMA) in renal transplantation patients treated with Simulect® was 2/138 in patients not exposed to muromonab-CD3 and 4/34 in patients who subsequently received muromonab-CD3. The available clinical data on the use of muromonab-CD3 in patients previously treated with Simulect® suggest that subsequent use of muromonab-CD3 or other murine antilymphocytic antibody preparations is not precluded.

Drug Interactions
No formal drug-drug interaction studies have been conducted. The following medications have been administered in clinical trials with Simulect® (basiliximab) with no incremental increase in adverse reactions: ATG/ALG, azathioprine, corticosteroids, cyclosporine, mycophenolate mofetil, and muromonab-CD3.

Carcinogenesis, Mutagenesis and Impairment of Fertility
No mutagenic potential of Simulect® was observed in the *in vitro* assays with Salmonella (Ames) and V79 Chinese hamster cells. No long-term or fertility studies in laboratory animals have been performed to evaluate the potential of Simulect® to produce carcinogenicity or fertility impairment, respectively.

Pregnancy Category B
There are no adequate and well-controlled studies in pregnant women. No maternal toxicity, embryotoxicity, or teratogenicity was observed in cynomolgus monkeys 100 days post coitum following dosing with basiliximab during the organogenesis period; blood levels in pregnant monkeys were 13-fold higher than those seen in human patients. Immunotoxicology studies have not been performed in the offspring. Because IgG molecules are known to cross the placental barrier, because IL-2 receptor may play an important role in development of the immune system, and because animal reproduction studies are not always predictive of human response, Simulect® should only be used in pregnant women when the potential benefit justifies the potential risk to the fetus. Women of childbearing potential should use effective contraception before beginning Simulect® therapy, during therapy, and for 2 months after completion of Simulect® therapy.

Nursing Mothers
It is not known whether Simulect® is excreted in human milk. Because many drugs including human antibodies are excreted in human milk, and because of the potential for adverse reactions, a decision should be made to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.

Pediatric Use
No adequate and well-controlled studies have been completed in pediatric patients. In an ongoing safety and pharmacokinetic study, pediatric patients [2-11 years of age (n=8), 12-15 years of age (n=4), median age 9.5 years] were treated with Simulect® via intravenous bolus injection in addition to standard immunosuppressive agents including cyclosporine, corticosteroids, azathioprine, and mycophenolate mofetil. Preliminary results indicate that 16.7% (2/12) of patients had experienced an acute rejection episode by 3 months post-transplantation. The most frequently reported adverse events were fever and urinary tract infections (41.7% each). Overall, the adverse event profile was consistent with general clinical experience in the pediatric renal transplantation population and with the profile in the controlled adult renal transplantation studies. The available pharmacokinetic data in children and adolescents are described in *CLINICAL PHARMACOLOGY and DOSAGE AND ADMINISTRATION.*

It is not known whether the immune response to vaccines, infection, and other antigenic stimuli administered or encountered during Simulect® therapy is impaired or whether such response will remain impaired after Simulect® therapy

Samsung Bioepis Exhibit No. 1002
Page 5262

Geriatric Use
Controlled clinical studies of Simulect® have included a small number of patients 65 years and older (Simulect® 15; placebo 19). From the available data comparing Simulect®- and placebo-treated patients, the adverse event profile in patients ≥65 years of age is not different from patients <65 years of age and no age-related dosing adjustment is required. Caution must be used in giving immunosuppressive drugs to elderly patients.

ADVERSE REACTIONS
The incidence of adverse events for Simulect® (basiliximab) was determined in two randomized comparative double-blind trials for the prevention of renal allograft rejection. A total of 721 patients received renal allografts, of which 363 received Simulect® and 358 received placebo. All patients received concomitant cyclosporine for microemulsion and corticosteroids.

Simulect® did not appear to add to the background of adverse events seen in organ transplantation patients as a consequence of their underlying disease and the concurrent administration of immunosuppressants and other medications. Adverse events were reported by 99% of the patients in the placebo-treated group and 99% of the patients in the Simulect®-treated group. Simulect® did not increase the incidence of serious adverse events observed compared with placebo. The most frequently reported adverse events were gastrointestinal disorders, reported in 75% of Simulect®-treated patients and 73% of placebo-treated patients.

The incidence and types of adverse events were similar in Simulect®-treated and placebo-treated patients. The following adverse events occurred in ≥10% of Simulect®-treated patients: ***Gastrointestinal System:*** constipation, nausea, diarrhea, abdominal pain, vomiting, dyspepsia, moniliasis; ***Metabolic and Nutritional:*** hyperkalemia, hypokalemia, hyperglycemia, hyperuricemia, hypophosphatemia, hypocalcemia, weight increase, hypercholesterolemia, acidosis; ***Central and Peripheral Nervous System:*** headache, tremor, dizziness; ***Urinary System:*** dysuria, increased non-protein nitrogen, urinary tract infection; ***Body as a Whole-General:*** pain, peripheral edema, edema, fever, viral infection, leg edema, asthenia; ***Cardiovascular Disorders-General:*** hypertension; ***Respiratory System:*** dyspnea, upper respiratory tract infection, coughing, rhinitis, pharyngitis; ***Skin and Appendages:*** surgical wound complications, acne; ***Psychiatric:*** insomnia; ***Musculoskeletal System:*** leg pain, back pain; ***Red Blood Cell:*** anemia.

The following adverse events, not mentioned above, were reported with an incidence of ≥3% and <10% in patients treated with Simulect® in the two controlled clinical trials: ***Body as a Whole:*** accidental trauma, chest pain, increased drug level, face edema, fatigue, infection, malaise, generalized edema, rigors, sepsis; ***Cardiovascular:*** angina pectoris, cardiac failure, chest pain, abnormal heart sounds, aggravated hypertension, hypotension; ***Nervous System:*** hypoesthesia, neuropathy, paraesthesia; ***Endocrine:*** increased glucocorticoids; ***Gastrointestinal:*** enlarged abdomen, flatulence, gastrointestinal disorder, gastroenteritis, GI hemorrhage, gum hyperplasia, melena, esophagitis, ulcerative stomatitis; ***Heart Rate and Rhythm:*** arrhythmia, atrial fibrillation, tachycardia; ***Metabolic and Nutritional:*** dehydration, diabetes mellitus, fluid overload, hypercalcemia, hyperlipemia, hypoglycemia, hypoproteinemia, hypomagnesemia; ***Musculoskeletal:*** arthralgia, arthropathy, bone fracture, cramps, hernia, myalgia; ***Nervous System:*** paraesthesia, hypoesthesia; ***Platelet and Bleeding:*** hematoma, hemorrhage, purpura, thrombocytopenia, thrombosis; ***Psychiatric:*** agitation, anxiety, depression; ***Red Blood Cell:*** polycythemia; ***Reproductive Disorders, Male:*** impotence, genital edema; ***Respiratory:*** bronchitis, bronchospasm, abnormal chest sounds, pneumonia, pulmonary disorder, pulmonary edema, sinusitis; ***Skin and Appendages:*** cyst, herpes simplex, herpes zoster, hypertrichosis, pruritus, rash, skin disorder, skin ulceration; ***Urinary:*** albuminuria, bladder disorder, hematuria, frequent micturition, oliguria, abnormal renal function, renal tubular necrosis, surgery, ureteral disorder, urinary retention; ***Vascular Disorders:*** vascular disorder; ***Vision Disorders:*** cataract, conjunctivitis, abnormal vision.

Samsung Bioepis Exhibit No. 1002
Page 5263

Incidence of Malignancies: The overall incidence of malignancies among all patients in the two 12-month controlled trials was not significantly different between the Simulect® and placebo treatment groups. Overall, lymphoma/lymphoproliferative disease occurred in 1 patient (0.3%) in the Simulect® group compared with 2 patients (0.6%) in the placebo group. Other malignancies were reported among 5 patients (1.4%) in the Simulect® group compared with 7 patients (1.9%) in patients treated with placebo.

Incidence of Infectious Episodes: Cytomegalovirus infection was reported in 14% of Simulect®-treated patients and 18% of placebo-treated patients. The rates of infections, serious infections, and infectious organisms were similar in the Simulect® and placebo treatment groups.

OVERDOSAGE

There have not been any reports of overdoses with Simulect® (basiliximab). A maximum tolerated dose has not been determined in patients. In clinical studies, Simulect® has been administered to renal transplantation patients in single doses of up to 60 mg without any associated serious adverse events.

DOSAGE AND ADMINISTRATION

Simulect® (basiliximab) is used as part of an immunosuppressive regimen that includes cyclosporine and corticosteroids. Simulect® is for central or peripheral intravenous administration only. Reconstituted Simulect® (20 mg in 5 mL) should be diluted to a volume of 50 mL with normal saline or dextrose 5% and administered as an intravenous infusion over 20 to 30 minutes.

Adult: In adult patients, the recommended regimen is two doses of 20 mg each. The first 20 mg dose should be given within 2 hours prior to transplantation surgery. The second 20 mg dose should be given 4 days after transplantation.

Pediatric: For children and adolescents from 2 up to 15 years of age, the recommended regimen is two doses of 12 mg/m^2 each, up to a maximum of 20 mg/dose. The first dose should be given within 2 hours prior to transplantation surgery. The second dose should be given 4 days after transplantation.

RECONSTITUTION OF 20 mg Simulect® (basiliximab) VIAL

To prepare the infusion solution, add 5 mL of Sterile Water for Injection, USP, using aseptic technique, to the vial containing the Simulect® (basiliximab) powder. Shake the vial gently to dissolve the powder.

The reconstituted solution is isotonic and should be diluted to a volume of 50 mL with normal saline or dextrose 5% for infusion. When mixing the solution, gently invert the bag in order to avoid foaming; DO NOT SHAKE.

Parenteral drug products should be inspected visually for particulate matter and discoloration before administration. After reconstitution, Simulect® should be a clear to opalescent, colorless solution. If particulate matter is present or the solution is colored, do not use.

Care must be taken to assure sterility of the prepared solution because the drug product does not contain any antimicrobial preservatives or bacteriostatic agents.

It is recommended that after reconstitution the solution should be used immediately. If not used immediately, it can be stored at 2°C to 8°C for 24 hours or at room temperature for 4 hours. Discard the reconstituted solution if not used within 24 hours.

No incompatibility between Simulect® and polyvinyl chloride bags or infusion sets has been observed. No data are available on the compatibility of Simulect® with other intravenous substances. Other drug substances should not be added or infused simultaneously through the same intravenous line.

HOW SUPPLIED

Simulect® (basiliximab) is supplied in a single use glass vial containing 20 mg of basiliximab. Each box contains 1 Simulect® vial (NDC 0078-0331-84). Store lyophilized Simulect® under refrigerated conditions (2°C to 8°C; 36°F to 46°F). Do not use beyond the expiration date stamped on the vial.

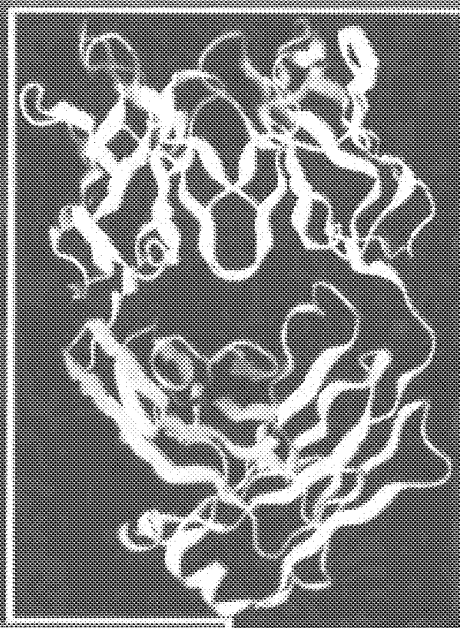
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Samsung Bioepis Exhibit No. 1002
Page 5264

Applications and Engineering of Monoclonal Antibodies



David J King

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Samsung Bioepis Exhibit No. 1002
Page 5265

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Samsung Bioepis Exhibit No. 1002
Page 5266

1

Preparation, Structure and Function of Monoclonal Antibodies

1.1 Introduction

Antibodies are proteins produced by an individual in response to the presence of a foreign molecule in the body. These foreign molecules are known as antigens, and they usually result from invading organisms such as bacteria, fungi or viruses. Antibodies bind to antigens and elicit a range of effector mechanisms to destroy the invading organism. Therefore, the generation of an antibody response is a key step in the immune system which has evolved to protect individuals from invading pathogenic organisms. However, antibodies are not restricted in specificity to pathogens, but can be formed to a huge variety of antigens including proteins, carbohydrates and organic compounds, including totally novel structures.

In 1975 Kohler and Milstein described a method for the 'production of antibodies of predefined specificity'. This technical breakthrough allowed, for the first time, the production of antibody molecules of a single specificity which could be characterised and defined. Such monoclonal antibodies immediately became valuable research tools, and applications in the diagnosis and therapy of human disease began to be widely investigated. Monoclonal antibodies (MAbs) have become increasingly important as diagnostic agents allowing precise molecular structures to be mapped and analysed. However, initial enthusiasm for their development as therapeutic agents was premature and many problems limited their use in humans (see Chapter 4). A second technical revolution has now arrived in the ability to manipulate antibody genes and to design and produce antibody molecules tailor-made for their application. Such redesigned antibody molecules are now rapidly becoming valuable reagents for therapy of human diseases as well as improved diagnostics and research reagents. The scope of these applications, and how antibody molecules are redesigned, or engineered, in the most suitable form for a particular application is the subject of this book. To understand the nature of this process requires an understanding of antibody structure and function and of how antibody specificities are generated.

Samsung Bioepis Exhibit No. 1002
Page 5267

1.2 The role of antibodies in the immune response

The immune system can be considered in two parts. Innate immunity is mediated by a variety of physical and biochemical barriers and by cells responding non-specifically to the foreign organism or molecule. Innate immunity is characterised by a similar reponse on re-exposure to the same foreign agent. Adaptive immunity, the second part of the immune system, is mediated by cells termed lymphocytes and is characterised by improved efficiency on re-exposure to the same foreign agent. It is in this system that antibodies play a major role.

Two major types of lymphocytes are involved in the adaptive immune response, T lymphocytes and B lymphocytes. T lymphocytes can be subdivided into cytotoxic T lymphocytes and 'helper' T lymphocytes. Cytotoxic T lymphocytes bind to foreign or infected cells through a surface antigen receptor, the T cell receptor, and lyse them. Helper T lymphocytes play a regulatory role in controlling the response of both T and B lymphocytes. B lymphocytes exert their effect through producing antibody molecules which bind to the foreign agent and invoke specific mechanisms for its elimination.

Antigen is recognised by B lymphocytes through the use of antibody molecules on the surface of the cell. Each B lymphocyte carries antibody molecules on its surface with a single specificity as a consequence of the rearrangement of immunoglobulin genes by the individual cell during its development in a random, antigen-independent process. Each individual will have many millions of B cells at one time thus comprising a 'polyclonal' population. When an antigen enters the body it will be recognised by any B cell which has antibody molecules able to bind to antigenic determinants (epitopes) present on the antigen. This recognition leads to activation of the cell leading to proliferation and differentiation (Figure 1.1).

In the case of protein antigens, bound antigen molecules are internalised into the B cell and degraded into peptides. Some of these antigen peptides are then bound to major histocompatibility complex (MHC) class II molecules to form a complex which is transported to the cell surface and 'presented' by the B cell. Although B cells are relatively weak at antigen presentation compared to other cell types such as dendritic cells, these complexes can be recognised by receptors present on T lymphocytes, the T cell receptors. If appropriate recognition takes place then the T cell may deliver 'help' in the form of signals back to the B cell to stimulate proliferation and differentiation. This process therefore gives rise to a 'T-cell-dependent B-cell response'.

Proliferation results in a clone of identical cells which can differentiate to form either plasma cells capable of secreting large amounts of soluble antibody of the same specificity as the original activated B cell, or memory cells which mount an accelerated immune response on re-exposure to the original antigen. The memory cell-mediated 'secondary' response results in the production of higher affinity antibodies due to hypermutation in the immunoglobulin gene loci followed by antigen driven selection, a process known as affinity maturation. This process of B cell clonal selection and the generation of antibody responses is covered in more detail in an excellent review (Rajewsky, 1996).

1.3 Structure and function of antibodies

Antibody molecules have two principal functions, firstly to bind to antigen and secondly to trigger its elimination from the body. Antibodies are therefore 'adaptor' molecules which have both the ability to bind to the antigen molecule and the ability to bind and

Samsung Bioepis Exhibit No. 1002
Page 5268

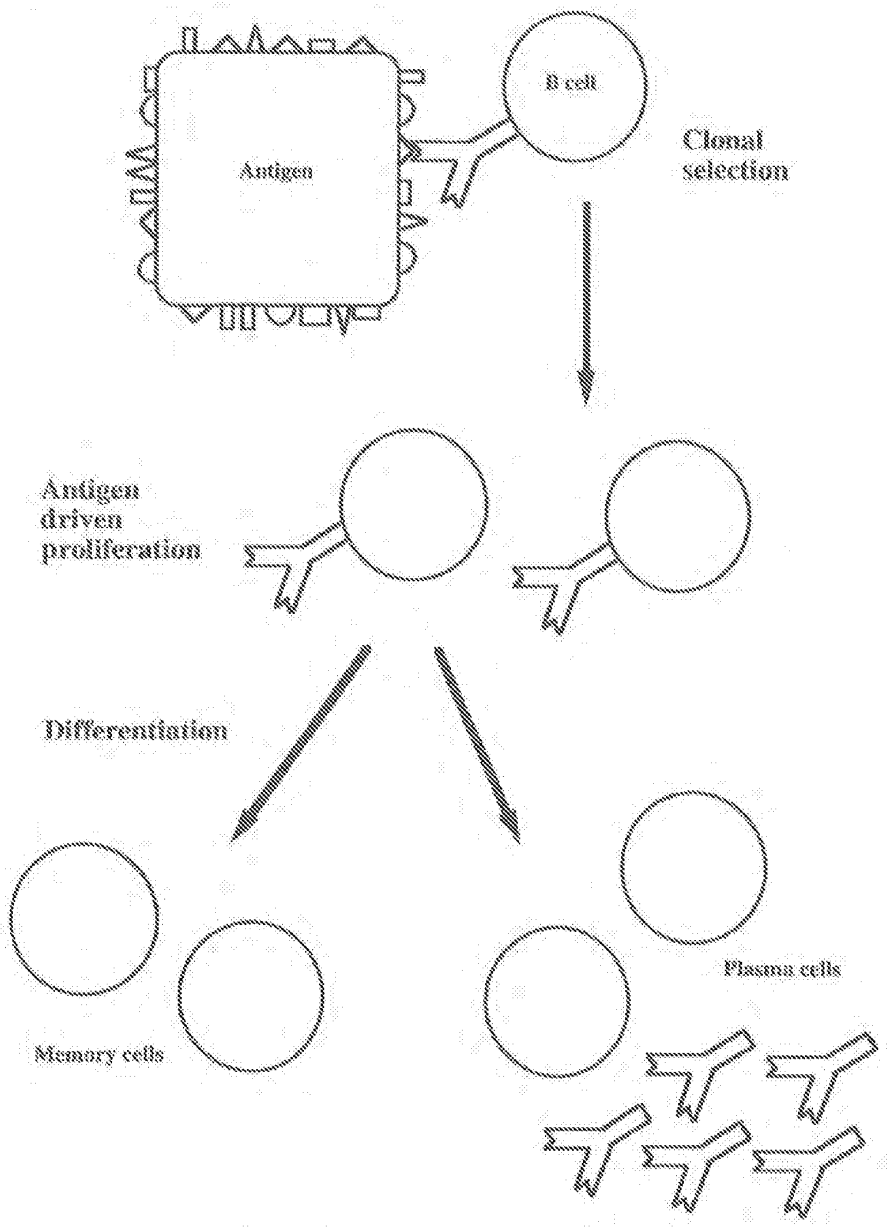


Figure 1.1 Differentiation of B cells to antibody-secreting plasma cells

bring into action molecules of the effector system which can then remove the foreign material. For effective defence systems to operate it is essential that an individual is able to recognise a wide variety of foreign material and thus antibody molecules of many different binding specificities are required. However, in each case the antibody must also

Samsung Bioepis Exhibit No. 1002
Page 5269

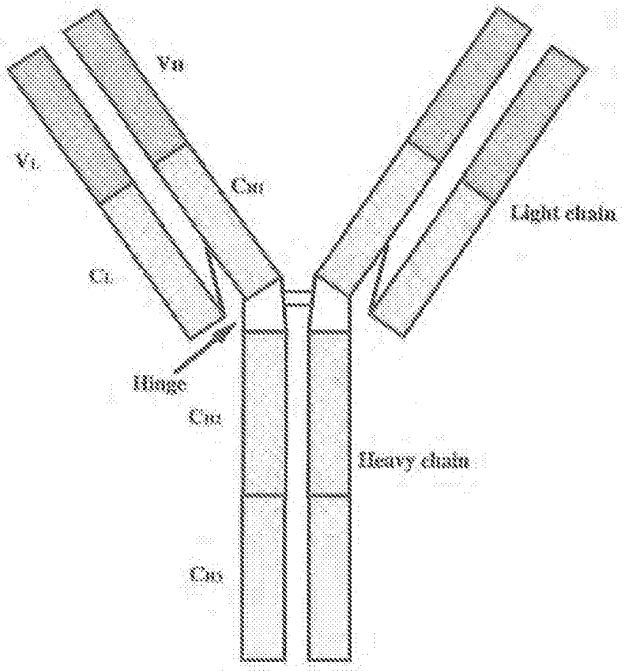


Figure 1.2 Representation of the organisation of protein chains of an IgG molecule

be able to trigger the same effector systems. To achieve this the antibody molecule has evolved variable regions which vary in protein sequence and structure to accommodate the development of binding specificities to a wide range of different antigens, and constant regions which are largely the same in each antibody. The constant regions maintain a common structure of the molecule and allow interaction with the effector systems such as complement binding and binding to Fc receptors on macrophages to activate phagocytosis.

Antibody molecules are also known as immunoglobulins. The term 'immunoglobulin' applies to the antibody protein whether or not the binding specificity of the molecule is characterised, whereas an antibody is an antigen-specific immunoglobulin. In practice the two terms are usually used interchangeably.

Higher mammals have five classes (isotypes) of immunoglobulin, termed IgG, IgM, IgA, IgE and IgD. The most abundant of these, which is used in most applications of antibodies, is the IgG class, the main class of antibody generated by the secondary immune response. The IgG molecule consists of four polypeptide chains, two heavy chains of approximately 50 kDa and two light chains of approximately 25 kDa (Figure 1.2). Each of these is divided into discretely folded structural domains of approximately 110 amino acids stabilised by an internal disulphide bond. These are linked together by short regions of comparatively flexible protein chain which allow movement of the domains relative to one another. Each light chain comprises a variable domain (V_L) and a constant domain (C_L), and each heavy chain a variable domain (V_H) and three constant domains (C_{H1}, C_{H2} and C_{H3}). The heavy and light chain variable domains associate to form the antigen-binding site. The IgG molecule thus has two antigen-binding sites and is capable of

Samsung Bioepis Exhibit No. 1002
Page 5270

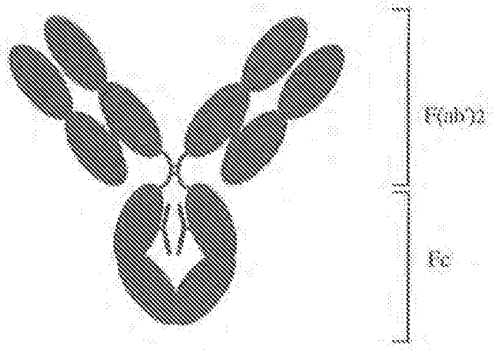


Figure 1.3 Domain structure of IgG, demonstrating the antigen-binding $F(ab')_2$ region and the Fc region with carbohydrate attached at the C_{H2} domain

binding antigens divalently to allow high binding avidity (see Section 1.5). The heavy and light chains are linked by a disulphide bond between the C_L and C_{H1} domains. A flexible region, known as the hinge, links the C_{H1} and C_{H2} domains of each heavy chain and it is at this point that the two heavy chains are linked by disulphide bonds. The C_{H2} domain is normally glycosylated and the C_{H3} domains of each heavy chain associate with each other by non-covalent interactions.

Much early work on the structure and function of antibodies made extensive use of proteolysis of the antibody protein and this has led to commonly used terminology for the different regions of the antibody (Figure 1.3). The area around the antibody hinge is more susceptible to proteolysis than the tightly folded domains and thus this is the point at which proteolytic cleavage usually takes place. Proteolysis above the disulphide bonds in the hinge region results in monovalent Fab (fragment antigen-binding) fragments which comprise light chain together with the N-terminal two domains of the heavy chain. The Fab fragment thus contains one functional antigen binding site. Proteolysis immediately below one or more of the hinge disulphide bonds results in the divalent $F(ab')_2$ fragment. The C_{H2} and C_{H3} domains together make up the Fc fragment (fragment crystalline) which contains the sites for binding effector molecules. These fragments were the starting points for crystallographic determinations of antibody structure, as the flexibility of the intact IgG molecule prevented crystallisation until fairly recently. Thus while there are more than 50 structures for Fab fragments in the literature and several for hinge deleted immunoglobulins (reviewed by Padlan, 1994) there is only one fully defined structure of an intact IgG (Harris *et al.*, 1997).

Crystallographic structure determinations of many Fab fragments and several Fc fragments of antibodies have revealed that the folded domains have similar overall structures. This structure comprises two stacked β-sheets twisted into a characteristic fold, termed the immunoglobulin fold, and stabilised by a disulphide bond (Poljak *et al.*, 1973). In the constant domains one sheet has three and the other four antiparallel beta strands, while in the variable domains there are nine strands.

The primary sequences of several thousand antibody molecules are known, representing the largest number of known sequences in one protein family. Analysis of these sequences has revealed that the variability of antibody variable domains is largely restricted to three 'hypervariable' regions in each of the heavy and light chain variable domains, with comparatively little variation in the intervening 'framework' regions (Kabat

Samsung Bioepis Exhibit No. 1002
Page 5271

et al., 1991). These hypervariable regions, also known as complementarity determining regions, or CDRs, form loops at the tip of the Fab structure making up an antigen-binding surface of approximately 2800 Å^2. Structures of several Fab:antigen complexes have confirmed that antigen binding takes place at this surface; the hypervariability of these loops is therefore responsible for the different binding specificities of different antibody molecules. The CDR loops vary in both amino acid sequence and length between antibodies. This results in the variety of antigen-binding surfaces, some of which contain grooves or clefts in the surface to accommodate antigen binding whereas others are comparatively smooth. In some cases three-dimensional structures have been determined for antibody fragments with and without bound antigen which show that the antigen-binding site is not always completely rigid and may move to allow tight antigen binding in an 'induced fit' mechanism (Bhat *et al.*, 1990; Rini *et al.*, 1992). In some cases this may also lead to some conformational changes at sites in the antibody further away from the antigen-binding region (Guddat *et al.*, 1994).

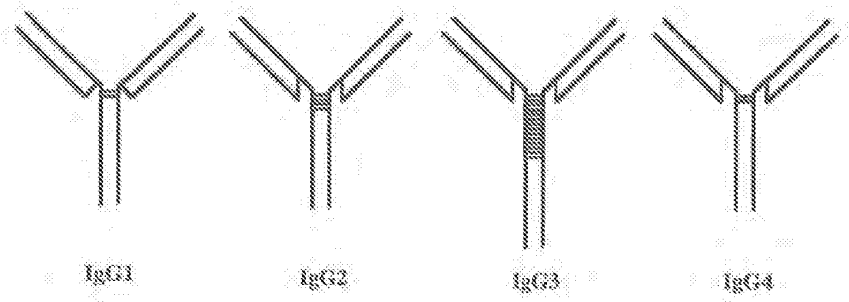
There are four different subclasses (or isotypes) of IgG, called IgG1, IgG2, IgG3 and IgG4 in humans and IgG1, IgG2a, IgG2b and IgG3 in mice. Although all within the IgG class, these subclasses vary to some extent in their structure and function. The subclasses have different heavy chains termed γ1, γ2, γ3 and γ4 in humans and each of these can use light chains from one of the two light chain isotypes, κ or λ. The structures of the subclasses vary in their pattern of disulphide bonding (Figure 1.4). The number of disulphide bonds between the heavy chains varies from two in human IgG1 and IgG4 to 15 in human IgG3. Also, the position of the disulphide bond between the heavy and light chains varies from between the C_L and C_{H1} region to between the C_L and V_H/C_{H1} interdomain region.

The antibody effector functions are mediated through the constant regions. The effector functions can be mediated through complement activation or by cellular interactions through specific Fc receptors expressed on a range of cell types, which can result in the process of antibody-dependent cellular cytotoxicity (ADCC). Complement activation via the classical complement cascade is initiated through binding of the IgG to the complement component C1q. This is subject to conformational restraints which are only partially understood, and requires two molecules of IgG bound to an antigenic surface to bind C1q efficiently. The binding site for C1q on IgG has been known for some time to be localised to the C_{H2} domain, with evidence that the N-terminal region of C_{H2} is important for binding C1q in the case of human IgG1 (Morgan *et al.*, 1995) and also that the C-terminal region of C_{H2} is required for efficient complement lysis (Tao *et al.*, 1993; Greenwood *et al.*, 1993). Mutagenesis studies have suggested that three amino acid residues on the surface of the C_{H2} domain of murine IgG2b, numbers 318, 320 and 322, are involved in the binding interaction (Duncan and Winter, 1988), although these residues are also present on antibodies which cannot activate complement, suggesting that these residues are necessary but not sufficient to activate complement. Mutagenesis studies with human IgG1 have also suggested that C1q binding alone is not sufficient for complement activation but that interactions of the IgG molecule with other steps in the complement pathway are also required (Tao *et al.*, 1993).

Fc receptors have been identified for all the classes of immunoglobulin, IgG, IgM, IgD, IgE and IgA. Of these the best characterised are those for IgG and IgE. Three types of Fc receptors have been identified for IgG in humans; FcγRI (also known as CD64), FcγRII (also known as CDw32), and FcγRIII (also known as CD16). These receptors are structurally related and distributed on various blood cell types. FcγRI is a high affinity receptor, capable of binding monomeric IgG, which plays a key role in ADCC and is

Samsung Bioepis Exhibit No. 1002
Page 5272

Human IgG



Murine IgG

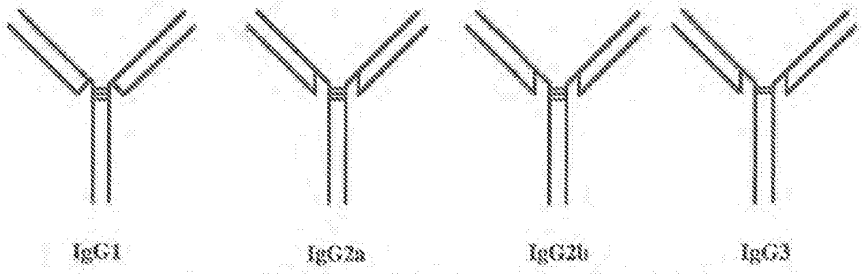


Figure 1.4 Variation in the disulphide bonds between heavy chains in IgG subclasses

found on macrophages, monocytes and neutrophils. FcγRII and FcγRIII are low affinity receptors which bind aggregated IgG. FcγRII is found on most leukocytes including monocytes, macrophages and neutrophils and FcγRIII is found on neutrophils, macrophages and NK cells. These low affinity receptors are also capable of eliciting ADCC and phagocytosis. The binding of a single antibody to Fc receptor is reversible and does not elicit a response. If several antibodies are clustered together Fc receptor clustering will take place and a cellular response will be elicited (Figure 1.5). All three Fcγ receptors recognise sites on the lower hinge/C_{H2} domain of IgG but not in an identical fashion. In particular, the sequence of heavy chain residues at positions 234–237 is involved in the FcγR1 receptor binding site (Duncan *et al.*, 1988; Sarmay *et al.*, 1992).

The ability to elicit effector functions varies between isotypes of IgG (Table 1.1), and this may reflect important differences in the functions of the individual isotypes. For example, human IgG1 and IgG3 are highly active isotypes with respect to complement activation and elicitation of ADCC responses whereas IgG2 and IgG4 are relatively inactive, being only poorly able to activate complement through C1q binding with little binding to Fc receptors.

The presence of carbohydrate attached to the C_{H2} domain has been shown to be required for both Fc receptor binding and complement activation (Tao and Morrison,

Samsung Bioepis Exhibit No. 1002
Page 5273

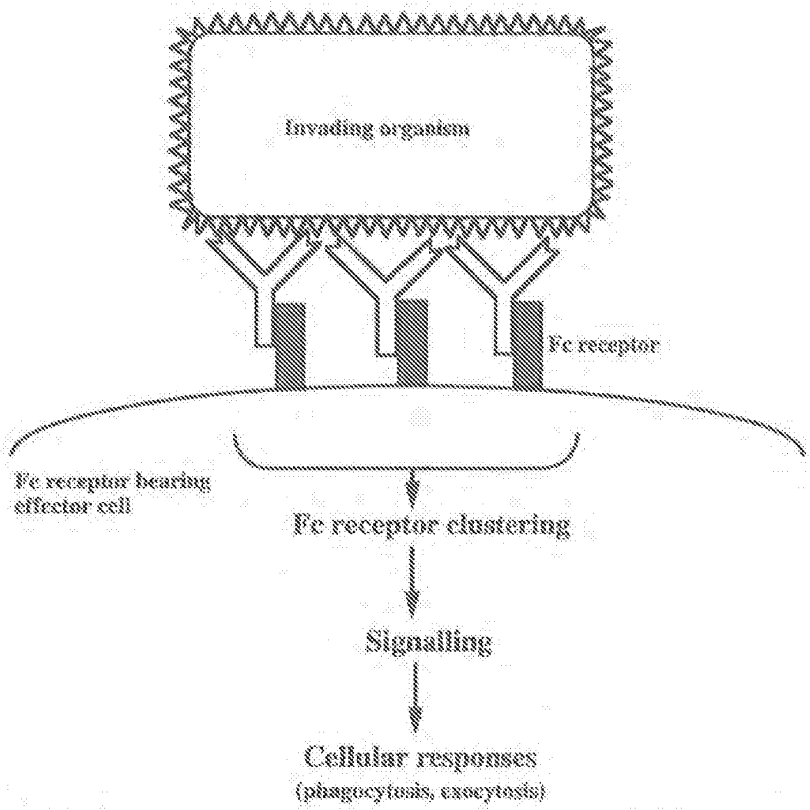


Figure 1.5 Fc receptor clustering due to multiple antibody molecules binding to antigen leading to cellular responses

Table 1.1 Effector functions of human and mouse IgG subclasses

	Human IgG				Mouse IgG			
	IgG1	IgG2	IgG3	IgG4	IgG1	IgG2a	IgG2b	IgG3
Complement activation	+++	+	++	—	+	++	+++	++
Human FcγRI (CD64)	+++	—	+++	+	—	++	—	++
Human FcγRII (CDw32)	++	+	++	—	+	+	+	—
Human FcγRIII (CD16)	++	—	++	—	—	+	+	++

Samsung Bioepis Exhibit No. 1002
Page 5274

Table 1.2 Human immunoglobulins

Antibody	Approximate molecular weight (kDa)	Heavy chain	Approximate concentration in normal human serum (mg/ml)
IgG1	146	γ1	9
IgG2	146	γ2	3
IgG3	170	γ3	1
IgG4	146	γ4	0.5
IgM	970	μ	1.5
IgA1	160	α1	3
IgA2	160	α2	0.5
IgD	200	δ	0.03
IgE	200	ε	0.0001

1989). The role of the carbohydrate is not fully understood but it is believed to be important in maintaining the tertiary structure and disposition of the C_{H2} domains. IgG without carbohydrate has been shown to have an altered conformation in the lower hinge region and may result in disruption of the interaction sites (Lund *et al.*, 1990).

IgG represents approximately 70–75% of the total immunoglobulin in human serum, the remainder being molecules of the other classes mentioned above, IgM, IgA, IgD and IgE. Although the IgG structure is a good general model for antibody structure, these immunoglobulin classes differ in their structure, reflecting differences in their normal functions. The different classes of immunoglobulin use light chains of the same type (κ or λ) and thus differ predominantly in their heavy chains (Table 1.2).

IgM is the predominant antibody raised in primary responses to many antigens and represents approximately 10% of the immunoglobulin in human serum. IgM antibodies have a pentameric structure of the basic four chain unit such that a total of ten antigen-binding sites per molecule are present (Figure 1.6). The μ heavy chain does not have a hinge region but has an extra constant region domain, which is inserted between the C_{H1} and C_{H2} domains in the analogous IgG structure, such that the C_{H2} domain of IgM is analogous to the C_{H3} domain of IgG. There is also an extra tail of 19 amino acids on the heavy chain involved in polymer assembly. The heavy chains are held together by disulphide bonds between C_{H3} domains and there is also an extra polypeptide chain, the J chain, whose role is not fully understood although it may assist the process of assembly of the pentameric IgM molecule. The μ heavy chain is also more heavily glycosylated than the γ heavy chain, with sugar attached at five glycosylation sites. Although it is presented as a pentameric structure in Figure 1.6, this is not always the case and the occurrence of IgM molecules with different numbers of binding units is now well established. The production of hexamers and tetramers has been reported (Eskeland and Christensen, 1975) and in some cases hexamers may be a predominant structure (Cattaneo and Neuberger, 1987; Davis *et al.*, 1988). This multivalent structure provides the IgM molecule with a high binding avidity, to help compensate for the relatively low affinity of each binding site. Another consequence of the multivalent structure is the ability of one molecule of IgM to activate complement, which is thought to occur via binding to the IgM C_{H2} domain. Therefore IgM molecules are well adapted for their role in the primary immune response.

Samsung Bioepis Exhibit No. 1002
Page 5275

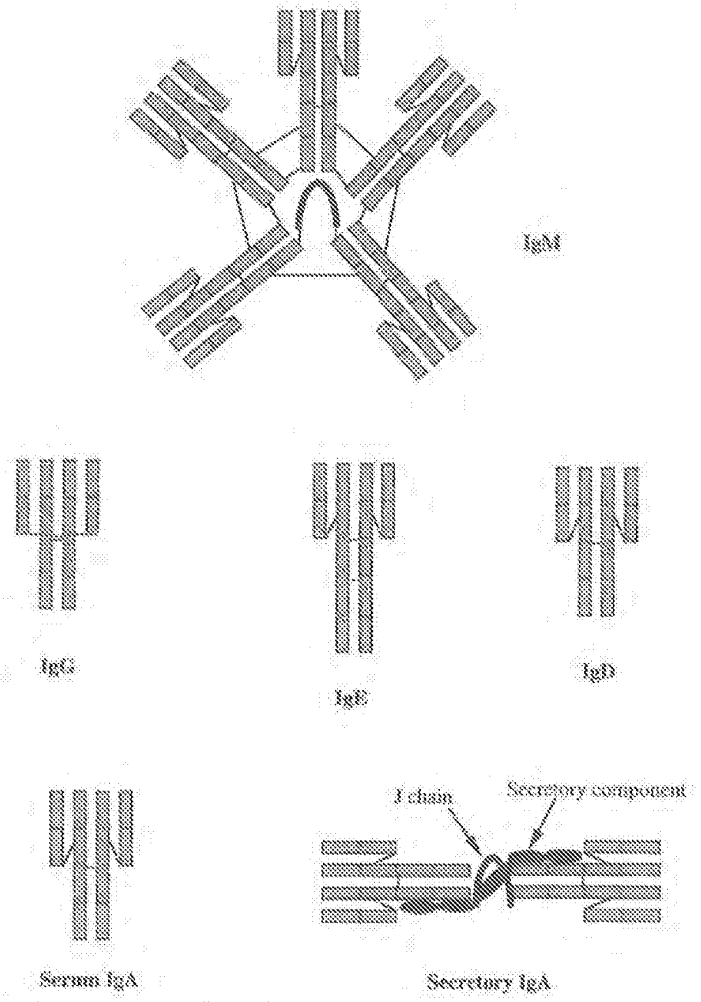


Figure 1.6 Organisation of protein chains in the immunoglobulin classes

There are two isotypes of IgA present in humans (IgA1 and IgA2) which together make up 15–20% of the total serum immunoglobulin. In addition, a form of IgA, termed secretory IgA or sIgA, is the predominant immunoglobulin present in external secretions such as saliva, milk, tears, genitourinary secretions and tracheobronchial secretions (Tomasi, 1992). The mucosal surfaces bathed in these secretions are a major site of exposure to the environment and thus secretory IgA is an important defence mechanism against invading organisms. In terms of quantity, sIgA is the major type of Ig produced, with estimates of 2g per day being produced in humans, more than other Ig forms. The IgA present in serum is largely monomeric and consists of two heavy chains and two light chains assembled in a similar manner to IgG. IgA1 is the predominant isotype, making up approximately 90% of the serum IgA. IgA heavy chains have three constant domains with IgA1 having a longer hinge region than IgA2. In addition, IgA heavy chains also

Samsung Bioepis Exhibit No. 1002
Page 5276

have a similar tail to IgM which allows polymerisation, and binding of the J chain to the α chain, through a C-terminal cysteine residue in a similar manner to IgM. sIgA exists predominantly in dimeric form with a molecular weight of approximately 385,000. sIgA also contains a secretory component which is an extra polypeptide chain of approximately 70 kDa, synthesised by epithelial cells and not the plasma cells. The secretory component is part of the receptor involved in transport of IgA into mucosal secretions which is cleaved during transport across endothelial surfaces to release the 70 kDa secretory component. This then becomes bound to the IgA by disulphide bonds (Fallgreen-Gebauer *et al*., 1993). sIgA is more stable to proteolytic attack than the serum form and part of this increased stability may result from the association of the secretory component which protects the immunoglobulin from degradation (Tomasi, 1992).

Human IgD and IgE are relatively poorly understood although the overall structures are known (Figure 1.6). IgD has been known to exist in a membrane-bound form on B cells for some time, where along with membrane-bound IgM it plays an important role in initiating the antibody response as described above. In the circulation, IgD is present at low levels, less than 1% of the circulating immunoglobulin, and may also be involved in regulation of the immune response (Roes and Rajewsky, 1993). IgD has a structure similar to IgG except that the heavy chain is more heavily glycosylated and there is only one disulphide bond between the two heavy chains even though the hinge region is extended and similar in length to that of IgG3 which has 11 inter-heavy chain disulphide bonds. IgE is present at very low levels in normal human serum although levels are raised significantly in allergic diseases and parasitic infections. IgE is believed to have evolved for protection against parasitic diseases, though in industrialised countries its role in allergy and asthma is now more important (Sutton and Gould, 1993). IgE also contains two heavy chains and two light chains in an assembled IgG-like structure. The ε heavy chain, however, contains four constant domains and multiple glycosylation sites (Figure 1.6).

1.4 The organisation of antibody genes

Antibody heavy and light chains are encoded in the genome at separate genetic loci; the two light chain loci, κ and λ, and the heavy chain locus, IgH. In humans and mice these loci are found on different chromosomes. Within each locus there are exons which encode the constant region domains and clusters of exons which are used to form the unique variable domains by each B cell. There are three families of repeated gene segments which are used to form the variable region at the heavy chain locus, variable (V), diversity (D) and joining (J), while the light chains use only V and J gene segments. During the development of the B cell a series of recombination events takes place to form the variable domain exon. In the mouse, the light chain variable region is recombined from one V out of a choice of approximately 200, and one J out of a choice of 4. The heavy chain chooses one V out of a choice of approximately 100, one D out of approximately 50 and one of 4 J region gene segments. The variable region along with the signal sequence exon (to direct secretion of the antibody) and the promoter/enhancer (involved in transcription) is then recombined with the constant region exons for subsequent expression (Figure 1.7). In this way each B cell forms a unique antibody from a total of approximately 10^{10} possible combinations. This is a random process and requires antigen itself to select out B cells producing antibodies of useful specificity (Section 1.2). Each individual mouse probably has B cells expressing about 10^8 specificities at one time. Further diversity in

Samsung Bioepis Exhibit No. 1002
Page 5277

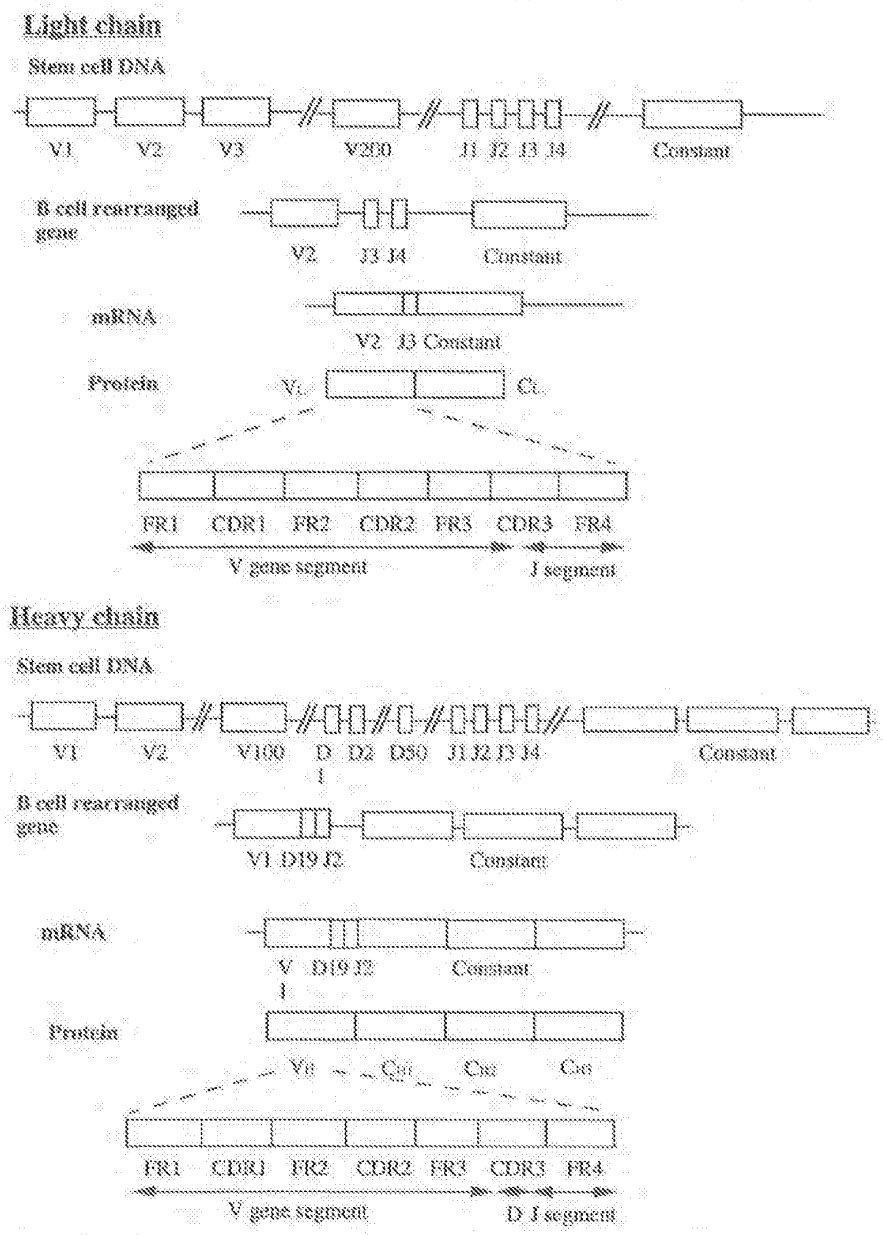


Figure 1.7 Gene organisation of IgG1, demonstrating an example of gene rearrangement to produce light and heavy chains.

the antibody genes is generated because of imprecision in joining the V, D and J regions, as well as occasional incorporation of extra nucleotides at the junctions (Lieber, 1996).

The constant region exons next to the variable regions determine the isotype of the antibody produced. For the heavy chain this is initially μ, leading to the production of

Samsung Bioepis Exhibit No. 1002
Page 5278

IgM. As the immune response progresses, B cells rearrange the genes such that other classes of immunoglobulin are produced: for example, switching the μ exons for γ1 exons to produce an IgG1. This phenomenon is known as class switching. Also antigen selects for B cells producing antibody of high binding affinity. This is achieved through hypermutation of the variable region sequences and selection of the cells producing antibody of the highest affinity. These cells then follow the same pathway as before, some differentiating into plasma cells and some into memory cells. This is the genetic basis of the process known as affinity maturation.

There is also some genetic variation in the constant region determined isotypes between individuals. These result from small variations in the constant region sequences of a particular isotype between populations, usually between different racial groups, and are known as allotypes. In humans, allotypes have been identified for IgG1, IgG2 and IgG3 as well as IgA, IgE and κ light chains.

1.5 Antigen-binding affinity and avidity

1.5.1 Affinity

A measure of the strength of the antibody:antigen interaction is essential in comparing MAbs to the same or different antigens. The strength of the antibody:antigen interaction is measured through the binding affinity. This can be considered as the sum of all the non-covalent interactions between antibody and antigen involved in the binding reaction. The antibody affinity is quantified through the association constant K_a.

For the binding reaction:

$$Ab + Ag \rightarrow AbAg\ complex$$

K_a is the equilibrium constant of this reaction, and can be determined from the equilibrium concentrations of the reactants and complex using the law of mass action:

$$K_a = \frac{[AbAg\ complex]}{[Ab]\,[Ag]}$$

The units of K_a are therefore M^{-1}.

Frequently the dissociation constant K_d of the reaction is used in preference to the association constant. The dissociation constant is the affinity constant of the reverse reaction, and is therefore given by:

$$K_d = \frac{[Ab]\,[Ag]}{[AbAg\ complex]}$$

K_d is also, of course, the reciprocal of the association constant: $K_d = 1/K_a$ and the units of K_d are M.

The popularity of K_d as a measure of antigen-binding strength stems from the determination of affinities through measuring the concentration of antibody required to complex with half of the antigen. At the 50% bound point the concentration of Ag = AbAg complex by definition. These therefore cancel each other out in the equation above, and K_d = concentration of antibody required to complex 50% of the antigen. High affinity antibodies will require a low concentration of antibody to achieve this, e.g. 10^{-9}–10^{-12} M.

In some circumstances the kinetic rate constants of the binding reaction, the binding on-rate k_{on} and the dissociation rate or off-rate k_{off} may also be important. These can be

Samsung Bioepis Exhibit No. 1002
Page 5279

considered measures of the rate of binding and dissociation whereas the overall strength of the reaction is given by K_a or K_d. The rate of the binding reaction is dependent on the concentrations of Ag and Ab and the rate constant k_{on}. Units for k_{on} are $M^{-1}\ s^{-1}$. The rate of dissociation is independent of the concentration of AbAg complex and depends only on the dissociation rate constant k_{off}. Units for k_{off} are s^{-1}. At equilibrium, by definition, the rate of binding is equal to the rate of dissociation, $k_{on} = k_{off}$.

$$k_{off}\,[\text{AbAg complex}] = k_{on}\,[\text{Ab}]\,[\text{Ag}]$$

Rearranging this equation gives:

$$\frac{k_{off}}{k_{on}} = \frac{[\text{Ab}]\,[\text{Ag}]}{[\text{AbAg complex}]}$$

Therefore the rate constants can be used to give K_a or K_d.

$$K_a = \frac{[\text{Ab}]\,[\text{Ag}]}{[\text{AbAg complex}]} = \frac{k_{off}}{k_{on}}$$

The values of k_{on} and k_{off} can be measured directly as initial rates using instrumentation such as the optical biosensor BIAcore device (see Chapter 3; Malmqvist, 1993). This allows rapid determination of K_a values without waiting for the reaction to come to equilibrium. Several methods for determination of affinity and kinetic parameters, either equilibrium-based or initial rate-based, are available and have been reviewed (Goldberg and Djavadi-Ohanliance, 1993; Malmqvist, 1993).

1.5.2 *Avidity*

The antibody affinity is a measure of the strength of binding of an individual antibody binding site to a single antigenic site. However, antibody molecules usually have more than one binding site (e.g. 2 for IgG, 10 for IgM), and many antigens contain more than one antigenic site (e.g. a cell surface or antigen immobilised on a solid support), and therefore multivalent binding may be possible. The strength with which a multivalent antibody binds antigen is termed avidity. The avidity of the antibody depends on the affinities of the individual antibody binding sites, but is greater than the binding affinity as all the antibody:antigen interactions must be broken simultaneously for the antibody to dissociate completely. Thus, although the affinities of the individual binding sites are the same, multivalency leads to a large effect on the dissociation rate resulting in high binding avidity. Avidity is also referred to as functional affinity.

1.6 Generation of monoclonal antibodies

1.6.1 *Hybridoma technology*

The techniques conventionally used for the production of MAbs are based on the original report by Kohler and Milstein (1975). This technology, known as hybridoma technology, has proved to be capable of producing rodent antibodies of predetermined specificity to a wide variety of different antigens, for example proteins, nucleic acids, carbohydrates and haptens. Since each individual B cell produces antibody of a single specificity the

Samsung Bioepis Exhibit No. 1002
Page 5280

production of antibodies of a single specificity requires isolation of individual B cells. However, B cells are not normally capable of growth in culture, and thus the production of MAbs directly from B cells is not possible. Hybridoma technology allows the production of hybrid cell lines from B cells which secrete a single, monoclonal, antibody with one binding specificity which can potentially be produced in unlimited quantitities.

The general scheme for the production of MAbs by hybridoma technology is shown in Figure 1.8. Mice are immunised by injection of the antigen to which a MAb is required. Rats are also commonly used to generate MAbs, and in some cases (particularly when antibodies to mouse antigens are required) other species have been used such as hamsters (Houiden *et al*., 1991), chickens (Nishinaka *et al*., 1991) and rabbits (Speiker-Polet *et al*., 1995). When an immune response has been raised, B cells are harvested from the rodent, usually from the spleen, and these are fused with myeloma cells by promoting fusion of the cell membranes with, for example, polyethylene glycol. Myeloma cells are derived from a mutant cell line of a B cell tumour, and cells are used which do not secrete any immunoglobulin, and which are essentially immortal and can be easily grown in culture. Fusion results in the production of immortalised hybrid cells or hybridomas. The remainder of the process is then one of isolation and propagation of hybridoma cells which have retained the ability of the B cell to produce antibody and the good growth characteristics of the myeloma cell. The process of hybridoma production can therefore be considered in three parts: immunisation, fusion and selection of the required hybridoma. Many protocols for immunisation and generation of hybridomas are available (e.g. Harlow and Lane, 1988; Donohoe *et al*., 1995), and should be referred to for practical guidance.

Immunisation of the animal to raise an immune response is of key importance. Immunogens of different molecular types can be used and they do not need to be pure materials, as a hybridoma secreting a MAb of the required specificity can be selected out later. Different molecules vary greatly in their immunogenicity and thus require different immunisation protocols for an optimal response. Factors such as the form of the antigen, number and route of immunisations, carrier, adjuvant and the species and strain of animal used need to be considered. It is important to consider the conformation of the molecule which the MAb is desired to bind to. For example, will a protein be in its native conformation or denatured? MAbs of exquisite specificity can be generated, and therefore a desired isomer or molecular form of a particular antigen might be required.

There are two important exceptions to the range of immunogens which can be used. Antibodies will not normally be raised to 'self' antigens, and small antigens (less than approximately 1000 in molecular weight) will not raise a response unless conjugated to a high molecular weight carrier protein. For this purpose proteins are used, such as bovine serum albumin or keyhole limpet haemocyanin, which are very immunogenic in rodents, easily available and readily conjugated to small molecules. The generation of high affinity antibodies normally requires a T-cell-dependent B-cell response as described in Section 1.2, and thus both T and B cell epitopes are needed to allow the memory response and class switching to take place. T cells respond to proteolytically produced peptides of the antigen presented by MHC class II molecules and thus in general non-self proteins and glycoproteins elicit high affinity responses after multiple immunisations. Without protein, carbohydrates and other non-proteinaceous materials elicit only a T cell-independent response with little or no memory or class switching.

The type of antibody produced is also dependent on the number of immunisations. If IgM is required, only one immunisation is carried out as only a primary response is required, whereas IgG antibodies require multiple injections (usually three or four) at intervals of approximately 3–4 weeks to allow an effective secondary response. Adjuvants,

Samsung Bioepis Exhibit No. 1002
Page 5281

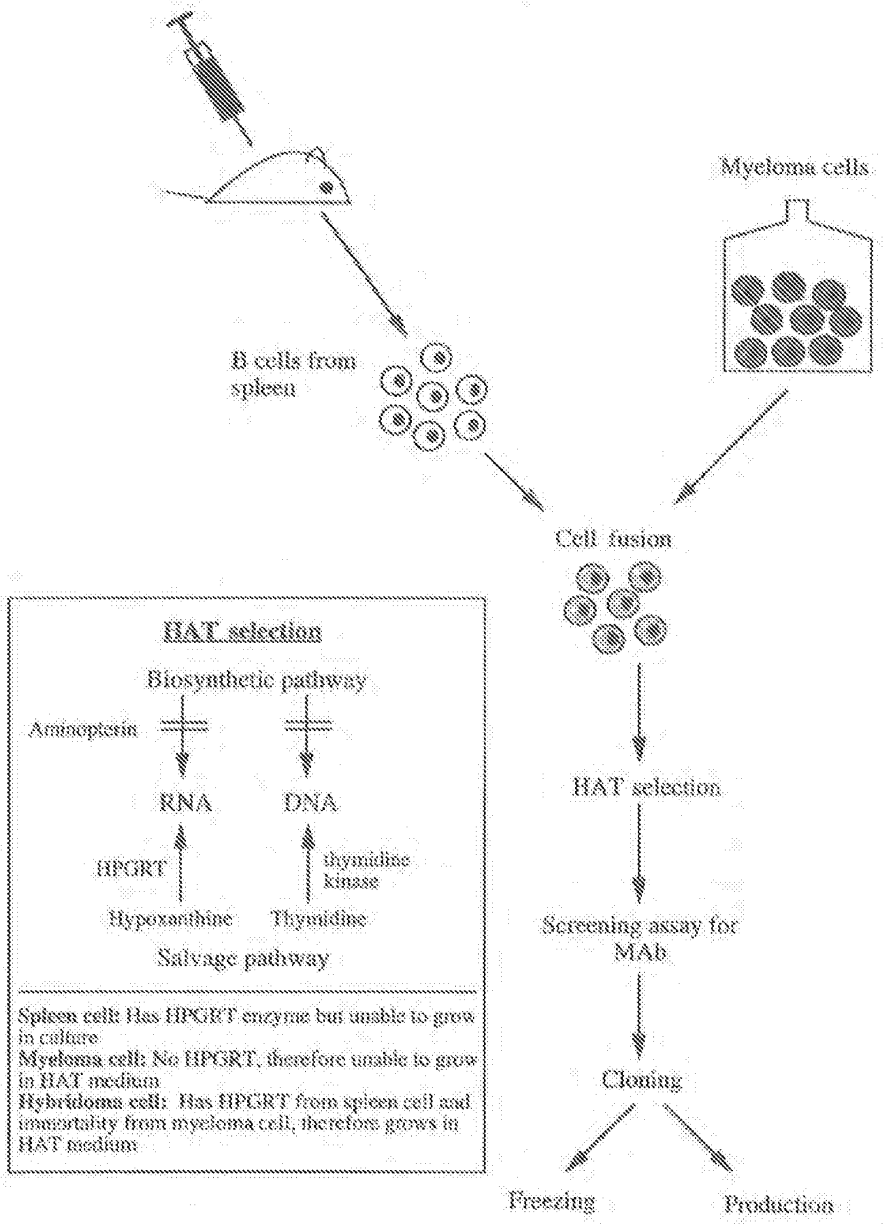


Figure 1.8 Production of monoclonal antibodies by hybridoma technology – see text for details

such as Freund's adjuvant, are widely used to aid the generation of an immune response to soluble antigens injected via the intraperitoneal, intradermal, intramuscular or subcutaneous routes which are usually used for primary immunisations (Harlow and Lane, 1988). Adjuvants are usually colloidal or oils, which are mixed with the soluble antigen to

Samsung Bioepis Exhibit No. 1002
Page 5282

produce an emulsion which is injected into the animal and provides a long-lasting local 'depot' of antigen which is slowly released and thus continually boosts the response. In addition, other components are sometimes included in the adjuvant mixture to activate the immune system non-specifically. For example, complete Freund's adjuvant contains killed mycobacteria for activation as well as oil and an emulsifying agent. Complete Freund's adjuvant is used for the first immunisation and subsequent booster immunisations are carried out in incomplete Freund's adjuvant (without the mycobacteria component) to maximise response to the antigen and avoid excessive inflammatory responses to mycobacteria. Insoluble antigens can often be used without adjuvant, as these form insoluble 'depots' on their own.

Genetic immunisation has recently been developed to allow immunisation with DNA encoding a protein of interest. The DNA is injected directly into mouse tissue, in a form which allows expression of protein from the DNA by the mouse tissue. An immune response is then generated against the protein which allows MAbs to be produced (Paddock *et al.*, 1994). This approach may be particularly useful when the protein is difficult to produce or purify sufficiently to allow direct immunisation. In addition, it may be possible to boost the immune response to poorly immunogenic proteins by producing fusion proteins with a highly immunogenic sequence.

To ensure that an immune response has been made to the antigen before harvesting B cells, blood samples can be obtained from the immunised animals and tested for the presence of specific antibodies. If positive, final boosts of soluble antigen are often made intravenously to increase the number of immune B cells present in the spleen prior to harvest. The spleen is the most common source of immune B cells, although cells can also be harvested from lymph nodes, particularly if soluble antigen is not available and a final intravenous boost cannot be given.

Several myeloma cell lines are available for fusion purposes from mice and rats. As these lines are derived from tumours of antibody-producing B cells, it is important to try to choose a line which does not produce any antibody of its own. If the resulting hybridoma produces two types of light and heavy chains these will probably associate randomly resulting in a mixture of antibody molecules produced comprising the original myeloma antibody, the desired MAb and various mixed combinations of the two types of heavy and light chains. This mixing can be advantageous for the production of bispecific antibodies (see Chapter 2), but will reduce the efficiency of the initial isolation of a MAb and make purification of the desired MAb difficult. Another requirement of the myeloma cells is that they are deficient in the enzyme hypoxanthinephosphoribosyltransferase and will allow selection of the fused hybridoma cell over the parent myeloma cell (see below).

Fusion of the myeloma cells and the harvested B cells is usually accomplished using polyethylene glycol (PEG) as the agent to induce membrane fusion. Alternative methods are also available, for example the use of Sendai virus as used in early research or electrofusion. However, PEG fusion is achieved simply, cheaply and reliably and thus has become the most common method. Initially only the cell membranes fuse and two nuclei are present in the cell. Nuclear fusion takes place during mitosis and subsequent generations of the hybridoma cell often result in loss of some chromosomes. After fusion a mixture of hybridoma cells, spleen cells and myeloma cells are present and the next stage of the process is the selection of the hybridoma cells over the other cell types. Spleen cells will not grow in culture and thus growth of the cell mixture for a few days will readily remove residual spleen cells. Myeloma cells, on the other hand, will grow rapidly and would make isolation of any hybridoma cells very difficult. For this reason myeloma cells are used for the fusion which are deficient in the enzyme

Samsung Bioepis Exhibit No. 1002
Page 5283

hypoxanthinephosphoribosyltransferase (HPRT) and consequently are not able to use the salvage pathway for RNA synthesis. Selection is then achieved by using HAT medium (Figure 1.8). HAT medium contains hypoxanthine (H), aminopterin (A) and thymidine (T). Aminopterin is an effective inhibitor of RNA and DNA synthesis and thus will block growth of the myeloma cells. However, hybrid cells which have the HPRT enzyme from the spleen cells will be able to use the added hypoxanthine and thymidine to produce RNA via the salvage pathway and survive.

After selection in HAT medium, hybrid cells are grown on and tested to determine which colonies of cells produce antibodies of interest. Not all of the spleen cells which form hybrids are B cells, and thus there will be some hybrids which do not produce antibody at all, and also many cells will be present which produce antibodies which are not of interest. An important step is therefore the screening of clones of cells to identify those which produce the MAbs required. A large number of clones may need to be screened quickly to allow selection of those required for further culture. A suitable screening assay should therefore be rapid, able to screen many samples and capable of giving a result with the small amount of antibody produced by early clones of cells. There are many types of suitable assays to measure antigen-binding properties including ELISA (enzyme linked immunoadsorbent assay), radioimmunoassay, dot blots and western blots, immunohistochemistry and immunofluorescence. In addition, it may be desirable to screen for other properties such as isotype or specific functional properties such as complement activation or the ability to elicit ADCC. If possible, the assay which uses the antibody in the closest way to that required for the intended end use of the antibody is the most suitable, as this will ensure that the optimal properties of the antibody are selected.

Once suitable hybridomas have been identified, these must be cloned several times to ensure a stable, homogeneous colony of cells and to ensure that the antibody is indeed monoclonal. To do this requires the growth of a colony from a single cell. This is achieved by subculturing the cells by limiting dilution so that each well of the culture plate contains an average of less than one cell. Alternatively cloning can be carried out by plating out cells in semisolid agar or by single cell manipulation techniques. Individual clones of cells can than be screened for antibody production as before. Once cloned, the hybridoma cell is ready to be used for the production of large amounts of antibody, but it is also important to ensure that samples of cells are preserved by freezing in liquid nitrogen at each stage so that valuable hybridomas are not lost!

1.8.2 *Human monoclonal antibodies*

The generation of human MAbs is often desirable for clinical applications. This is because murine antibodies are recognised as foreign when administered to humans and therefore elicit an immune response directed against the administered MAb: a human anti-mouse antibody response or HAMA. This prevents repeat administration of MAb as the HAMA results in formation of immune complexes which are rapidly cleared, rendering the antibody ineffective. In addition, HAMA responses may result in adverse reactions by the patient such as allergic reactions, hepatic dysfunction and in some cases anaphylactic shock. Attempts to overcome this problem have driven much research both in reconstructing rodent antibodies by genetic engineering (see Chapter 2) and in attempts to generate human MAbs. Human antibodies may also be advantageous in that they are compatible with human effector mechanisms such as complement and ADCC which are often not fully activated by rodent antibodies.

Samsung Bioepis Exhibit No. 1002
Page 5284

Table 1.3 Problems in human MAb production using conventional techniques

Immunisation *in vivo* or *in vitro*
Source of human B lymphocytes
Immortalisation: EBV/choice of fusion partner cell line
Stability of human hybridoma/heterohybridoma cell lines
Selection of isotypes and high affinity MAbs
Scale up to produce large quantities of human MAbs

The production of human MAbs has not been straightforward and attempts have driven many technical innovations and improvements to antibody technology. Production of human MAbs by hybridoma techniques is usually difficult for several reasons (Table 1.3). For many antigens of interest, such as human disease markers, it is not appropriate to immunise humans directly with the antigen for ethical and moral reasons. Therefore it can be difficult to obtain suitably immune lymphocytes. The source of lymphocytes is also a major problem. It is usually not possible to harvest these from the spleen or lymph node and the most available and commonly used source is peripheral blood. B cells are comparatively poorly represented in peripheral blood and the majority of those present express surface IgM. Fusion therefore often results in cells which largely produce low affinity IgM antibodies. The choice of cell line to be used as a fusion partner is also problematic. Human myeloma lines have proved to be comparatively poor with respect to their growth characteristics and fusion efficiencies and usually secrete immunoglobulin of their own which complicates production and purification. Attempts to use murine cell lines, which do not produce any immunoglobulin of their own, as fusion partners have met with some success, although the hybrid cells produced can be unstable and may preferentially lose human chromosomes and thus the ability to secrete human antibody. More successful has been the development of mouse–human heteromyeloma cell lines as fusion partners. These are formed by fusing a human lymphoid tumour line with a mouse myeloma cell line and using the resulting hybrid cell line to fuse with the required B cells. The resulting fused cells are termed heterohybridomas or triomas and these often produce more antibody than cell lines produced with murine fusion partners alone and are relatively stable.

An alternative approach to immortalise human B cells has been the transformation of these cells with Epstein–Barr virus (EBV). EBV is a virus which preferentially infects B cells and activates them to divide and produce antibody. Some of the infected cells may be immortalised but this technique is relatively inefficient and cell lines are often unstable and lose the ability to produce antibody. Combinations of this approach with the use of heteromyeloma fusion are more efficient. For example, stimulating B cells with EBV followed by fusion with a mouse–human heteromyeloma has been used to produce several human MAbs (e.g. Kozbor *et al.*, 1982; Gustafsson and Hinkula, 1994).

To overcome the problem of being often unable to immunise humans *in vivo*, approaches to the *in vitro* immunisation of human B cells in culture have been developed (Borrebaeck, 1989; Koda and Glassy, 1990). Although developed primarily for use in human antibody production, *in vitro* immunisation was first used for the production of mouse antibody and may offer advantages for the production of rodent antibodies in some circumstances, for example when trying to raise antibodies to weakly antigenic epitopes which might be masked by dominant epitopes *in vivo*, or when trying to raise antibodies to hazardous antigens which it would not be possible to administer *in vivo*. *In*

Samsung Bioepis Exhibit No. 1002
Page 5285

vitro immunisation is an attempt to achieve primary activation of B cells in culture and as such requires a complex *in vitro* protocol including co-cultivation with a variety of cytokines and mitogenic factors to stimulate B cells (Danielsson *et al.*, 1987; Darveau *et al.*, 1993). Suppressor cells also need to be eliminated from the population by cell separation or by treatment with agents such as leucyl leucine methyl ester which is cytotoxic to CD8+ T cells and natural killer cells (Borrebaeck *et al.*, 1988). The major drawback of *in vitro* immunisation approaches developed to date is that the antibodies produced are characteristic of a limited primary response and are therefore often of the IgM subclass with low to intermediate affinities for antigen.

Mice with a mutation resulting in severe combined immune deficiency which lack functional B and T cells (SCID mice) have been used in attempts to produce higher affinity human antibodies. Because of their immune deficiency, SCID mice can accept grafts of human lymphocytes isolated from peripheral blood which can be used to re-create a partial human immune system in the mouse (Mosier *et al.*, 1988). Cells can be transplanted from immunised donors or after *in vitro* immunisation and re-immunised in the mouse to generate secondary responses (Duchosal *et al.*, 1992; Carlsson *et al.*, 1992). Immune human B cells can then be harvested from the spleen or lymph nodes to allow antibody production.

In addition, it is possible to use SCID mice with human immune cells to generate primary responses using cells from donors not previously immunised (Sandhu *et al.*, 1994). This raises the prospect of producing a wide range of human antibodies in mice, and research towards developing suitable methods is under way (Walker and Gallagher, 1994). So far only a limited number of antigens have been investigated using this system and the limits to the ability of this system to produce high affinity antibodies to a range of different antigens will be a subject of intensive research over the next few years.

One approach which can successfully overcome the instability of human antibody-producing cell lines once they have been generated and the difficulties involved in their large-scale culture is gene rescue. In this approach the antibody genes are cloned from the cells and expressed in a stable alternative host cell such as a murine myeloma. For example, genes coding for human antibodies to tetanus toxoid and pseudomonal exotoxin A have been rescued from a mouse–human heterohybridoma and an EBV transformed B cell line respectively and re-expressed in a murine myeloma cell line (Gillies *et al.*, 1989b; Nakatani *et al.*, 1989). In both cases stable murine cell lines were obtained which secreted high levels of human antibody, similar to the yields of antibody produced by murine hybridoma cells. This technique also allows engineering of the human antibody, such as changing the isotype of the antibody if required. Engineering of antibodies is discussed in detail in Chapter 2.

1.6.3 Human MAbs from transgenic mice

An alternative approach to the generation of human antibodies is the genetic manipulation of mice to disable the production of mouse immunoglobulin and to introduce human immunoglobulin loci. The resulting transgenic mice are capable of producing human antibodies in reponse to challenge with antigen which can be isolated by conventional mouse hybridoma technology (Lonberg *et al.*, 1994; Green *et al.*, 1994). The human immunoglobulin loci are large and contain the V, D and J genes of the variable domains

Samsung Bioepis Exhibit No. 1002
Page 5286

as well as the constant regions as described in Section 1.4. The transfer of the entire locus is technically extremely difficult and thus miniloci have been constructed containing a limited number of the heavy and light chain genes. These genes are introduced into mouse embryonic stem cells and implanted into a surrogate mother to develop. The resulting mouse strain therefore contains both human and mouse antibody genes. A separate mouse strain is produced from embryonic stem cells in which the mouse immunoglobulin heavy and κ light chain genes are disrupted and this is then bred with the strain containing mouse and human genes. Progeny containing only functional human immunoglobulin genes can then be isolated. These mice are capable of immune responses to administered antigen and although the entire diversity of V, D and J regions has not been reproduced, those present are utilised to produce diverse antibody molecules. In some instances somatic mutation has also been demonstrated, suggesting that an extensive diversity can be achieved, with both IgM and IgG antibodies being produced (Lonberg *et al.*, 1994). High avidity antibodies have been isolated, with a panel of human antibodies produced in this way to the human T cell marker CD4, K_a values of 10^8–10^{10} M^{-1} were produced (Fishwild *et al.*, 1996). Progress is also being made on the introduction of larger segments of the human Ig gene loci into mice, with transfer of large DNA segments or chromosome fragments containing the Ig loci now possible (Mendez *et al.*, 1997; Tomizuka *et al.*, 1997). This technology is very much in its infancy and, in the next few years, will no doubt develop to the point where a strain of mice can be produced which is capable of generating a range of useful human antibodies.

Attempts have been made to overcome many of the problems summarised in Table 1.3, and many human MAbs have been generated, but the technology is far from routine and relatively few high affinity antibodies have been produced. Recently an alternative technology for the production of antibodies which can bypass hybridoma technology has been developed, known as phage display. This can be used to produce human antibodies and is probably the method which will dominate human antibody production in the future.

1.6.4 Isolation of antibodies by phage display

An alternative approach to isolation of MAbs is to isolate the genes for the antibody directly and thus bypass hybridoma technology altogether. As the difference in antigen-binding specificities between antibodies lies entirely within the variable domains, it is only necessary to isolate genes for the variable regions which can then be joined to constant region genes by recombinant DNA techniques to allow expression of the required antibody molecule (see Chapter 2). Strategies for isolating antibody specificities using this approach have been developed over the past few years (Winter *et al.*, 1994; Burton and Barbas, 1994). These strategies attempt to mimic the key features of antibody generation by the immune system. To achieve this, repertoires of large numbers of antibody genes are isolated and expressed as antibody fragments displayed on the surface of bacteriophage. Ideally each phage displays a single antibody fragment. The phage with the appropriate antibody fragment displayed are then selected by binding to antigen attached to a solid support. Usually several rounds of selection are carried out to isolate the highest affinity antibody fragments. The selected phage can then be used to express soluble antibody fragment and to recover the antibody genes which are used to construct the desired form of antibody (Figure 1.9).

Samsung Bioepis Exhibit No. 1002
Page 5287

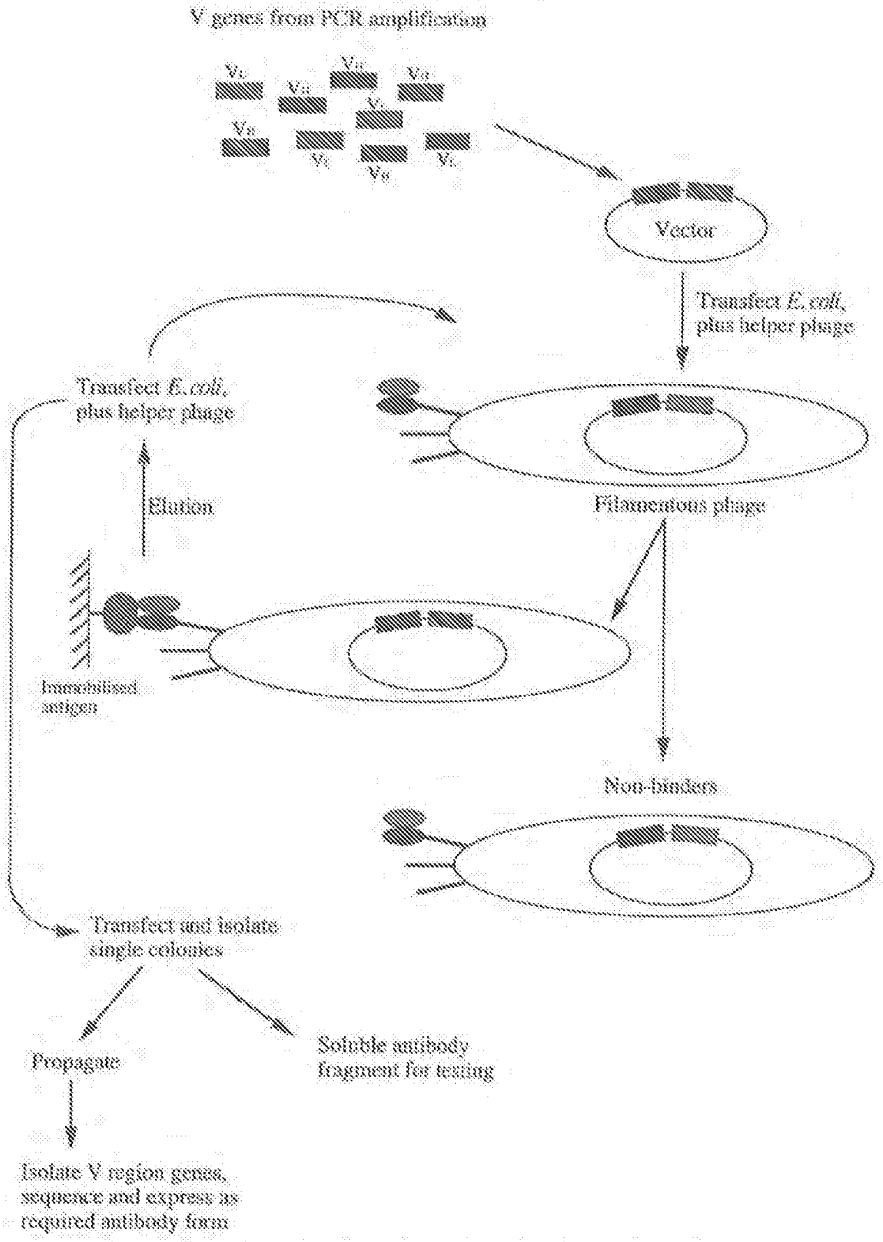


Figure 1.9 Production of monoclonal antibodies by phage display – see text for details

The first requirement is thus a repertoire of antibody genes. Several sources have been used. Genes can be isolated directly from B cells recovered from the spleen, bone marrow or peripheral blood of either immunised or non-immunised animals. Immunised animals contain larger numbers of genes encoding antibodies to the antigen of interest, and may

Samsung Bioepis Exhibit No. 1002
Page 5288

also contain higher affinity antibody genes as a result of secondary immune responses. Therefore it is easier to produce high affinity antibodies from immunised animals. The technique is also applicable to non-immunised donors and has therefore attracted great interest as a technique for the production of human antibodies. As hybridoma technology is bypassed completely, phage display can also circumvent many of the other difficulties in producing human antibodies, such as immortalisation and instability of cell lines, discussed above (Table 1.3). The most common source of variable region genes is cDNA derived from mRNA and amplified by polymerase chain reaction (PCR) technology, although genes can also be recovered by PCR directly from genomic DNA. PCR technology amplifies DNA, producing many copies of the required sequences. Oligonucleotide primers are prepared for binding at the 5′ and 3′ ends of the target DNA on opposite strands which are then extended with a thermostable DNA polymerase. Several cycles of (a) DNA denaturation to single strands, (b) primer annealing and (c) extension by DNA polymerase are carried out in a thermal cycler that cycles between the three temperatures required for these processes. The amount of DNA increases exponentially with each cycle as each new DNA molecule can act as a template to produce new molecules. The flanking sequences of antibody variable region genes have been well characterised and sets of primers have been designed to allow both murine and human antibody genes to be amplified (Orlandi *et al.*, 1989; Marks *et al.*, 1991).

To allow selection of high affinity antibodies a large library of antibody genes is required in order to represent as much of the total repertoire of antibody genes as possible. The larger the library, the greater is the gene diversity and therefore the higher the chance of isolating high affinity specific antibodies. After immunisation of animals, or with humans who have been exposed to the required antigen, for example as a result of infection, the library constructed will have a large proportion of rearranged genes from immune B lymphocytes, and thus smaller numbers of antibody genes need to be screened to identify specific antibodies. Libraries from non-immunised individuals require screening of larger numbers but have the advantage that antibodies to different antigens can be isolated from a single library. Initial libraries were relatively small (10^5–10^6) and resulted in the isolation of weakly binding antibodies. Over the past few years rapid technological advances in library isolation have taken place, including the introduction of synthetic libraries in which random CDR sequences are introduced (Barbas, 1995). Library sizes of 10^{10} have now been achieved and allowed selection of high affinity, specific antibodies (Griffiths *et al.*, 1994; Vaughan *et al.*, 1996).

Two different types of antibody fragment have been widely used for display of antibody specificities on phage, Fab and single-chain Fv (scFv, see Chapter 2). Antibody fragments are used as these are efficiently expressed in bacterial systems (see Chapter 5) and they can be well presented on the surface of the phage. Phage display was first achieved using the phage λ (Huse *et al.*, 1989), but subsequently filamentous phage such as M13, fd or f1 have been found to be more suitable systems (McCafferty *et al.*, 1990; Clackson *et al.*, 1991). These phages are single-stranded DNA phages which consist of a long single strand of DNA coated with approx. 2700 copies of the major coat protein pVIII and a low number of copies of four minor protein components, including the pIII protein which is normally present at 3–5 copies. The pIII protein is located at one end of the phage and is normally involved in infection of a bacterial cell to allow replication. Display at the phage surface is achieved by fusion of the antibody genes to one of the phage surface proteins. It was demonstrated by Smith (1985) that peptides could be presented on the surface of filamentous phage by fusion to the N-terminus of the pIII protein. This was extended to the fusion of antibody fragments resulting in their surface

Samsung Bioepis Exhibit No. 1002
Page 5289

presentation in a form which could bind specifically to antigen (McCafferty *et al*., 1990; Clackson *et al*., 1991). Fusions to the pIII protein can be made by direct cloning into the phage genome, although this results in phage presenting an antibody fragment on each of its pIII proteins and results in phage of low infectivity due to preventing the normal function of the pIII protein. Also the presentation of multiple copies of the antibody fragment on each phage is not ideal as multivalent binding may then take place, where the low affinity binding of several copies may result in relatively high avidity interactions which may mask the binding of a single copy of a high affinity antibody fragment (Barbas *et al*., 1991). A preferred system is therefore the use of phagemid vectors, plasmid vectors containing a viral origin of replication. Phagemids are co-introduced into the bacterial cell with helper phage which provide the genes for native phage proteins. Under appropriate conditions this results in a large number of phage particles containing a single antibody fragment (Garrard *et al*., 1991). Several different vector systems have now been developed using either the whole or part of the pIII protein fused to either Fab or scFv fragments (Winter *et al*., 1994; Burton and Barbas, 1994). The pVIII protein has also been used for phage display, although the large number of copies of this protein in the viral coat means that display of antibody fragments results in many antibody molecules on each phage, and thus this system has been relatively little used.

After assembly of the phage display library, selection must be carried out. This is done by binding to antigen which is attached to a solid phase. Antigen is often coated onto microtitre plates for this purpose or can be used in many other formats such as coated to beads or column matrices. Cells containing surface antigen can also be used or antigen in a form which can be readily captured such as biotinylated antigen, which can be captured on streptavidin-coated beads via the high affinity biotin–streptavidin interaction. Phage bound to the solid phase are then eluted either by competition with soluble antigen or by a change in pH or with a chaotropic agent to disrupt the antibody–antigen interaction in much the same way as used in immunoaffinity chromatography (see Chapter 3). Several rounds of selection are usually carried out to ensure isolation of specific binding phage.

After selection, the antibody genes are recovered and may be sequenced and manipulated as required. In some phage vector systems an amber codon is inserted between the pIII protein gene and that for the antibody fragment (Hoogenboom *et al*., 1991). When grown in a supE supressor strain of *E. coli* the codon is read as glutamine and the antibody fragment is displayed on the phage surface, but when grown in a non-supressor strain the amber codon is read as a stop codon and soluble antibody fragment is secreted from the bacterial cell. This facilitates rapid analysis of the antigen-binding properties of the isolated antibody sequences without further genetic manipulation.

An interesting variation of phage display has been developed using phage which are only infective when carrying the desired antibody specificity (Spada and Pluckthun, 1997). Such selectively infective phage (SIP) are particularly useful in selecting antibody specificities from large libraries as they improve the specificity of the isolation of desired phage, reducing non-specifically binding phage from the system. Selective infectivity is conferred by using a truncated version of the pIII protein for display of the antibody fragment which is not sufficient to allow infectivity on its own. The pIII protein comprises three domains and the two N-terminal domains are required for phage infectivity. Antibody fragments can be displayed on the surface of phage through fusion to the C-terminal domain alone, resulting in non-infectious phage. Infectivity can then be restored through binding antigen fused to the N-terminal domains of pIII. Antigen–pIII fusions can be prepared either as fusion proteins or chemically, therefore allowing both protein and non-protein antigens to be used. As phage are only infective when antibody fragments

Samsung Bioepis Exhibit No. 1002
Page 5290

capable of binding antigen are present, background phage are reduced such that isolation of antibodies is simplified. Using this approach antibodies to a range of different antigens have been described (Spada and Pluckthun, 1997).

Although high affinity antibodies can be isolated directly from large libraries, it is often necessary to improve the antigen-binding affinity of antibodies isolated by phage display. Several strategies for this have been developed. This can be considered to be analogous to the process of affinity maturation undergone in the immune system to generate high affinity secondary response antibodies (see Section 1.2). One approach is to use mutagenesis strategies where sequences in the CDR regions are mutated either randomly or in a more directed fashion and antibodies with higher binding affinities selected by phage display (Yang *et al.*, 1995). This can be achieved either by several rounds of

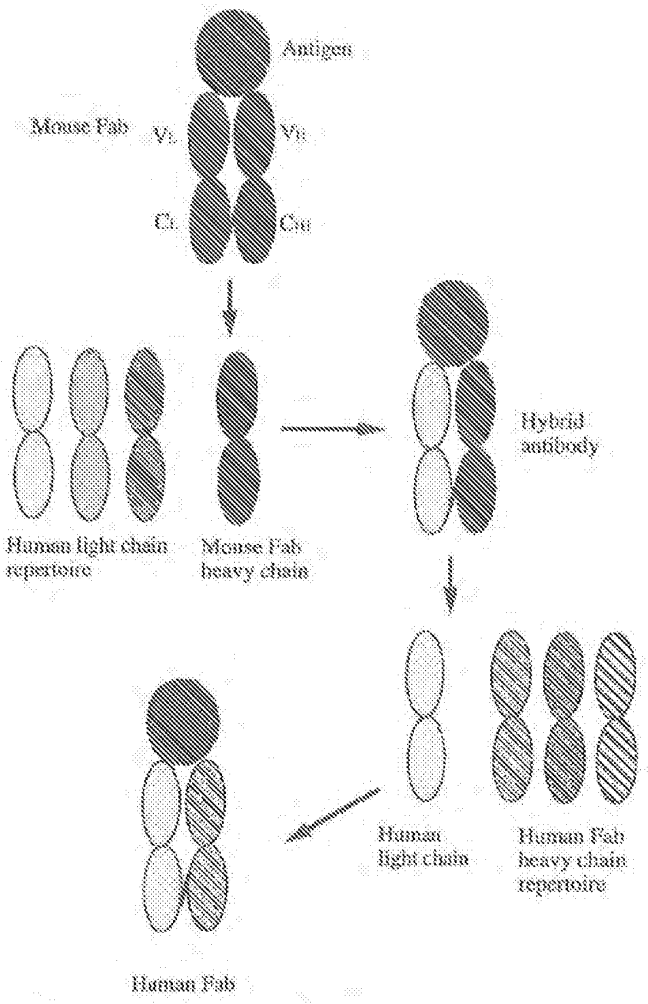


Figure 1.10 Epitope imprinted selection of human Fab from a mouse Fab to the same antigen (adapted from Jespers *et al.*, 1994)

Samsung Bioepis Exhibit No. 1002
Page 5291

sequential mutagenesis of CDR regions or by mutating them in parallel. To date sequential approaches have yielded the best results, with particular improvements coming from mutagenesis of CDR3 regions of the heavy and light chains. Antibodies to both HIV gp120 and the tumour-associated antigen $p185^{HER2}$ have been improved to picomolar affinities by this approach (Yang *et al*., 1995; Schier *et al*., 1996). Residues outside the CDR regions may also have an impact on antigen-binding affinity (see Chapter 2) and mutagenesis strategies for these sequences may also need to be taken into account.

An alternative approach is to use chain shuffling for affinity maturation. In this technique one of the two antibody chains (e.g. V_H) is used to recombine with a library of light chain genes and the higher affinity binding combination retrieved. The new antibody V_L is then used to recombine with a library of heavy chain genes to find a higher affinity heavy chain. This can be repeated, combined with mutagenesis strategies or libraries of synthetic V gene sequences, to introduce further diversity and isolate higher affinity antibodies. In this way antibody affinities have been improved from K_a values of 10^{-7} M to 10^{-9} M (Marks *et al*., 1992).

An interesting technique for isolating human antibodies to antigens for which mouse antibodies already exist has been described based on chain shuffling (Jespers *et al*., 1994). This technique, known as epitope imprinted selection, starts with an existing mouse antibody heavy chain and selects light chains from a human light chain library which pair with the mouse heavy chain and bind to antigen. The light chain is then recombined with a library of human heavy chains and a human heavy chain is selected (Figure 1.10). This technique has been used to isolate a human antibody to TNFα with similar binding affinity to the original mouse antibody (Jespers *et al*., 1994).

The phage display approach offers several potential advantages over hybridoma technology. New antibody specificities can be isolated rapidly, with or without prior immunisation, and high affinity antibodies obtained. Human antibodies can be generated, including to self antigens which is difficult to achieve by other means (Griffiths *et al*., 1993) and antibodies to weak epitopes can be deliberately selected by masking immunologically dominant epitopes (Ditzel *et al*., 1995). However, it should not be seen as only a competing technology to hybridoma technology. It is possible to use both techniques to generate antibodies with different properties such that the most appropriate antibody for a specific application can be generated. In addition it is possible to use antibody chains isolated by hybridoma techniques in chain shuffling experiments with phage libraries and isolate novel high affinity antibody chains (Ames *et al*., 1995). Mutagenesis or chain shuffling techniques combined with phage display can also be used to increase the affinity or even change the epitope recognised by a pre-existing antibody, allowing manipulation of the antibody specificity (Jackson *et al*., 1995; Ohlin *et al*., 1996).

Samsung Bioepis Exhibit No. 1002
Page 5292

2

Antibody Engineering: Design for Specific Applications

2.1 Introduction

Antibody molecules are very amenable to manipulation both chemically and by genetic engineering. As described in Chapter 1, the antibody molecule is made up of a series of domains which are capable of folding into their native structure independently. This has enabled many manipulations of the antibody molecule to be carried out without affecting the conformation of adjacent domains, and allows domains to be moved in position, or substituted with other molecules relatively easily. In addition, the beta sheet structure of the immunoglobulin fold allows some substitutions to be made within domains without loss of activity provided care is taken to ensure the integrity of the domain. In particular, as the antigen binding activity of the V domains is largely conferred by the CDR loops alone, it is possible to make changes in these loops, or switch them from one antibody to another, without disturbing the structure of the framework supporting them. This ease of genetic manipulation has led to the technology of antibody engineering which now allows the specific design of antibody molecules for particular applications.

Several factors must be considered in the design of antibody molecules, and these will be determined by the particular application for which the antibody is to be used. Some major issues to consider are listed in Table 2.1. A large range of modifications to the basic structure are possible, although the choice of one feature may limit the choices in other areas. Firstly, the antibody to be used must be of suitable specificity and affinity for the intended use, and subsequent manipulation should not usually result in alteration of the specificity, as this may lead to undesirable cross-reactivity with other, non-target, antigens. The affinity of the antibody for its antigen should not be drastically reduced, although in some instances this may be an advantage, for example in affinity purification applications. In some cases it has proved possible to improve affinity to a small extent and, in some therapeutic applications particularly, this may be advantageous. The valency of the antibody should also be considered and can be tailored for different applications. The normally divalent antibody can be made into monovalent antibody fragments or multiple binding sites linked together to increase the avidity of antigen binding. In addition, bispecific or even trispecific antibodies can be prepared with binding sites to more than one antigen. Antibody fragments of different sizes can be prepared which may have

Samsung Bioepis Exhibit No. 1002
Page 5293

Table 2.1 Issues in the design of antibodies for specific applications

Specificity and affinity of the binding site
Valency
Size
Requirement for effector function
Attachment of effector or reporter molecules
In vivo properties: immunogenicity
pharmacokinetics & metabolism
Cost of production

more appropriate properties *in vivo*, for example increased penetration into tissue or altered pharmacokinetics. The native effector or signalling functions of the antibody might be exploited for therapy, or specifically disabled if undesirable. Alternatively, a wide range of potentially therapeutic effector molecules, such as drugs, toxins, radionuclides or cytokines, can be attached to the antibody, as can reporter molecules for diagnostic uses. Specific properties can be designed into the molecule for *in vivo* use, such as appropriate pharmacokinetics and low immunogenicity to allow repeat usage. And of course, for industrial users the costs of producing the molecule required must be economic, and this can also be addressed by engineering. Forms of the antibody can be produced which are suitable for low-cost manufacturing and the use of high-yielding, low-cost expression systems can reduce costs further.

2.2 Isolation of variable region genes

At present it is not possible to design antibody variable domains capable of specific antigen binding *de novo*. Therefore a prerequisite for antibody engineering is the production of a suitable MAb and cloning of the variable region genes. With MAbs isolated by phage display, the gene is isolated directly, and variable region genes can be retrieved from hybridoma cells by several different methods. Early cloning of variable regions was carried out by isolating the rearranged genes from genomic clones (Seidman and Leder, 1978). Libraries of genomic clones constructed in a λ phage vector were size fractionated and screened with gene fragments or synthetic oligonucleotide probes to identify variable region heavy and light chain genes. This approach has the advantage that the Ig promoter and in some cases the Ig enhancer sequences can also be recovered as a single DNA fragment using suitable restriction sites which may be useful in subsequent expression studies (see Chapter 5). Another approach is to recover the genes from mRNA through cDNA cloning (e.g. Bothwell *et al.*, 1981). As there is abundant mRNA for the antibody in most hybridoma lines, only small libraries of cDNA are required (approx. 5000–10 000 clones). The libraries are also screened with gene fragments or oligonucleotides to isolate the required genes. Care must be taken however, as hybridoma cell lines often contain aberrant variable region gene segments, derived from the fusion partner, some of which are transcribed. During both genomic and cDNA cloning these extra antibody sequences can be cloned inadvertently (Cabilly *et al.*, 1984; Carroll *et al.*, 1988; Neumaier *et al.*, 1990), and thus all cloned sequences should be checked to ensure cloning of full length antibody variable regions for both heavy and light chains. In some cases the sequence of the antibody may be known or the N-terminal protein sequence can be determined, although in many cases identification of novel sequences is the goal. The extra

Samsung Bioepis Exhibit No. 1002
Page 5294

light chains most commonly encountered as full length genes from mouse hybridomas are the κ1C38 or MOPC21 light chain (Cabilly and Riggs, 1985).

These cloning methodologies have now been largely replaced by cloning of variable region genes by PCR using the methods described in Section 1.7. This technique allows rapid isolation of variable region genes through PCR amplification, and can be carried out from very few cells (Liu *et al.*, 1992). An excellent practical guide to isolation of variable region genes by PCR has been published (Adair, 1997). One problem using this technique is the introduction of errors into the sequence due to replication errors or cross-contamination. This is usually overcome by sequencing multiple independent clones. Also aberrant and extra gene sequences originating from the fusion partner may be identified as described above. These can be rapidly screened against with primers that anneal specifically to known sequences in these antibody chains. With this technique, as well as conventional cloning procedures, the best test of accuracy is a functional test of the antigen binding ability of the antibody expressed from the cloned sequence. This can be done rapidly by expressing the antibody either as murine antibody or directly as a chimeric antibody (see Section 2.3.1) in a transient expression system such as COS cells or CHO cells to produce small amounts of recombinant antibody for analysis (Whittle *et al.*, 1987; see Chapter 5).

2.3 Overcoming immunogenicity

The application of MAbs for human therapy and diagnosis *in vivo* has been limited by many factors. One of the major problems encountered with the administration of murine MAbs to humans has been the generation of an immune response to the administered antibody protein known as human anti-mouse antibody response or HAMA. For antibody therapy in many types of diseases, repeat treatment will be required to achieve effective results. However, HAMA prevents repeat administration of antibody due to the formation of immune complexes which are rapidly cleared from the body, rendering repeat therapy ineffective. Also, in some cases, HAMA responses may result in adverse reactions such as allergic reactions or anaphylatic shock. Because of the problems in generating high-affinity human MAbs in many cases (discussed in Section 1.6), alternative solutions to this problem have been sought. These include the use of antibody engineering techniques to produce chimeric or fully humanised antibodies, chemical modification with polymers such as poly(ethylene glycol), the use of antibody fragments and immunosuppressant therapy.

2.3.1 Chimeric and humanised antibodies

One of the most successful approaches to overcoming immunogenicity has been the 'humanisation' of rodent MAbs by genetic engineering. A simple approach to making an antibody more human is the replacement of the constant domains of the antibody with constant domains of a human antibody (Morrison *et al.*, 1984). The resulting chimeric antibody (Figure 2.1) contains only the variable regions of murine origin and would therefore be expected to be less immunogenic in people. Many chimeric antibodies have been prepared and shown to retain the full antigen binding ability of the parent murine antibody as well as taking on the constant region effector functions of the human antibody used.

Samsung Bioepis Exhibit No. 1002
Page 5295

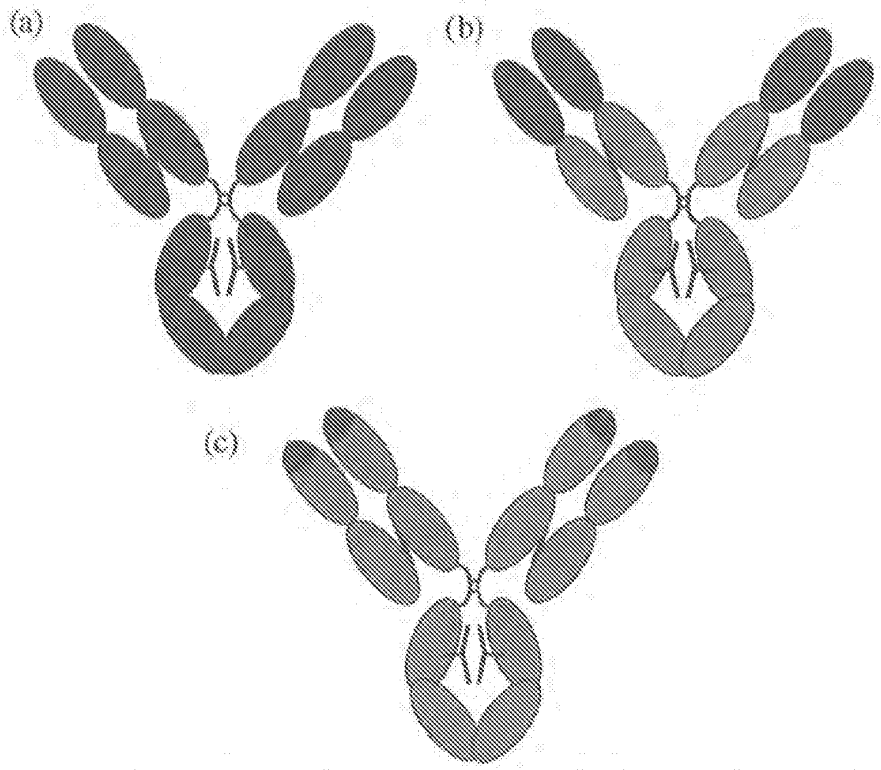


Figure 2.1 Humanisation of IgG: (a) parent murine antibody; (b) mouse/human chimeric antibody with the variable domains retained from the murine antibody and constant regions of human origin; (c) CDR-grafted antibody in which only the antigen-binding loops are retained from the murine antibody, the remainder of the sequence being of human origin

Construction of chimeric antibodies is relatively straightforward and requires simple ligation of the variable region genes 5′ to the constant region DNA and transfer into one of many available expression vectors. The constant region DNA can be in the form of genomic DNA (Whittle *et al.*, 1987) or cDNA (Liu *et al.*, 1987). If cloned by genomic cloning, a restriction site in the intron between the variable domain and constant domain can be used to assemble the required construct. As the intron sequences are removed during RNA splicing, alterations in the intron sequence are of little consequence. Alternatively cDNA clones require site-directed mutagenesis to introduce restriction sites at the V–C junctions to make the chimeric construct, taking care not to alter the required protein sequence. Also oligonucleotide linkers can be used to recreate variable region sequences from a convenient restriction site near the 3′ end of the V regions and introduce a splice donor site immediately after the coding sequence. A convenient restriction site is included to allow assembly of the chimeric gene. For PCR derived genes, primer design can allow incorporation of convenient sites for subsequent manipulation of the cDNA into an appropriate expression construct. PCR approaches using primers at the 3′ end of the coding region will not isolate the signal sequence, and thus this will need to be

Samsung Bioepis Exhibit No. 1002
Page 5296

added to allow secretion of the expressed antibody. Construction of an appropriate vector for expression of a chimeric antibody in mammalian cells requires assembly not just of the antibody heavy and light chain genes themselves but also of a suitable promoter and regulatory elements (see Chapter 5).

Chimeric antibodies have also been produced by homologous recombination in hybridoma cells (Yarnold and Fell, 1994). Homologous recombination is achieved by targeting a plasmid with the human constant region genes of choice to the immunoglobulin locus in the hybridoma cell, such that the murine constant regions are replaced with the targeted human sequences. This approach can be used to chimerise both heavy and light chains but at relatively low efficiency. The other drawbacks of this approach include the limited range of antibody engineering which can be achieved (for example, it is not possible to produce fully humanised antibodies by this technique) and the limitation of the expression level to that seen with the hybridoma, which may be significantly lower than expression levels achieved with many recombinant cell lines.

Transgenic mice have now been developed which can produce chimeric antibodies directly. A bacteriophage derived recombination sysytem known as *Cre-loxP* can be used to replace the mouse antibody constant domain gene segments in embryonic stem cells with human sequences (Zou *et al.*, 1994). Mice producing chimeric light chains have been produced as well as γ1 heavy chain recombinants and crossed together to produce offspring capable of producing γ1 antibodies with both chimeric heavy and light chains at similar levels to endogenous γ1 antibody in wild type mice. These mice are therefore theoretically capable of producing chimeric γ1 antibodies to any antigen to which the mice can respond. This technique has the advantage that chimeric constructs do not need to be made for each individual antibody, as it should be possible to recover chimeric antibodies directly by hybridoma or recombinant techniques; however, the disadvantages are similar to the use of recombination in cell lines, in that a relatively restricted amount of engineering is possible.

The HAMA response to murine MAbs is induced in a large proportion (50–100%) of patients receiving antibody (Khazaeli *et al.*, 1994). Table 2.2 summarises some of the data obtained from clinical trials with chimeric antibodies. Although care should be taken in interpreting the figures absolutely, as these are compiled from different investigators using different clinical protocols and different assays to measure the immune response, it is clear that the response to chimeric antibodies is reduced compared with HAMA responses to murine antibodies. The immune responses detected to chimeric antibodies have been directed toward the murine variable domains, and not to the human constant regions. In some cases responses to novel epitopes created at the interface of the murine variable and human constant regions have also been observed (Khazaeli *et al.*, 1991; Baker *et al.*, 1991). The chimeric antibodies vary in their extent of immunogenicity, although the reasons for this are not understood. It is possible that differences seen simply reflect the different immunogenicity of the antibody variable domains, and this has enabled predictions of the immunogenicity of individual chimeric antibodies in man to be made by testing immunogenicity in animals (Cartner *et al.*, 1993). The antibody responses observed towards the chimeric antibody variable domains include responses to both the framework regions and the antigen-binding loops themselves. Antibodies against the antigen-binding loops (the CDRs) are known as anti-idiotype antibodies, and can usually block antigen binding.

An alternative type of chimeric antibody produced from a monkey antibody variable region linked to human constant regions, termed a 'primatised' antibody, has also been produced (Newman *et al.*, 1992). Primate antibody sequences are more homologous to

Samsung Bioepis Exhibit No. 1002
Page 5297

Table 2.2 Immunogenicity of mouse/human chimeric antibodies in clinical studies

Antibody	Source	Indication	Dosing	Immune response	Reference
c17–1A (γ1)	Centocor	Colorectal carcinoma	1–3 doses, 4–40 mg	1/16, 6%	Lothaglio *et al.*, 1989 Meredith *et al.*, 1991
cB72.3 (γ4)	Celltech	Colorectal carcinoma	1–3 doses, 3–7 mg	16/24, 67%	Khazaeli *et al.*, 1991 Meredith *et al.*, 1992a
cB72.3 (γ4)	Celltech	Colorectal carcinoma	1–4 doses, 10–20 mg	3/6, 50%	Baker *et al.*, 1991
cAnti-CD4 (γ1)	Becton-Dickinson	Mycosis Fungoides	6 doses, 10–40 mg	2/7, 29%	Knox *et al.*, 1991
ch14.18 (γ1)	Damon Biotech	Melanoma	1 dose, 5–100 mg	5/13, 62%	Saleh *et al.*, 1992
c14.18 (γ4)	Damon Biotech	Neuroblastoma	1–5 doses, 7–144 mg	3/5, 60%	Untereuther-Fischer *et al.*, 1995
cL6 (γ1)	InVitron/ Bristol Myers	Carcinoma (lung, breast or colon)	1–4 doses, 35–700 mg/m^2	4/18, 22%	Goodman *et al.*, 1993
NR-LU-13 (γ1)	NeoRx	Colorectal carcinoma	1 dose, 42 mg	6/8, 75%	Wesolek *et al.*, 1993
C2B8	IDEC	B-cell lymphoma	1 dose, 10–500 mg/m^2	0/15, 0%	Maloney *et al.*, 1994
cAnti-CEA (γ4)	Ciba Geigy	Colorectal carcinoma	1 dose, 2–4 mg	2/9, 22%	Buchegger *et al.*, 1995
cMOv18 (γ1)	Centocor	Ovarian carcinoma	1 dose, 3 mg	0/24, 0%	Bunst *et al.*, 1995

Samsung Bioepis Exhibit No. 1002
Page 5298

human than murine sequences and thus it is possible that these may prove less immunogenic than mouse:human chimeric antibodies.

Humanisation of rodent antibodies can be taken further to produce fully humanised antibodies, in the form of reshaped, CDR-grafted or engineered human antibodies, in which much of the variable domain sequences are also replaced by human antibody sequence (Figure 2.1). In these approaches the antigen-binding loops are derived from the rodent antibody and much of the supporting framework is human. The relatively conserved structure of the human and rodent variable domain frameworks allow grafting of the hypervariable loops (CDRs) from one antibody onto another without loss of the overall conformation and structure of the loops and therefore allows antigen-binding ability to be retained. This should reduce any immune response against the variable domain framework. The remaining murine parts of the antibody, the CDRs, are also hypervariable in sequence in human antibodies and therefore more sequence variation may be permitted without generating immune responses.

The simplest form of CDR grafting involves the transfer of the CDR residues from the murine antibody to a human antibody variable domain framework. However, full antigen-binding activity is not achieved by this simple transfer, probably due to small conformational differences in the orientation of the antibody CDRs on the new framework structure. It has therefore proved necessary to introduce a small number of framework residue substitutions from the murine antibody to the humanised antibody, as well as the CDR residues, to restore full binding activity. Successful humanisation therefore requires several key choices to be made, including definition of the CDR residues, identification of human antibody framework sequences, and which if any framework residues to change to murine residues. Several different approaches to humanisation have been developed which largely reflect different approaches to making these choices. The first humanisation in this way was the heavy chain of the hapten binding antibody B1-8 (Jones *et al.*, 1986), and the humanisation of antibodies to complex protein antigens followed soon after (Verhoeyen *et al.*, 1988; Riechmann *et al.*, 1988a). Although difficulty in achieving full antigen binding activity was encountered early in the development of this technology, humanisation of murine antibodies is now relatively routine and many antibodies have been successfully humanised. A review of humanised antibodies, compiled in 1995, revealed over 100 then known examples in which humanisation of the variable domains had been successfully achieved (Adair and Bright, 1995).

The CDR residues are those residues most hypervariable in sequence between antibodies, and have been defined from a comparison of all known antibody sequences (Kabat *et al.*, 1991). All of the six antigen-binding loops are included within the six CDRs, which in several cases extend a short way beyond the structural loops. The choice of human framework is made from the known human antibody variable domain sequences. A human framework can be chosen for which a crystal structure is available, for example the heavy chains from the human antibodies KOL or NEW and the light chain from REI, so that the conformation of the loops can be modelled onto the existing structure and predictions made as to which framework residue substitutions may be required. Alternatively, the heavy and light chains from a single human antibody can be used, such as EU or LAY, or a consensus human antibody sequence. In many cases the human antibody of highest homology to the murine antibody to be humanised is used, as this requires fewer choices to be made with regard to possible framework substitutions. In addition, variable region sequences have been divided into a number of subgroups (Kabat *et al.*, 1991) which are useful in determining the choice of homologous framework.

Samsung Bioepis Exhibit No. 1002
Page 5299

The remaining choices are which framework residues from the mouse sequence to transfer to the human framework. Some residues not in the CDRs may contribute to antigen binding directly or indirectly by affecting the conformation of the loops, by allowing stable packing of the domain structure or by interacting with the other variable domain at the heavy:light chain variable domain interface (Foote and Winter, 1992). The ideal way to determine which framework residues would be required to maintain an accurate conformation of the CDRs would be by analysis of an X-ray crystallographic structure of the binding site. However, the time taken to generate a crystal structure for each antibody to be humanised is usually prohibitive. Alternatively, these residues can be identified by generating a three-dimensional model of the humanised antibody variable regions and then evaluation of the role of particular residues (e.g. Queen *et al.*, 1989) or by alignment and analysis of the sequences to determine their likely structural location on the basis of known structures and thus enable estimation of the consequences of substitutions (e.g. Riechmann *et al.*, 1988a). In this way some unusual amino acids at particular positions in the individual human framework sequences can also be identified and changed. These approaches to humanisation have been alternatively termed CDR-grafting (Jones *et al.*, 1986), reshaping (Riechmann *et al.*, 1988a), hyperchimerisation (Junghans *et al.*, 1990) and civilisation (Kurle *et al.*, 1990).

In some cases comparisons of different humanisation methods have been carried out with the same antibody. The antibody 0.5β recognises the gp120 envelope protein of HIV, and has been humanised using the human framework regions from REI for the light chain and from NEW for the heavy chain, both antibody chains for which structural information is available (Maeda *et al.*, 1991). Alternatively the heavy chain was humanised using the consensus sequence of human group I heavy chains with the same light chain. Although several variants were tested, the consensus sequence framework gave significantly more active antibody, probably as a result of its higher degree of homology with the 0.5β murine antibody (Maeda *et al.*, 1991). A similar result was observed with humanisation of the antibody CAMPATH-9 (Gorman *et al.*, 1991). In this case two heavy chain grafted variants were tested based on the framework sequences from NEW and KOL. Again the version using the antibody framework of highest homology was more active, in this case based on KOL. Other examples are the heavy chain of the anti-CD18 antibody 1B4 (Singer *et al.*, 1993), which was humanised using three different frameworks (NEW, GAL and JON), and the anti-interleukin 6 receptor antibody AUK12-20, which was humanised using two different framework sequences, HAX and consensus group I (Sato *et al.*, 1994). In each case the best retention of antigen-binding activity was achieved using the most homologous human antibody framework, be it a consensus sequence or from an individual human antibody.

A further approach to humanisation has been developed, known as veneering or resurfacing (Padlan, 1991). In this approach it is proposed that the immunogenicity of murine framework regions is determined by the surface residues alone and thus identification of the surface residues not involved with antigen binding and their substitution with 'human' residues will reduce immunogenicity. This approach has been successfully applied with retention of antigen-binding activity (Roguska *et al.*, 1994) and also combined with the 'traditional' CDR-grafting technique to humanise an antibody in an approach referred to as framework exchange (Benhar *et al.*, 1994).

Once designed, the humanised antibody variable domain can be assembled by several procedures. Site-directed mutagenesis of a single stranded DNA template (Riechmann *et al.*, 1988a), or by gene synthesis with alternating overlapping oligonucleotides, filling the gaps with DNA polymerase (Queen *et al.*, 1989), or by PCR procedures to gap fill

Samsung Bioepis Exhibit No. 1002
Page 5300

and amplify the DNA (Daugherty *et al.*, 1991). Of these the PCR approach is the most rapid and efficient although errors may be introduced, so several PCR assembled variable region genes may need to be sequenced to identify the correct product. The humanised variable region genes are then joined to constant region genes as described for chimeric antibodies.

For all humanisation approaches it is usually useful to produce several humanised variants of the antibody and produce small amounts of each for analysis of antigen binding or bioactivity. This can be achieved rapidly by transient expression of small amounts of antibody in mammalian cell systems such as COS or CHO cells (Whittle *et al.*, 1987; Baker *et al.*, 1994 – see Section 5.2). In theory it is only necessary to express the variable region genes as an Fv or Fab fragment to produce material for analysis (e.g. Benhar *et al.*, 1994). This can be achieved rapidly in *E. coli* expression systems; however, the analysis of the antigen-binding ability of these monovalent antigen-binding species is complicated by the possibility of formation of small amounts of dimeric or aggregated material which can greatly influence the outcome of the antigen-binding affinity measurement. For example, it has been shown that the presence of only 0.5% of $F(ab')_2$ in a monovalent Fab′ preparation can overestimate the antigen-binding measurement fivefold (Desplancq *et al.*, 1994). For this reason it is preferable to measure the antigen-binding ability of stable dimeric molecules such as IgG.

In some instances unexpected increases in antigen-binding affinity have been reported on humanisation (Co *et al.*, 1992; Carter *et al.*, 1992a, Baker *et al.*, 1994). This may be due to removal of a glycosylation site from the variable region (e.g. Co *et al.*, 1993), although in other cases the reason is unknown and may reflect subtle changes to the conformation of the binding site (e.g. Baker *et al.*, 1994). However, care must be taken to characterise the engineered antibody produced to ensure that properties of the antigen-binding site are not altered in an undesirable fashion, such as leading to a reduced specificity or some unexpected property. Subtle alterations in the recognition of the antigen by the engineered antibody are possible which may only be revealed by a detailed analysis (Kelley *et al.*, 1992).

Several humanised antibodies have now been examined in clinical studies and thus information on the success of humanisation in overcoming immunogenicity is beginning to emerge. Direct comparisons of immunogenicity of humanised antibodies with the parent murine antibody have not been carried out in patients, but several reports have appeared on the immunogenicity of humanised antibodies in monkeys compared to murine antibody (Hakimi *et al.*, 1991; Singer *et al.*, 1993; Stephens *et al.*, 1995). In each case, the immune response was much reduced compared to the parent murine antibodies, with an anti-idiotype response (i.e. directed to the CDRs) developed on repeat dosing. In the case reported in greatest detail (Stephens *et al.*, 1995), the CDRs appear less immunogenic when presented in the human framework since the anti-idiotype response to the humanised antibody was greatly reduced compared to the anti-idiotype component of the immune response to the murine antibody.

The immunogenicity of humanised antibodies in patients is also low, as summarised in Table 2.3. The earliest studies were carried out with the humanised antibody CAMPATH-1H. Two non-Hodgkin lymphoma patients treated with 1–20 mg doses of this antibody for up to 43 days showed no anti-CAMPATH-1H response during the course of treatment (Hale *et al.*, 1988). However, these results should not be over-interpreted, because such patients are somewhat immunocompromised prior to treatment and because the treatment itself is likely to be immunosuppressive. In subsequent studies CAMPATH-1H was administered to eight rheumatoid arthritis patients (Isaacs *et al.*, 1992) and four

Samsung Bioepis Exhibit No. 1002
Page 5301

Table 2.3 Immunogenicity of humanised antibodies in clinical studies

Antibody	Source	Indication	Dosing	Immune response	Reference
CAMPATH-1H	Univ. Cambridge/ Wellcome	Non-Hodgkin lymphoma		0/2	Hale *et al.*, 1988
CAMPATH-1H	Univ. Cambridge/ Wellcome	Rheumatoid arthritis	5 x 4 mg + 5 x 8 mg	0/8, 3/4 on retreatment	Isaacs *et al.*, 1992
CAMPATH-1H	Univ. Cambridge/ Wellcome	Systemic vasculitis	3 doses, 2–40 mg	1/4	Lockwood *et al.*, 1993
Humanised anti-Tac	PDL/Hoffman La Roche	Graft v. host disease	1–2 doses, 0.5–1.5 mg/kg	0/20	Anasetti *et al.*, 1994
Humanised M195	PDL/MSKCC	Myeloid leukemia	6 or 12 doses, 0.5–10 mg/m^2	0/13 (3 non-specific responses)	Caron *et al.*, 1994
CDP571	Celltech	Healthy volunteers	1 dose, 0.1–10 mg/kg	4/4 at 0.1 mg/kg 0/4 at 2, 5 and 10 mg/kg	Stephens *et al.*, 1995
BAYN-14	Immunomedics	CEA producing cancer	1–3 doses, 1–20 mg	0/6 (without exposure to murine ab)	Sharkey *et al.*, 1995
rhuMAb HER2	Genentech	Breast cancer	250 mg + 10 doses 100 mg	0/48	Baselga *et al.*, 1996

Samsung Bioepis Exhibit No. 1002
Page 5302

patients with systemic vasculitis (Lockwood *et al.*, 1993). In the rheumatoid arthritis study, 4–8 mg doses were given over 10 days. Clinical benefit was seen in seven of the patients, and anti-CAMPATH-1H antibodies were not detectable in any after one course of treatment. Of four patients given a second course of treatment, three showed a detectable anti-CAMPATH-1H response. In the study in systemic vasculitis, one out of four patients developed an anti-idiotype response after treatment. No data are yet available concerning the nature of this response, whether it interferes with efficacy, and if so, whether such interference can be overcome using larger doses. A study with a humanised anti-Tac antibody in patients with graft versus host disease noted no immune responses in any of the patients (Anasetti *et al.*, 1994); however, caution must also be exercised in the interpretation of this study due to the severe immunodeficiency of these patients. The humanised anti-CD33 antibody hM195 has been evaluated in 13 patients with myeloid leukemia at 0.5–10 mg/m^2 with 6 or 12 doses per patient without the onset of any immune response (Caron *et al.*, 1994). The immune response to the humanised anti-tumour necrosis factor antibody CDP571 has been examined after administration of a single dose of 0.1 to 10 mg/kg to fully immunocompetent human volunteers (Stephens *et al.*, 1995). Administration of the lower doses led to the development of a weak anti-idiotype response, predominantly of the IgM isotype, although at higher doses responses were very low or undetectable. The weak IgM anti-idiotype antibodies raised at low doses did not appear to result in rapid clearance of the circulating antibody or to neutralise the ability of the circulating CDP571 to bind TNF, suggesting that this type of response would not be problematic. This type of weak immune response is also seen with the administration of human MAbs (e.g. Steiss *et al.*, 1990) and may be indicative of the humanised antibody behaving in a similar manner to administered human antibody. In a study with a humanised anti-p185^{HER2} antibody, rhuMAb HER2, 46 patients were administered 250 mg of antibody followed by 10 weekly doses of 100 mg each (Baselga *et al.*, 1996). No immune responses were observed. The absence of immune responses after administration of these large doses of antibody is consistent with the results of the other studies. The humanised anti-tumour antibodies hMN-14 and Hu2PLAP have also been studied in humans, but in these cases the analysis of immune response data is difficult due to responses to chemical groups attached to the antibody (Kalofonos *et al.*, 1994) or the administration of murine antibody to the same patients (Sharkey *et al.*, 1995). Nevertheless, in patients receiving hMN-14 alone no immune response was detected (Sharkey *et al.*, 1995). These results taken together demonstrate that fully humanised antibodies have low immunogenicity, and should allow prolonged or repeat therapy in man.

2.3.2 *Antibody fragments to reduce immunogenicity*

The use of fragments of antibodies has also been advocated as a possible means to reduce the immunogenicity of rodent antibodies in man. Antibody fragments such as Fab, F(ab')$_2$ and Fv can be produced from IgG by proteolytic digestion, or directly by recombinant means (see Section 2.4). Several comparative studies have shown that antibody fragments have reduced incidence of HAMA formation compared to IgG (e.g. Breitz *et al.*, 1992; Buist *et al.*, 1995), presumably as a result of the absence of the immunogenic rodent constant regions. However, there is still a sufficient immune response to murine Fab to prevent repeat dose therapy. To reduce responses, recombinant mouse/human chimeric and humanised antibody fragments have been produced. Similarly to studies with IgG, presentation of the murine variable region in a chimeric Fab fragment leads to less

Samsung Bioepis Exhibit No. 1002
Page 5303

immune reponse than when the murine fragment is used. For example, the Fab fragment of the antibody 7E3 which is directed against the platelet glycoprotein IIb/IIIa receptor has been evaluated in clinical studies as both a murine Fab and a mouse:human chimeric Fab. Immune responses to the chimeric Fab in a large phase III trial of approximately 1400 patients were seen in 5.8% of cases, whereas in similar patients treated with murine Fab, immune responses were detected in approximately 45% of patients (Knight *et al.*, 1995). This was seen even though most of the reponses towards the murine Fab were directed toward the variable region which is identical to that of the chimeric Fab. This result therefore suggests that the presentation of the variable region attached to a human constant region results in a significant advantage over murine antibody fragments.

2.3.3. *Chemical modification to reduce immunogenicity*

An alternative strategy which has been used to reduce the immunogenicity of many different proteins including MAbs is chemical modification. Addition of polymers such as poly(ethylene glycol) (PEG) and low molecular weight dextran have been shown to be able to reduce the immunogenicity of antibodies in animal model experiments (Kitamura *et al.*, 1991; Fagnani *et al.*, 1990).

PEG can be attached to antibody using a variety of different chemical linkages, usually by attachment to amine groups of lysine residues on the surface of the protein. Some of the methods which have been used for attachment of PEG to antibody are shown in Figure 2.2. Attachment via cyanuric chloride was the first method developed for PEG attachment to protein (Abuchowski *et al.*, 1977), but is relatively harsh and often leads to inactivation of the biological function of the protein. Attachment via *N*-hydroxysuccinimide esters is now preferred, as it is a more gentle procedure (Zalipsky and Lee, 1992). Other lysine-directed attachment methods such as tresyl chloride-activated PEG are now available and have been used for antibody fragments (Delgado *et al.*, 1996). A two-step method for attachment to lysine via thiol groups has also been described (Pedley *et al.*, 1994). In this method stable attachment is achieved under mild conditions by modification of lysine residues with 2-iminothiolane (Traut's reagent) to generate free thiol groups on the antibody, followed by reaction with a thiol-specific, PEG–maleimide reagent. This method has the advantage that the number of thiol groups introduced can be accurately measured and the consequent coupling of PEG to the antibody closely controlled.

PEG conjugation to antibodies results in reduced immunogenicity and also changes in other biological properties such as resistance to proteolysis and increased circulating half-life (see Section 2.7). The magnitude of the effect may be related to the degree of substitution with PEG achieved, i.e. the number of PEG molecules per antibody molecule. Attachment of large numbers of PEG molecules often leads to reduced ability to bind antigen, particularly if harsh attachment methods are used which may also damage other sites in the protein. Therefore, production of suitable conjugates is a compromise between attaching as much PEG as possible and retaining antigen-binding ability. For example, conjugates of a murine MAb prepared by Kitamura *et al.* (1991) showed reduced antigen-binding ability with 10 and 15 PEG molecules per antibody, although a conjugate with 5 PEG molecules attached was relatively unaffected. The conjugate with 10 PEG molecules attached did not elicit an immune response after multiple administrations to rabbits, although the unmodified antibody readily raised an immune response.

An alternative approach has been to use highly substituted IgG–PEG conjugates as tolerogens. A human IgG–PEG conjugate, with 20 PEG molecules per antibody, administered to mice was able to prevent 85–90% of the immune response to subsequently

Samsung Bioepis Exhibit No. 1002
Page 5304

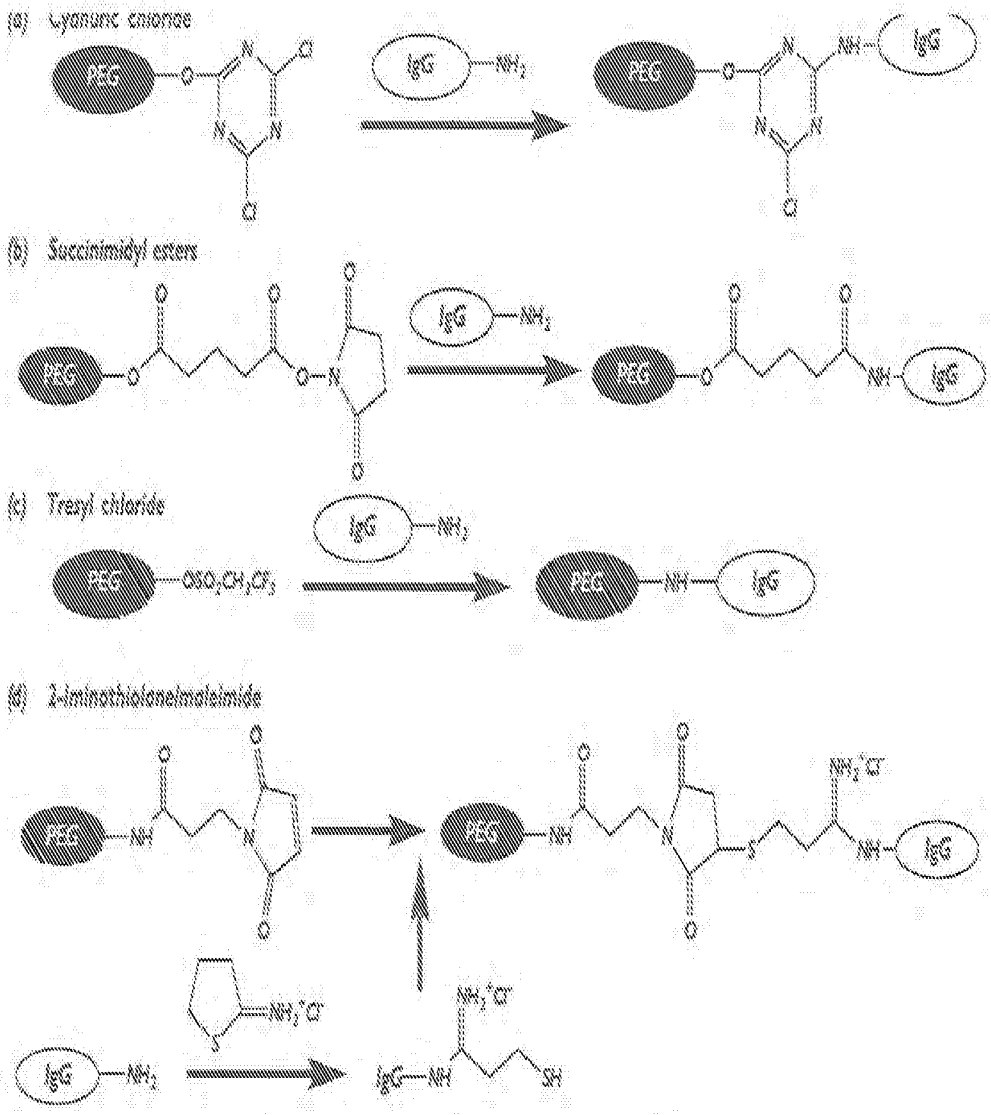


Figure 2.2 Examples of chemical methods for attachment of polyethylene glycol to antibody: (a) attachment via cyanuric chloride-activated PEG; (b) attachment via succinimidyl active esters, e.g. succinimidyl glutarate-activated PEG; (c) attachment via tresyl chloride-activated PEG; (d) two-stage attachment via 2-iminothiolane and PEG–maleimide

Samsung Bioepis Exhibit No. 1002
Page 5305

administered unmodified human IgG (Wilkinson *et al.*, 1987). Subsequent studies have demonstrated that specific immunosuppression of HAMA can be achieved using this approach, such that reponses to the determinants of individual antibody protein chains are suppressed without affecting responses to other antigens (Bitoh *et al.*, 1993).

The covalent attachment of low molecular weight dextrans has also been shown to reduce immunogenicity of IgG in a similar manner to PEG attachment. Conjugates with up to 17 dextran molecules per antibody have been prepared which retain >80% of their immunoreactivity (Fagnani *et al.*, 1990). Tests of immunogenicity in animals reveal a significant reduction in immune response compared to the unmodified antibody.

2.3.4 *Immunosuppressive therapy*

A further approach to reduce the incidence of HAMA response has been to treat patients with immunosuppressive drugs. For example, cyclosporin A has been used to allow repeat dosing of a murine anti-carcinoembryonic antigen (CEA) antibody to patients with CEA producing tumours (Ledermann *et al.*, 1988). A significant reduction in HAMA was observed which allowed up to four administrations of murine antibody to be given. However, cyclosporin A was effective for only a limited time and secondary responses were observed in some patients. Cyclosporin A and similar drugs are general immunosuppressants, and while their use may be possible in cancer and other severe diseases, their routine use for antibody therapy is unlikely.

2.4 Antibody fragments

Fragments of MAbs have been of interest for many years as small antigen targeting molecules. Antibody fragments are also useful 'building blocks' for the assembly of molecules designed to carry other agents to a desired antigen such as diagnostic reagents or therapeutic conjugates. For *in vivo* applications, fragments of antibodies have been of interest due to their altered pharmacokinetic behaviour which has been investigated for cancer therapy with cytotoxic agents, and for their rapid penetration into body tissues which may offer advantages for imaging and therapy techniques. Also, effector functions can be disabled by removal of the Fc portion, and cell internalication may be disabled by making a monovalent binding fragment. Initially antibody fragments, notably $F(ab')_2$ and Fab fragments and occasionally functional Fv fragments, were produced by proteolysis of IgG molecules. More recently recombinant techniques have extended the range of antibody fragments available by allowing novel fragments to be produced, and have extended the range of potential applications by opening up the possibilities of economical large-scale manufacturing.

2.4.1 *Antibody fragments from proteolysis of IgG*

The production of Fab and $F(ab')_2$ fragments of antibodies by proteolysis of IgG has been established for many years (Figure 2.3). As described in Chapter 1, the area around the antibody hinge is more susceptible to proteolysis than the tightly folded domains and thus this is the point at which cleavage usually takes place. Proteolysis above the disulphide bonds in the hinge region with, for example, papain results in Fab fragments which are monovalent for antigen binding, whereas proteolysis below the hinge disulphide bonds,

Samsung Bioepis Exhibit No. 1002
Page 5306

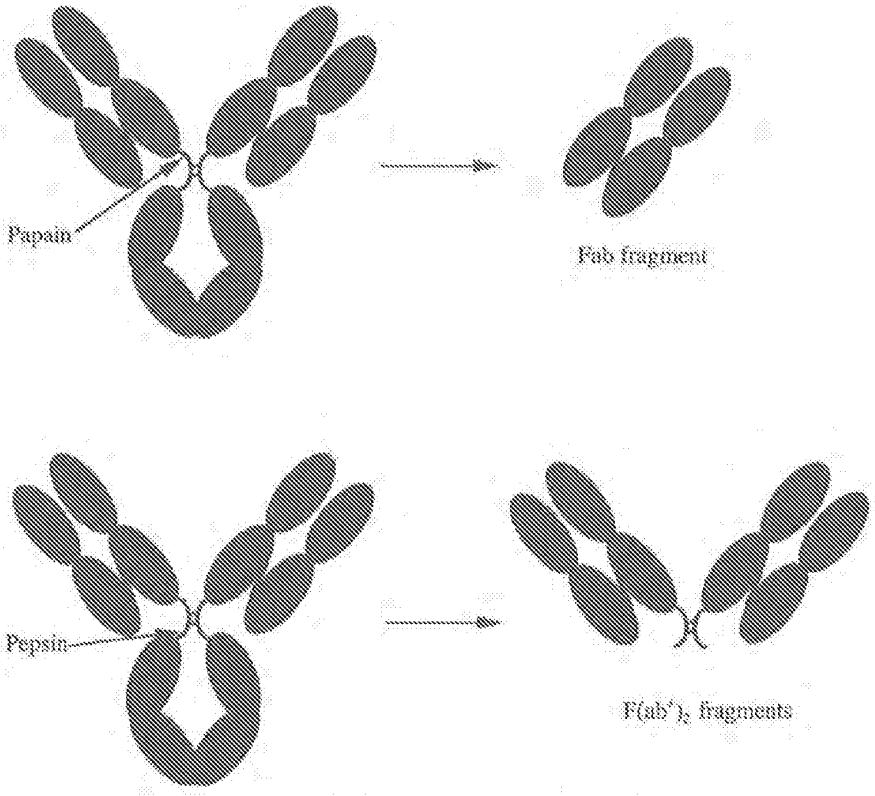


Figure 2.3 Diagram to represent the difference in cleavage point between the proteases pepsin and papain: papain cleaves IgG above the hinge region cysteines leading to the production of monovalent Fab fragments; pepsin cleaves IgG below the hinge region cysteines leading to the divalent $F(ab')_2$ fragment

with enzymes such as pepsin, results in the divalent $F(ab')_2$ fragment. Fc fragment is also produced during the production of Fab fragment by papain digestion, although during the production of $F(ab')_2$ fragments with pepsin the Fc portion is substantially degraded. Typical enzymes used to produce Fab and $F(ab')_2$ fragments from murine IgG are given in Table 2.4. Fab′ fragments, which are Fab fragments with an attached hinge region, can be produced by selective reduction of the hinge disulphide bonds of $F(ab')_2$ fragments. The digestion of IgG is very subclass-dependent and different experimental conditions are required for the production of fragments from different antibody subclasses (Parham, 1986). Individual antibodies within a subclass can also vary in their susceptibility to digestion (e.g. Rea and Ultee, 1993). Therefore, to maximise the yields of active fragments achieved, it is usually necessary to carry out small-scale experiments to optimise the enzyme used, buffer and pH, incubation time and the ratio of enzyme to antibody.

The production of Fab fragments with the non-specific thiol protease papain or other related thiol proteases, such as ficin, is generally successful across a range of antibody types, but the production of active $F(ab')_2$ fragments is more variable. Murine monoclonal IgG1 $F(ab')_2$ can be produced in good yield with bromelain or ficin (Milenic *et al.*, 1989;

Samsung Bioepis Exhibit No. 1002
Page 5307

Table 2.4 Enzymes commonly used for the production of murine antibody fragments by proteolysis of monoclonal IgG.

	Fab	$F(ab')_2$
IgG1	Papain	Bromelain, ficin, pepsin, papain
IgG2a	Papain	Pepsin
IgG2b	Papain, pepsin	Lysyl endopeptidase*
IgG3	Papain	Pepsin

*Based on one report, general applicability currently unknown.

Mariani *et al.*, 1991) and in a few cases with appropriate buffer conditions with pepsin (Parham, 1986; Rea and Ultee, 1993). $F(ab')_2$ fragments can usually be generated from murine IgG2a and IgG3 antibodies with pepsin. However, the production of $F(ab')_2$ from murine IgG2b has not proved possible with pepsin or other enzymes, with rapid degradation to Fab/c and smaller fragments taking place (Parham, 1986), although one report suggests that careful digestion of a murine IgG2b with lysyl endopeptidase can allow $F(ab')_2$ formation (Yamaguchi *et al.*, 1995). Conditions for $F(ab')_2$ production from the rat IgG subclasses have been described (Rousseaux *et al.*, 1983), and human IgG1 and IgG4 $F(ab')_2$ can be produced readily with pepsin and bromelain respectively. It is also possible to produce fragments of IgM antibodies including Fab and $F(ab')_2$ with, for example, pepsin and trypsin (Parham, 1986), although as the individual binding site affinities of IgM antibodies are generally of low affinity (normally compensated for by the high avidity of the pentameric molecule) there has been relatively little use of these for other than experimental investigations. Similarly, murine IgA Fab, IgD Fab and IgE $F(ab')_2$ have been produced by proteolysis (Parham, 1986).

The production of other types of antibody fragment by proteolysis is more difficult. There has been much interest in the production of smaller antigen-binding fragments which may be more effective at penetrating tissues. The Fv fragment comprises only the paired heavy and light chain variable domains and is thus approximately half the size of a Fab fragment. Fv fragments were first prepared by pepsin digestion of the murine IgA myeloma protein, MOPC315 (Inbar *et al.*, 1972). However, attempts to produce Fv fragments of other antibodies have proved largely unsuccessful, being restricted to a small number of cases of digestion of IgM (Lin and Putnam, 1978), IgA myeloma protein (Sharon and Givol, 1976), IgG containing λ light chain (Ornatowska and Glasel, 1991) and IgG2a with a deleted C_H1 domain (Takahashi *et al.*, 1991).

Although they are of great utility for experimental applications, the problems of expense, heterogeneity of product, difficulty of purification and limited transferability from one antibody to another have limited the applications of antibody fragments produced by proteolytic means. Also, the range of fragments which can be produced in this way is restricted, and thus much effort has recently been invested to investigate the potential of recombinant antibody fragments.

2.4.2 *Recombinant antibody fragments*

The direct expression of recombinant antibody fragments allows a range of antibody fragments to be produced using any monoclonal antibody specificity without the restrictions imposed by proteolysis of the native antibody structure. $F(ab')_2$, Fab', Fab and Fv can all

Samsung Bioepis Exhibit No. 1002
Page 5308

be readily produced by expression of the appropriate gene segments in a suitable host, as can a range of novel fragment types. Antibody fragments can be expressed in mammalian cells and also in microbial systems, particularly *E. coli* (see Chapter 5). Two modes of expression are available for use with *E. coli*, intracellular expression and secretion to the periplasm. In common with other types of protein expressed in *E. coli*, intracellular expression of antibody fragments usually results in the accumulation of the expressed protein in a denatured form as an insoluble inclusion body which requires solubilisation and refolding to obtain active material (e.g. Bird *et al.*, 1988; Field *et al.*, 1989). This is often an expensive and low yielding procedure. Alternatively, attachment of a signal sequence to each protein chain can result in secretion of the antibody fragment to the periplasm, from which soluble active protein can be recovered (Skerra and Pluckthun, 1988; Better *et al.*, 1988). The amount of antibody fragment secreted varies between antibodies and between different types of antibody fragment. In general smaller antibody fragments are produced more efficiently than larger fragments, and therefore *E. coli* has become the most widely used expression host for Fv-based fragments, whereas with Fab fragments the most efficient expression system appears to depend on the particular antibody studied.

Fab-based fragments

Fab and Fab′ fragments are produced by co-expression of the light chain and Fd or Fd′ fragment of the heavy chain. These vary only in the presence of the hinge region in Fd′. Fab fragments are relatively stable due to extensive non-covalent interactions between the protein chains and also the presence of a disulphide bond anchoring the chains together. Assembly of expressed light chain and truncated heavy chain (Fd or Fd′) has generally been efficient. However, expression of Fd′ with light chain results in Fab′ production, with little or no $F(ab')_2$ produced. Expression in both mammalian cells and *E. coli* has been shown to result in high yields of Fab and Fab′ fragments and may result in more homogeneous molecules than those obtained by proteolysis.

For many applications the increased avidity of the bivalent molecule may be important and therefore attempts to produce bivalent antibody fragments have been made. Direct expression of $F(ab')_2$ can be achieved, but requires design of a hinge region containing multiple disulphide bonds. Hinge regions with single cysteine residues have resulted in very little $F(ab')_2$ production (Carter *et al.*, 1992a; King *et al.*, 1994) and hinge regions containing two cysteine residues results in only 10–30% $F(ab')_2$ formation (King *et al.*, 1992a; Better *et al.*, 1993). Engineering a hinge region with three potential disulphide bonds has resulted in the production of approx. 70% $F(ab')_2$ from Fab′ expressed in *E. coli* (Rodrigues *et al.*, 1993) and inclusion of five disulphide bonds in the hinge region has resulted in quantitative $F(ab')_2$ formation (King *et al.*, unpublished data). Alternatively, recombinant $F(ab')_2$ can be produced by *in vitro* re-oxidation of the hinge thiols of the expressed Fab′ (King *et al.*, 1992a) or chemically cross-linked $F(ab')_2$ can be produced by specifically cross-linking the hinge thiols (Carter *et al.*, 1992a; King *et al.*, 1994) (Figure 2.4). If chemical cross-linking is used a single hinge thiol is desirable to reduce side-reactions and allow high cross-linking yields. This is easily designed into the Fab′ construct to be expressed. Cross-linking is achieved using a cross-linker with two thiol reactive groups, usually maleimide groups, and can be used to generate $F(ab')_2$-like molecules with two Fab's of the same antibody or using two different Fab's to produce bispecific antibody fragments (Glennie *et al.*, 1987; see Section 2.5). Chemical cross-linking also allows the possibility of incorporating extra moeities into the cross-linking reagent and therefore the site-specific attachment of reagents away from the antigen-binding site (Figure 2.4).

Samsung Bioepis Exhibit No. 1002
Page 5309

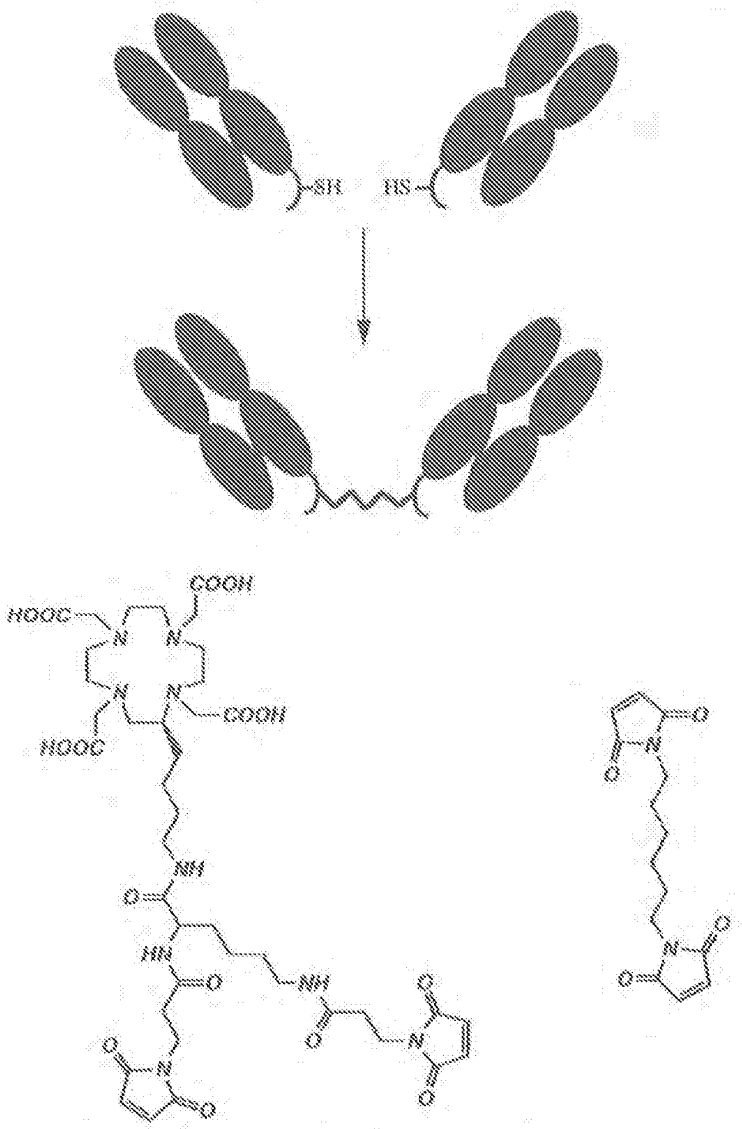


Figure 2.4 Chemical cross-linking of Fab' fragments to form chemically cross-linked di-Fab, using either two Fab's of the same specificity or two of different specificity, in which case a bispecific fragment is produced. Also shown are the structures of two linkers which can be used for this purpose. Hexane bis-maleimide is a simple linker which allows di-Fab formation through cross-linking cysteine thiol groups. Alternatively linkers can be used which contain sites for site-specific attachment. In this case a 12N4 macrocycle is incorporated for site-specific labelling with radioactive metals (King *et al.*, 1994).

Samsung Bioepis Exhibit No. 1002
Page 5310

For example, cross-linkers containing macrocyclic ligands for the stable attachment of radioisotopes have been used to allow formation of radiolabelled antibody fragments without the need for further derivatisation of the antibody (King *et al.*, 1994).

Fv-based fragments

The two chains of the Fv fragment are less stably associated than the Fd and light chain of the Fab fragment with no covalent bond and less non-covalent interaction, but nevertheless functional Fv fragments have been expressed for a number of different antibodies. Characterisation of Fv fragments has revealed that although the antigen-binding ability of the Fab fragment is retained, dissociation of the two domains takes place at low protein concentration or under physiological conditions. Thus strategies for stabilising Fv fragments have been of great interest. Two strategies have been investigated with a number of different antibodies: firstly, mutating a selected residue on each of the V_H and V_L chains to a cysteine to allow formation of a disulphide bond between the two domains; and secondly, the introduction of a peptide linker between the C-terminus of one domain and the N-terminus of the other, such that the Fv is produced as a single polypeptide chain known as a single-chain Fv (Figure 2.5).

The production of single-chain Fv's (scFv's) was first described in 1988 (Bird *et al.*, 1988; Huston *et al.*, 1988). Since then a large number of scFv's have been produced which retain antigen-binding characteristics, although in some cases antigen-binding affinity is reduced. Expression in *E. coli* has been the most frequently used production method, with both intracellular expression and secretion enabling high yields of scFv to be made. The production of scFv molecules requires the identification of a suitable peptide linker to span the 35–40 Å distance between the C-terminus of one domain and the N-terminus of the other and allow correct folding and assembly of the Fv structure. Several different types of linker have been used and shown to result in functional scFv. Linkers have been selected from searching existing protein structures for protein fragments of the appropriate length and conformation, or have been designed *de novo* based on simple, flexible structures with perhaps the most commonly used being the 15 amino acid sequence



Figure 2.5 The organisation of domains in a Fab' fragment compared to Fv, disulphide-linked Fv and single-chain Fv fragments: disulphide-linked Fvs are formed when a selected amino acid residue on both V_L and V_H is mutated to cysteine such that a disulphide bond can form between the variable domains; single-chain Fvs are formed as a result of the introduction of a peptide linker into the sequence, linking the C-terminus of one variable domain to the N-terminus of the second

Samsung Bioepis Exhibit No. 1002
Page 5311

$(Gly_4Ser)_3$. Active single-chain Fv molecules have been produced in both of the two possible orientations, V_H–linker–V_L or V_L–linker–V_H; however, for some antibodies one particular orientation may be preferable as a free N-terminus of one domain, or C-terminus of the other, may be required to retain the native conformation and thus full antigen binding. For example, V_L–linker–V_H scFv's of the anti-tumour antibody B72.3 have been shown to be more active than V_H–linker–V_L with a range of different linker lengths (Desplancq *et al.*, 1994).

Many scFv's have been shown to be susceptible to aggregation with dimers and larger species formed in a concentration-dependent manner (reviewed by Raag and Whitlow, 1995). This is because the interactions between V_H and V_L are relatively weak and there appears to be an equilibrium set up between assembled and unfolded scFv in solution. This leads to domain interchange with neighbouring molecules taking place at high concentrations (Figure 2.6). This is particularly prevalent if strain is introduced into the conformation of the scFv molecule by the use of short linker peptides. Conversely, the use of longer peptide linkers relaxes any strain in the conformation and encourages monomer formation (Whitlow *et al.*, 1993). For example, the B72.3 scFv is in the form of mostly dimers and high molecular weight aggregates with linker lengths of 0, 5 and 10 amino acids but mostly monomeric with linkers of 15, 20, 25 and 30 amino acids (Desplancq *et al.*, 1994). In addition, the tendency towards dimer formation at high concentration decreases as the linker length increases (Figure 2.6). The potential of forming dimers with very short linkers, or no linker at all, can be exploited to produce stable dimeric structures which have been termed "diabodies" (Holliger *et al.*, 1993). Such an approach can also be used to create molecules with two different binding specificities, bispecific diabodies, by fusing the V_H of an antibody of one specificity to the V_L of another and vice versa (see Section 2.5). The crystal structure of a diabody recognising a phospholipase enzyme has been solved and it has been demonstrated that the V_H and V_L domains on the two polypeptide chains associate as expected, although in this case there is also non-covalent association between two diabody molecules to form a tetrameric structure (Perisic *et al.*, 1994).

Fv's stabilised by disulphide linkages appear to be more stable structures than scFv's (Glockshuber *et al.*, 1990). The introduction of a disulphide bond between the V_H and V_L domains to form a disulphide-linked Fv requires the identification of residues in close proximity on each chain which are unlikely to affect directly the conformation of the binding site when mutated to cysteine, and will be capable of forming a disulphide bond without introducing strain into the structure of the Fv. Sites have been identified in both CDR regions and framework regions which appear to result in the formation of such disulphide bonds and allow the production of stabilised Fv fragments which retain antigen-binding characteristics (Glockshuber *et al.*, 1990; Reiter *et al.*, 1994). The use of sites in the framework region is likely to be of more general utility, and several different antibody Fv fragments have now been stabilised in this way (reviewed by Reiter and Pastan, 1996).

A different approach would be to use a single immunoglobulin domain for antigen binding. In a small number of cases a single-antigen binding domain may have antigen-binding activity. For example, V_H domains with affinity for lysozyme in the 20 nM range have been isolated which have been termed single domain antibodies or dAbs (Ward *et al.*, 1989). Light chain domains may also have antigen-binding activity in some cases (Masat *et al.*, 1994). However, single domains have been difficult to express and are poorly soluble due to an exposed hydrophobic surface which normally associates with the other V domain, and thus such small antigen binding proteins have not been widely used.

Samsung Bioepis Exhibit No. 1002
Page 5312

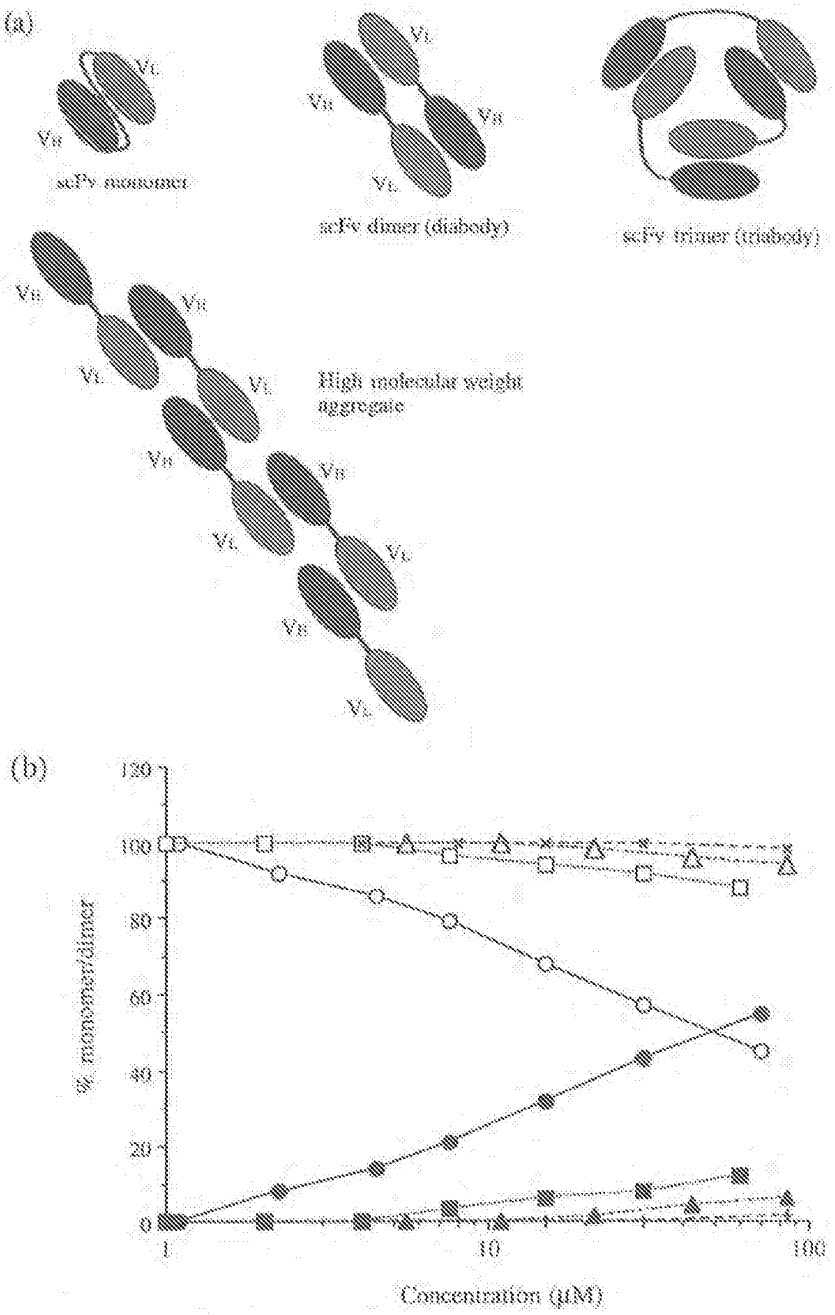


Figure 2.6 Aggregation of single-chain Fv: (a) representation of scFv monomer, dimer (diabody), trimer (triabody) and high-molecular weight aggregate; (b) extent of dimer formation with variation in linker length for scFv of the antibody B72.3, adapted from Desplancq *et al.* (1994). scFvs in the orientation V_L–V_H with linker lengths of 15, 20, 25 and 30 amino acids are shown. (○) Monomer, (●) dimer V_L–15–V_H; (□) monomer, (■) dimer V_L–20–V_H; (△) monomer, (▲) dimer V_L–25–V_H and (x) monomer, (+) dimer V_L–30–V_H.

Samsung Bioepis Exhibit No. 1002
Page 5313

Camels have been found to have naturally occurring antibodies without light chains, and thus attempts have been made to engineer the interface of the VH domain to mimic camel VH domains which may be more soluble (Davies and Riechmann, 1995). Although these attempts have been partially successful, in that antigen-binding specificities could be obtained from a library of such engineered VH domains, only low affinity binding has been achieved to date. This is probably a consequence of the reduction in available CDR regions for antigen binding from 6 to 3 compared to authentic Fv. In some cases it may be possible to increase the number of antigen contact residues, for example by engineering an extra antigen-binding loop which can be inserted into the framework 3 region of VH domains to increase binding affinity (Sinon and Rajewsky, 1992).

Multivalent antibody fragments

As monovalent binding entities, Fv fragments suffer from relatively low avidity binding and therefore there has been much interest in producing multivalent Fv fragments which still retain a relatively small size. The production of 'diabodies' is one approach as described above, and some diabodies can be produced to high levels in the form of stable dimers (Zhu *et al.*, 1996). However, the stability of such dimeric species varies from one antibody to another (Whitlow *et al.*, 1994). In some cases, particularly with direct fusions of VH and VL, stable trimeric species are produced which have been termed 'triabodies' (Iliades *et al.*, 1997). Some triabodies show improved avidity for antigen as expected from the increased number of binding sites, although this is not always the case. A crystal structure of a non-functional triabody produced from the variable domains of two anti-hapten antibodies has been solved and demonstrated a dramatic alteration in the conformation of the VH CDR3 antigen-binding loop (Pei *et al.*, 1997).

Several alternative approaches have been examined in attempts to produce dimeric species (Figure 2.7). A peptide linker can be introduced between two scFv fragments to produce a single polypeptide chain di-scFv molecule. However, attempts to produce such dimers have often resulted in reduced antigen binding for the two binding sites, even when anti-hapten antibodies were used (Mallender and Voss, 1994). One of the most successful strategies to produce dimeric species has been to introduce a cysteine residue which can be used to introduce a disulphide bond or a chemical cross-link between two scFv fragments. Either this can be introduced directly at the C-terminus of an scFv, via a linker sequence, or a natural antibody hinge region can be attached to form an scFv' fragment (Cumber *et al.*, 1992; Adams *et al.*, 1993; King *et al.*, 1994). Dimeric scFv can then be formed in a similar manner to that described for Fab' fragments above, and offers the same potential for site-specific attachment of radiolabels or other moieties (King *et al.*, 1994).

An alternative approach has been the fusion of Fv fragments with peptides that naturally form dimeric structures. Amphipathic helices can be fused to the C-terminus of scFv fragments to allow spontaneous dimerisation (Pack and Pluckthun, 1992; Pack *et al.*, 1993). These 'dimerisation domains' can be taken from, for example, a four-helix bundle structure or a leucine zipper from a naturally occurring protein. Leucine zippers are peptides of approximately 30 amino acids which naturally form amphipathic helices with leucine residues lining up on the hydrophobic face of the helix. The hydrophobic faces of two zipper peptides associate with each other through hydrophobic interactions. Therefore the association of these molecules occurs through non-covalent interactions, which require further stabilisation to form stable dimeric species. Stabilisation is achieved through engineering a disulphide bond to be formed between the two dimerisation domains when

Samsung Bioepis Exhibit No. 1002
Page 5314

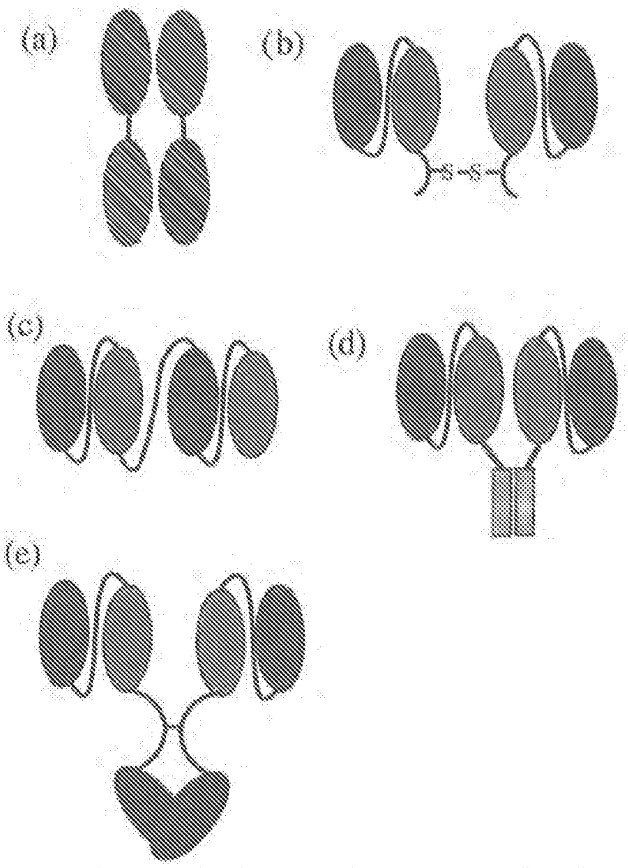


Figure 2.7 Strategies for formation of dimeric scFv species: (a) diabodies formed spontaneously from scFv with short peptide linkers; (b) formation of a disulphide bond or chemical cross-linking through attached hinge region peptides; (c) direct fusion of one scFv to another through an extra linking peptide; (d) the use of dimerisation domains such as leucine zippers; (e) minibody formed from the use of IgG C_{H3} domains and a hinge region peptide to drive, and stabilise, dimerisation

associated. This approach to dimer formation has the advantage that no *in vitro* manipulation is required after expression and purification of the molecule. However, for *in vivo* applications of Fv fragments, the added dimerisation domains may increase the immunogenicity of the molecule, thus limiting its usefulness. An alternative dimerisation domain is the IgG C_{H3} domain (Hu *et al.*, 1996). As described in Chapter 1, there are extensive non-covalent interactions between IgG C_{H3} domains which help drive assembly of the native paired heavy chains in IgG. This interaction can also be used to promote dimerisation of scFv–C_{H3} fusion proteins which can be stabilised by inclusion of a hinge region containing cysteine residues to allow disulphide bond formation (Figure 2.7).

The use of self-assembling structures can be extended to produce higher avidity antigen-binding proteins such as tetrameric scFv's. A modified amphipathic helix can result in tetrameric fragments (Pack *et al.*, 1995), as can fusions to the core region of streptavidin

Samsung Bioepis Exhibit No. 1002
Page 5315

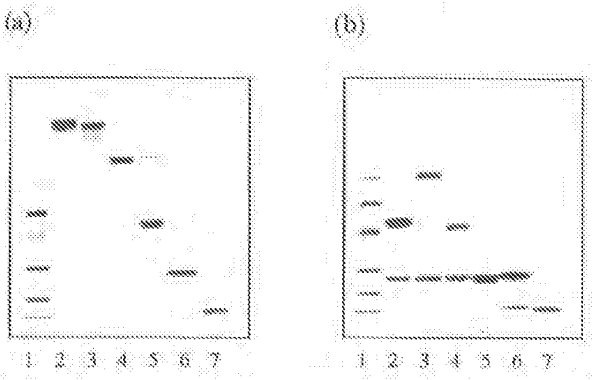


Figure 2.8 SDS-PAGE analysis of chemically cross-linked di-Fab and tri-Fab compared to other immunoglobulin forms: (a) (non-reducing conditions) lane 1, molecular weight markers; lane 2, IgG; lane 3, chemically cross-linked tri-Fab′; lane 4, chemically cross-linked di-Fab′; lane 5, Fab′ containing trace of $F(ab')_2$; lane 6, scFv; lane 7, Fv; (b) SDS-PAGE under reducing conditions, samples as for the non-reduced gel

which forms a natural tetrameric structure (Kipriyanov *et al.*, 1996). These fusion proteins can be difficult to express to high levels directly in soluble form, although procedures have been developed to enable recovery of active material from insoluble protein expressed in *E. coli* (Kipriyanov *et al.*, 1996). The tetramerisation domain of the human protein p53 has also been used to produce fusion proteins which assemble to tetrameric scFv's (Rheinnecker *et al.*, 1996). The use of human protein domains may have advantages for *in vivo* use as the human protein may be less immunogenic than entirely synthetic sequences or bacterial proteins such as streptavidin, although as a normally intracellular protein, p53 may not be the optimal choice for such reagents.

Trimeric and tetrameric antigen-binding proteins can also be produced by chemical cross-linking of Fab′ or scFv′ with tri- or tetra-maleimide cross-linking reagents (Schott *et al.*, 1993; King *et al.*, 1994), or by polyoxime chemistry (Werlen *et al.*, 1996). This approach can be easily used for a range of fragments from different antibodies, either recombinant or produced by proteolysis, and is perhaps the most attractive method for the production of multivalent fragments at present. Such cross-linked fragments can be produced in high yield and purified to homogeneity (Figure 2.8). Tri-scFv's and tri-Fab's produced in this way show increased avidity of binding to antigen and favourable *in vivo* properties (see Chapter 4).

2.5 Antibodies with multiple specificities

Bispecific antibodies have two different specific antigen-binding sites, one on each arm of the antibody molecule, and are potentially useful for a range of diagnostic or therapeutic applications. For example, a bispecific antibody can be prepared with one binding specificity to a target tissue antigen and a second specificity to peroxidase for use in immunohistochemistry (Milstein and Cuello, 1983). Alternatively, for therapy, a bispecific antibody could be used with one arm binding to a tumour cell antigen and one to a cell killing

Samsung Bioepis Exhibit No. 1002
Page 5316

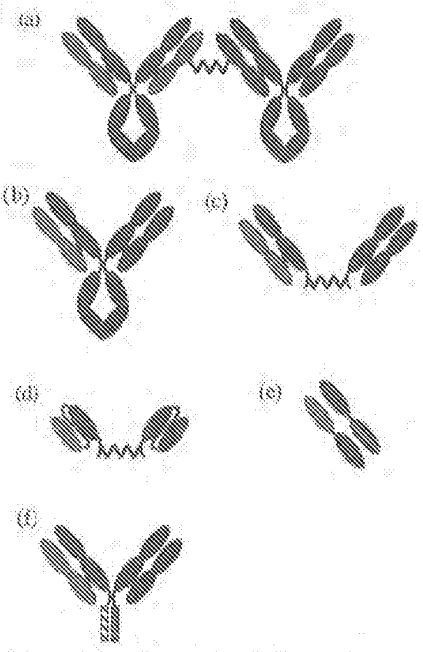


Figure 2.9 Approaches to bispecific antibody production: (a) chemical cross-linking of IgG, (b) production of IgG from a hybrid hybridoma, (c) chemical cross-linking of Fab's, (d) chemical cross-linking of scFvs, (e) bispecific diabody, (f) association through leucine zippers

agent such as a drug, toxin or effector cell (reviewed by Fanger and Guyre, 1991). There are now several approaches to the production of bispecific antibodies (Figure 2.9). All of these approaches have advantages and disadvantages with no one method being used predominantly at the present time.

Hybrid hybridoma cell lines, also known as quadromas, can be produced by the fusion of two antibody-secreting cells such that the resultant hybrid produces two individual heavy and light chains. This can be achieved using two hybridoma cells, or by direct fusion of a hybridoma cell and an immune spleen cell (trioma). Fusion of two existing hybridoma cell lines is usually preferable as the antibodies to be used can be characterised individually and thus the outcome is more predictable. The heavy and light chains associate with each other to produce IgG using the normal cellular mechanisms, but without any selectivity in the pairing of the chains ten combinations are possible, with the bispecific IgG making up only a small proportion of the total (Figure 2.10). Attempts to improve the proportion of bispecific produced have been made, for example, by using rat/mouse hybrid hybridomas wherein the light chains will only pair with the heavy chain of the same species (Lindhofer *et al.*, 1995). However, purification of the correct bispecific antibody from a mixture of very similar IgG molecules remains a considerable problem. If both antigens are available these can be used for sequential affinity purification although this is an expensive operation which is usually difficult to perform on a large scale. Alternatively, methods such as ion-exchange chromatography based on differences in charge of the individual IgG chains can be used (Allard *et al.*, 1992), or if the two

Samsung Bioepis Exhibit No. 1002
Page 5317

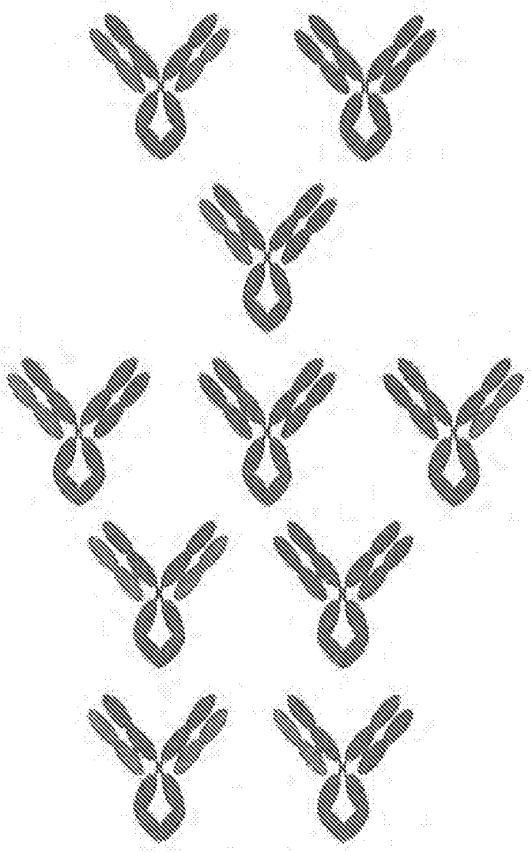


Figure 2.10 Combinations of heavy and light chains possible from a hybrid hybridoma

heavy chains are of different isotype, fractionation on protein A chromatography may be possible (Lindhofer *et al.*, 1995). A further problem with hybrid hybridomas is that they are usually unstable as a result of being polyploid due to carrying the chromosomes of each of the parent cells. Chromosome loss and loss of the ability to secrete bispecific antibody is a constant danger and maintenance of a productive cell line therefore requires frequent cloning to ensure that both sets of heavy and light chain genes are still present. An alternative method to overcome the instability problem is the introduction and expression of the genes for two heavy and light chains in the same cell by recombinant DNA techniques (Songsivilai *et al.*, 1989). This method also allows engineering of the antibody genes to introduce other desired features; for example, humanised antibody genes can be used.

A bispecific reagent can also be prepared simply by chemically cross-linking two IgG molecules (e.g. Karpovski *et al.*, 1984). This results in an undefined heteroaggregate of approx. 300 kDa, and one or both of the antigen-binding sites may very well be damaged during the non-specific chemistry required. This approach does have the advantage, however, that bivalent binding for both antigens may be retained in some cases resulting in high avidity binding to both antigens.

Samsung Bioepis Exhibit No. 1002
Page 5318

The production of bispecific IgGs by protein engineering techniques has also been investigated by designing specific protrusions, 'knobs' on one C_H3 domain and a corresponding 'hole' in the C_H3 domain of the second antibody heavy chain (Ridgeway *et al.*, 1996). This is achieved by swapping a small amino acid at the interface with a large one to make the 'knob' and vice versa to produce a complementary 'hole'. Assembly of the two engineered heavy chains is thus favoured while assembly of the homodimers containing two 'knobs' or two 'holes' is unfavourable. Coexpression of the two heavy chains resulted in mostly heterodimer produced. However, pairing of the correct light chains may require further engineering of the interface between the heavy and light chains, possibly using further 'knobs' and 'holes'.

A more attractive alternative is to prepare Fab′ fragments from the two antibodies required and then to cross-link the two Fab′ fragments via the hinge cysteine residues to produce bispecific $F(ab')_2$. This can be achieved by use of a thiol reactive cross-linker (Figure 2.4). Methods have been devised for the preparation of bispecific molecules without any homodimers produced, and such bispecific reagents have been shown to maintain the full functionality of each binding site (Brennan *et al.*, 1985; Glennie *et al.*, 1987). Chemically cross-linked $F(ab')_2$ produced with bis-maleimide reagents, which therefore contain thioether bonds between the two Fab's, are produced in higher yields and are more stable than disulphide-linked molecules (Glennie *et al.*, 1987). This approach can be used with Fab′ molecules produced by proteolysis or with recombinant Fab′ or other antibody fragments engineered to contain a free thiol group, such as scFv′. If Fab′ with multiple hinge thiols is used, or alternatively tri-functional linkers, it is also possible to produce bispecific tri-Fab with two antigen-binding arms to one of the antigens, allowing higher avidity binding (Tutt *et al.*, 1991a). This approach can be modified to allow the production of trispecific antibody fragments, which allows cross-linking of two independent antigens on an effector cell as well as target antigen (Tutt *et al.*, 1991b).

The formation of bispecific $F(ab')_2$ molecules can also be accomplished using 'dimerisation domains' (Kostelny *et al.*, 1992). Leucine zipper peptides have been taken from the transcription factor proteins Jun and Fos which preferentially associate with each other to form a heterodimer. These zipper peptides have been fused to the C-terminus of the Fd′ chains of two Fab′ fragments and expressed as fusion proteins. When co-expressed *in vivo* little heterodimer was produced, but when the individual Fab′-Jun and Fab′-Fos proteins were expressed and purified separately, they could be reduced and recombined *in vitro* to produce the bispecific molecule in high yield. The design of these molecules also incorporates hinge disulphides to stabilise the bispecific $F(ab')_2$ produced. The Jun and Fos peptides attached may increase the immunogenicity of the $F(ab')_2$, but further design could incorporate proteolytic cleavage sites between the Fab′ and the dimerisation domains to allow their subsequent removal once the disulphide bonds were formed.

Bispecific scFv molecules can also be produced by several means using techniques similar to those described for the formation of homodimers above (Section 2.4.2). Direct fusion of two scFv molecules to form single polypeptide molecules containing two different specificities has been attempted by several groups. Initial attempts to express such bispecific molecules in *E. coli* resulted in insoluble material, even if secretion to the periplasm was attempted (Gruber *et al.*, 1994; Mallender and Voss, 1994). Refolding of functional molecules was achieved but resulted in reduced antigen-binding activity of one or both antigen-binding sites. Subsequent expression of single-chain bispecific constructs in mammalian cells resulted in molecules in which both binding sites appeared to be functional (Mack *et al.*, 1995; Jost *et al.*, 1996).

Samsung Bioepis Exhibit No. 1002
Page 5319

Fusion of the V_L of one antibody to the V_H of a second antibody with a short linker and coexpression with the V_L of antibody two fused to the V_H of antibody one allows bispecific diabodies to be produced (Figure 2.9). Expression of high yields of bispecific diabodies in *E. coli* is possible, with yields of 935 mg/l reported after purification with approximately 75% as functional heterodimers (Zhu *et al.*, 1996). Engineering of the domain interfaces has also been investigated to improve assembly of dimeric molecules (Zhu *et al.*, 1997). Improvements in the proportion of functional heterodimer to 92% were reported by engineering the interface to contain 'knobs' and 'holes' as described for the pairing of two heavy chains above. Bispecific diabodies can also be combined with phage display technology to allow selection of diabodies with improved binding and association properties (McGuinness *et al.*, 1996). Although it is easier to produce in *E. coli*, non-specific association of diabodies has been demonstrated (Perisic *et al.*, 1994), and further work examining both the physical properties of these molecules and their *in vivo* characteristics is required.

Genetic fusions of anti-dansyl scFv's to the C-terminus of anti-dextran antibody, either as IgG or as $F(ab')_2$, has been described resulting in tetravalent bispecific molecules (Coloma and Morrison, 1997). Although produced at relatively low levels (0.5–5 mg/l), proteins capable of binding both antigens resulted. It was not clear however, whether the potential for bivalent binding by both specificities was achieved.

2.6 Engineering effector functions

For many applications a simple blocking or neutralising effect of antibody is not enough, and it is desirable to use the antibody to target a diagnostic or therapeutic effector mechanism. Therapeutics can utilise natural effector functions such as complement activation, phagocytosis or ADCC, or entirely novel functions can be introduced. For example, the antibody can be used to target radioisotopes, drugs or toxins to kill tumour cells. Novel functions can be introduced by chemical coupling to antibody molecules or in many cases by recombinant DNA techniques. Similarly, reagents useful as reporter molecules for diagnostic agents, such as enzymes or peptides, can be attached to antibodies either chemically or by means of recombinant DNA. Antibody engineering can thus be used to tailor the effector functions required for individual applications.

2.6.1 *Engineering natural effector functions*

Use of different human isotype constant regions for chimeric and humanised antibodies can confer different biological properties as expected. For example, Shaw *et al.* (1988) produced a set of mouse/human chimeric antibodies from the mouse MAb 17-1A which recognises a tumour-associated antigen. It was demonstrated that the chimeric molecules could activate the effector functions expected for each particular human IgG isotype. This has also been shown for several other sets of matched chimeric or humanised antibodies. Thus the choice of isotype for a recombinant antibody allows the selection of the desired effector function profile. For example, human IgG1 and IgG3 are the most active IgG isotypes in binding to Fc receptors and eliciting ADCC responses, whereas IgG2 or IgG4 can be chosen to minimise Fc binding and ADCC effects. The ability of human antibodies to mediate complement-dependent lysis is highly dependent on a number of poorly understood factors, including antigen access and density, antigen size and proximity to

Samsung Bioepis Exhibit No. 1002
Page 5320

the cell membrane. However, comparisons of sets of recombinant antibodies with identical variable regions have revealed that human IgG1 and IgG3 are also the most active isotypes for complement activation (see Table 1.1) and thus when an active isotype is required one of these two should be chosen. IgG3 molecules are difficult to purify and handle; their long hinge region results in susceptibility to proteolysis and aggregation, and therefore IgG1 is a common choice when an active antibody is required.

In some cases isotypes are chosen for chimeric and humanised antibodies to mimic the functional properties of the original rodent antibody. The effector functions of murine and human isotypes are compared in Table 1.1. Most mouse and rat antibodies are poorly effective at activation of human effector functions, although mouse IgG2a and rat IgG2b are more efficient than other isotypes. Active human isotypes can confer human effector functions to humanised mouse antibodies previously unable to mediate such effects (e.g. Junghans *et al.*, 1990; Caron *et al.*, 1992a), although this is not always the case due to restrictions of the antibody/antigen interaction mentioned above, and also due to the existence of specific cellular protective mechanisms. As more is understood of the molecular basis of Fc receptor binding and complement activation, specific mutations can be made to alter effector functions of a particular isotype. For example, in many *in vivo* situations antibodies are used as blocking or neutralising molecules and effector functions are undesirable as these could lead to unnecessary toxicity. In these cases human isotypes such as IgG2 or IgG4 have been used, although even these relatively neutral isotypes have low level functionality for both ADCC and complement lysis, which may be undesired. The recognition site for Fc receptor binding on the lower hinge/C_H2 domain has been mapped to residues Leu234–Ser329, and mutations in these residues have been shown to reduce Fc receptor binding (Lund *et al.*, 1991; Canfield and Morrison, 1991). The residue Leu235 in this motif has been changed to Glu in a humanised IgG4 and shown to remove the residual Fc receptor binding of this isotype (Alegre *et al.*, 1992). In engineering human antibodies for specific effects of this type, however, it must be remembered that mutations from the natural sequence may have unexpected effects on other functional properties or may increase the immunogenicity of the molecule, and therefore careful testing of the engineered molecule is required.

Other considerations must also be borne in mind when choosing an antibody isotype. The flexibility of the hinge region varies between the human IgG isotypes, and this may affect binding properties, particularly to cell surface antigens. For example, the isotype choice for a chimeric antibody against intercellular adhesion molecule 1 (ICAM-1) affected the antigen-binding properties of the antibody (Morelock *et al.*, 1994). In this case a chimeric IgG1 variant had equivalent antigen-binding ability to the murine antibody, yet less active molecules were produced with human IgG4 or IgG2 constant regions. The affinity of the individual binding sites was shown to be the same for all the chimeric antibodies, the difference being in the avidity due to differences in the ease of bivalent binding to this cell surface antigen. This correlated with the different flexibility of the hinge region in these antibodies, IgG1 being the most flexible with highest avidity and IgG2 the least flexible with the lowest avidity. Similarly, differences in antigen-binding properties between antibodies with identical variable regions have been identified in other antibody–antigen systems with both murine and human antibodies (Cooper *et al.*, 1993; McCloskey *et al.*, 1996).

Human IgG4 is a common isotype choice when an antibody with few effector functions is required. Natural human IgG4 contains a proportion of molecules in which the inter-heavy chain disulphide bonds do not form although the two halves of the molecule are still assembled through non-covalent interactions (King *et al.*, 1992a). During analysis by

Samsung Bioepis Exhibit No. 1002
Page 5321

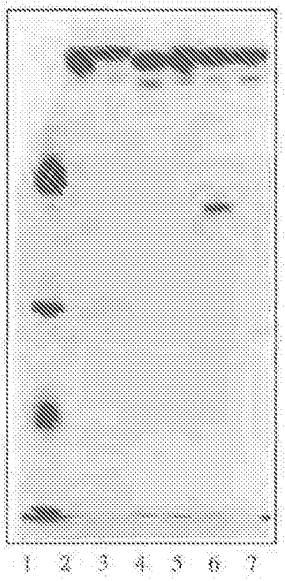


Figure 2.11 SDS-PAGE of an isotype series of the mouse:human chimeric antibody B72.3 under non-reducing conditions; the presence of a band of non-disulphide-linked molecules is observed at approx. 80 kDa for IgG4: lane 1, molecular weight markers; lane 2, murine B72.3; lane 3, chimeric IgG1; lane 4, chimeric IgG2; lane 5, chimeric IgG3; lane 6, chimeric IgG4; lane 7, chimeric IgG4P, Ser-Pro mutant (see text for details)

SDS-PAGE non-covalent interactions are broken such that two bands are seen under non-reducing conditions, one of disulphide bonded IgG at approx. 155 kDa and one representing IgG with no inter-heavy chain disulphide bond at approx. 80 kDa (Figure 2.11). This can lead to confusion during analysis of purified antibody as the 80 kDa band (which is present at 5–25% depending on the particular IgG4 antibody) may be viewed as an impurity, although it is present in all natural IgG4 antibodies studied as well as recombinant antibodies (King *et al.*, 1992a). The core hinge region of human IgG4 contains the sequence CPSC, compared to IgG1 which contains the sequence CPPC. The serine residue present in the IgG4 sequence leads to increased flexibility in this region and therefore a proportion of molecules form disulphide bonds within the same protein chain (an intrachain disulphide) rather than bridging to the other heavy chain in the IgG tetramer to form the interchain disulphide (Bloom *et al.*, 1997). Changing the serine residue to a proline to give the same core sequence as IgG1 allows complete formation of inter-heavy chain disulphides in the IgG4 molecule, hence removing the 80 kDa band seen on SDS-PAGE (Angal *et al.*, 1993). This altered isotype, termed IgG4P, has been used for several chimeric and engineered human antibodies to overcome the double-banded gel pattern (e.g. Owens and Robinson, 1995).

It is also possible to produce recombinant antibodies of other classes which are functional in Fc receptor binding. Mouse:human chimeric IgM, IgA1, IgA2 and IgD molecules have all been expressed and have shown functional binding to their respective receptors (Bruggemann *et al.*, 1987; Morton *et al.*, 1993; Shin *et al.*, 1992). The properties of these constant regions are therefore also available for incorporation into recombinant

Samsung Bioepis Exhibit No. 1002
Page 5322

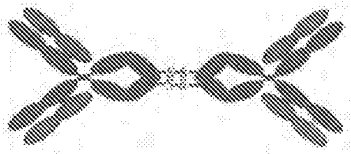


Figure 2.12 Dimeric IgG formed through mutation of a C_{H1} domain residue to cysteine (see text for details)

antibodies if required. In some cases these isotypes may offer advantages, for example a chimeric IgA2 has been used to translocate across an epithelial cell layer via the polymeric Ig receptor for IgA and therefore target tumour cells by reaching an antigen more abundant on the apical cell surface (Terskikh *et al*., 1994).

Although the amino acids involved in the interaction sites for Fc receptor binding and complement activation are beginning to be identified, at present the structural basis by which effector functions are elicited is not fully understood. Mutations in recombinant antibody molecules are playing a large role in elucidating recognition sites for individual functional effects. When the molecular basis of these interactions is understood it may be possible to tailor antibodies to bind to a chosen set of receptors and so design their *in vivo* properties.

The role of carbohydrate in maintaining the conformation of the IgG C_{H2} domains must also be considered. Removal of carbohydrate by mutation of the normal attachment site for N-linked carbohydrate at position 297 in the C_{H2} domain results in severe reductions in the ability of antibody to mediate effector functions (Tao and Morrison, 1989). This is probably a consequence of altered conformation of the antibody molecule (Lund *et al*., 1990). Recent studies have shown that mutation of amino acids involved in structural contacts with antibody carbohydrate can influence effector functions as well as the profile of sugar residues attached to the antibody itself (Lund *et al*., 1996). Manipulation of antibody carbohydrate structure may, therefore, represent another route towards engineering antibody effector functions.

Another approach to alter effector functions is the production of polymeric IgG antibodies. As described in Chapter 1, the ability of IgG to elicit complement activation is dependent on multiple molecules attaching to antigen to allow Fc clustering. Pentameric and hexameric IgM molecules contain multiple attachment sites for C1q binding which are revealed by a conformational change upon antigen binding and thus IgM molecules in general are more active at complement activation than IgG. Simple chemical cross-linking can be used to produce dimeric or polymeric species with increased antigen-binding avidity (Tsai *et al*., 1995). However, antibody engineering allows more controlled and reproducible site-specific linking strategies to be considered. Two approaches to engineering IgG molecules for increased effector function via multimerisation have been investigated. Substitution of a serine residue near the C-terminus of the C_{H3} domain (Ser444) to cysteine allowed the production of IgG dimer of a chimeric human IgG1 (Figure 2.12). This mutation resulted in 50% of the molecules forming dimeric IgG, reminiscent of IgA dimers, which were approx. 200-fold more potent in complement-mediated lysis (Shopes, 1992). The same mutation introduced into a humanised IgG1, huG1-M195, also resulted in the formation of IgG dimers which were more potent at both complement activation and ADCC (Caron *et al*., 1992a). In addition, dimers of an antibody to an internalising antigen such as huG1-M195 internalise into cells more rapidly than the IgG, which may offer advantages for delivery of cytotoxic agents to cells *in vivo*

Samsung Bioepis Exhibit No. 1002
Page 5323

(see Chapter 4). The second approach investigated has been the expression of IgG molecules with a tailpiece from the IgM μ chain which results in the production of IgM-like IgG polymers with up to six IgG units (Smith and Morrison, 1994; Smith *et al.*, 1995). All four human IgG isotypes have been shown to result in polymers using this approach, and as expected the ability of such multimeric molecules to mediate complement activation is increased, as is their ability to interact with the FcγRII receptor. Disadvantages include the heterogeneity of polymers produced, with species from monomers to hexamers present, activation of complement in the absence of antigen for some isotypes, and altered *in vivo* tissue distribution and clearance properties, with rapid loss of polymeric species from the circulation.

Another approach is the use of bispecific antibody fragments to redirect effector functions. Bispecific diabodies have been produced with one specificity for target antigen and the second for serum immunoglobulin (Holliger *et al.*, 1997). Such diabodies can retarget serum Ig to the target antigen and elicit a range of effector functions including complement activation, phagocytosis and ADCC. This potentially exciting strategy offers the potential to develop reagents capable of retargeting specific immunoglobulin types and thus their particular effector functions, or all immunoglobulins via a common epitope such as light chain constant domain (Holliger *et al.*, 1997). Similarly, more specific reagents may be produced through retargeting individual effector molecules. For example, a bispecific diabody with one specificity towards complement component C1q and the other against lysozyme as a model target antigen was capable of activating complement resulting in specific target cell lysis (Kontermann *et al.*, 1997).

2.6.2 Attachment of diagnostic or therapeutic agents

Chemical conjugates

Chemical conjugates of antibodies with drugs, radioisotopes, proteins or other molecules have been widely investigated, and a range of chemical approaches are available and widely used. Chemical conjugation is often the quickest route to the preparation of conjugates for many purposes, and in some instances conjugates with superior properties to those produced as fusion proteins may result (see below). Indeed, chemical conjugates of antibodies with enzymes or other reporter groups are a mainstay of the diagnostics industry (see Chapter 3). Chemical conjugation is, of course, the only option for the attachment of non-proteinaceous materials such as drugs to antibodies, and the ability to use a range of different linker chemistries allows variation in the properties of the reagents produced to be tested until the optimum is identified. The major issues in the production of such conjugates are the degree of substitution which can be achieved, the stability of the linkage and the biological activity of the resulting conjugate. Loss of some or all of the antigen-binding ability of the antibody is a common consequence of non-specific conjugation methods which may result from the modification of amino acids at or close to the CDR amino acid residues involved in antigen binding. This is more pronounced when antibody fragments are used, as there is more chance of randomly modifying a residue important for function simply due to the smaller number of amino acids present. Similarly, care must also be taken that the effector or reporter molecule attached is not damaged by the conjugation methodology. Therefore, choice of the conjugation method and control of the conjugation process to minimise such effects is a major

Samsung Bioepis Exhibit No. 1002
Page 5324

consideration. Other drawbacks of this approach include the heterogeneous nature of the conjugates produced which can lead to difficulties with batch-to-batch variation.

A variety of amino acid residues on the surface of antibody molecules can be used for chemical modification and coupling. Residues with useful functional groups for attachment include tyrosine (phenolic hydroxyl group), aspartic and glutamic acid (carboxyl group), lysine (amino group) and cysteine (thiol group). The tyrosine phenolic hydroxl group is reactive towards electrophilic agents, and its major role as a modification site is as a site for labelling of proteins with radioactive iodine. The carboxyl group of the acidic aspartate and glutamate residues can be used for coupling with carbodiimides such as EDC which react with both carboxyl and amine groups. However, such reagents are relatively non-specific and often lead to antibody aggregation and precipitation. The most useful and widely used sites for attachment to antibodies are the terminal amino group of surface lysine residues and the free thiol group of cysteine residues which can be generated by reduction of disulphide bonds in the hinge region. A large variety of reagents are available as linkers for attachment to these two groups under mild conditions which minimise damage to the antibody (Figure 2.13). Immunoconjugates which incorporate low molecular weight ligands are usually prepared using a derivative of the ligand with a suitable amino acid reactive group, whereas for protein conjugates a linker is required. Both homobifunctional linkers with two identical reactive groups and heterobifunctional linkers with reactive groups for different moieties are commercially available.

The most useful amine reactive groups include imidoesters and *N*-hydroxysuccinimide esters. Both of these groups react with the terminal amino group of lysine residues under relatively mild conditions (Figure 2.13), but many imidoesters require high pH (>9) for selective reactivity and can be reversible. Therefore *N*-hydroxysuccinimide esters have become the most popular reagents for linking to lysine residues due to their efficient coupling at physiological pH and the stability of the linkage. The amine groups at the N-terminus of the protein chains can also react with these linkers. Other lysine-reactive groups are not useful for protein–protein linkage but are commonly used for attachment of small chemical moieties such as drugs, or fluorescent reporter groups onto antibodies. For example, fluorescein and rhodamine isothiocyanates are commonly used for the attachment of these fluorescent groups to antibodies, and several amine-reactive groups have been used for polyethylene glycol attachment to antibodies (see Figure 2.2).

The cysteine thiol group is the most reactive amino acid side chain and is thus a useful site for specific conjugation. Thiol-reactive groups for antibody modification include maleimide, vinylsulphone, haloacetyl or pyridyl disulphide groups (Figure 2.13). Maleimides react selectively with cysteine residues at neutral pH, although there is reactivity with amine groups at higher pH values. A stable thioether bond is generated. Haloacetyl groups, usually containing iodoacetyl, are also useful reagents for introducing stable thioether bonds into immunoconjugates. These reagents are less selective than maleimides and at high concentrations can also react with histidine groups. Also, free iodine is generated under some conditions which may react with tyrosine, tryptophan or histidine residues causing unnecessary antibody damage. Pyridyl disulphides react with thiols to form a disulphide bond and are useful in situations when a less stable chemical linkage is required. A major advantage of coupling via pyridyl disulphides is the release of pyridine-2-thione which can be used to monitor the reaction spectrophotometrically at 343 nm. Antibody molecules do not normally contain a free thiol group as all of the available cysteine residues form disulphide bonds. Free thiols can be liberated by reduction of the hinge region of IgG, or of $F(ab')_2$ fragments to form Fab′ which is a method of site-specific labelling (see below). Alternatively, surface lysine residues can be modified to

Samsung Bioepis Exhibit No. 1002
Page 5325

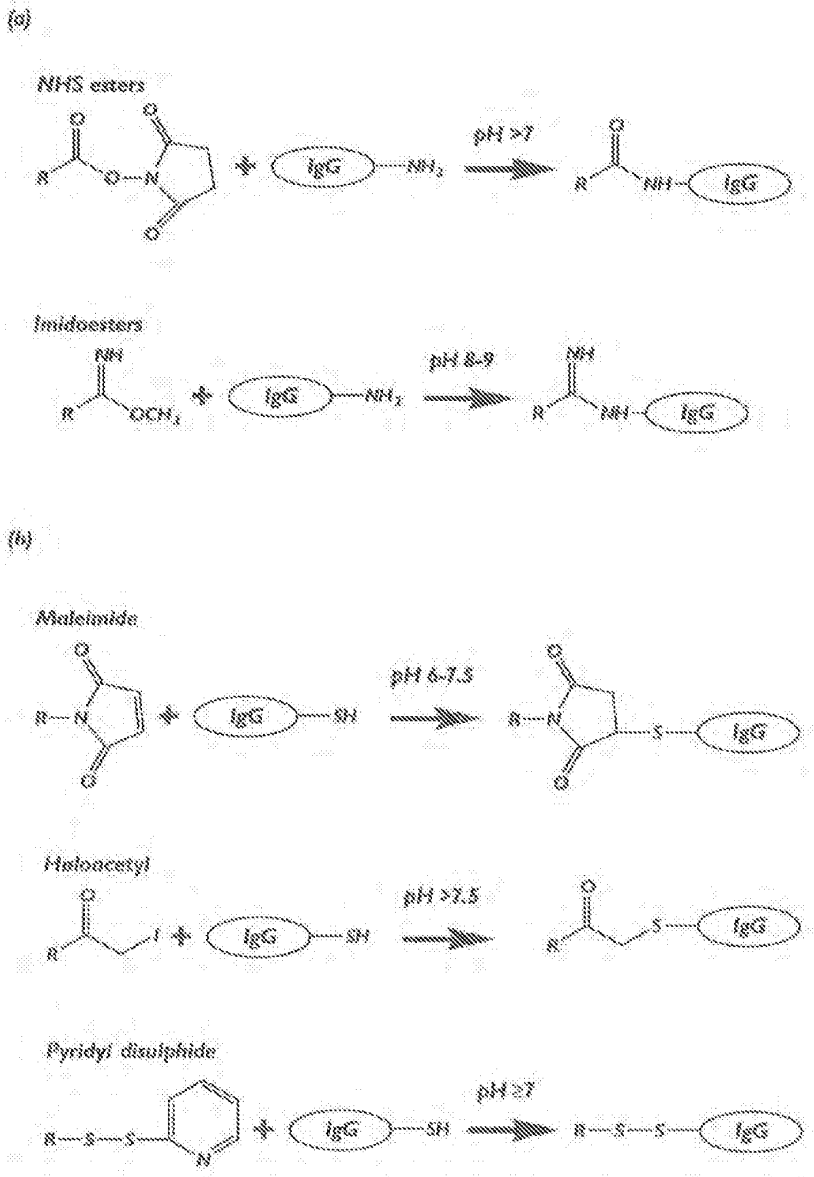


Figure 2.13 Examples of amine and thiol reactive linkers commonly used for chemical modification of antibodies: (a) lysine reactive linkers such as NHS esters and imidoesters; (b) thiol reactive linkers such as maleimide, haloacetyl and pyridyl disulphide

introduce a thiol group using reagents such as 2-iminothiolane, SATA or SPDP (Figure 2.14), which in turn can be used as an attachment point. One advantage of modifying lysine residues with thiol groups in this way is to achieve good control over the conjugation reaction, and thus minimise loss of biological properties of the conjugate. While

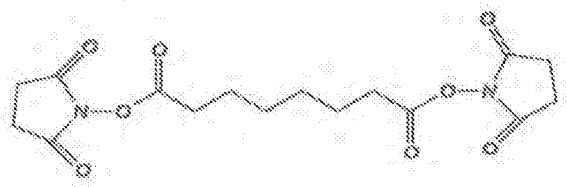
Samsung Bioepis Exhibit No. 1002
Page 5326

(a) Homobifunctional

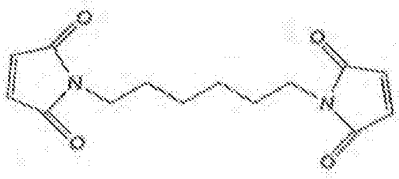
Glutaraldehyde



DSS

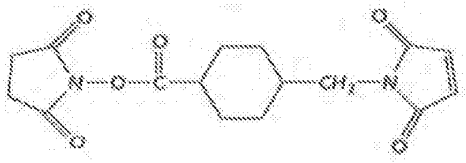


BMH

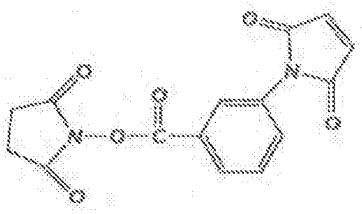


(b) Heterobifunctional

SMCC



MBS



SPDP

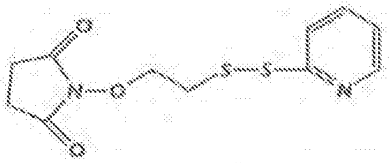


Figure 2.14 Some useful commercially available protein cross-linking reagents: (a) homobifunctional reagents glutaraldehyde, disuccinimidyl suberate (DSS) and bis-maleimidohexane (BMH); (b) heterobifunctional reagents, succinimydl 4-N-maleimidomethylcyclohexane-1-carboxylate (SMCC), maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) and N-succinimidyl-3-(2-pyridyldithiol)propionate (SPDP)

Samsung Bioepis Exhibit No. 1002
Page 5327

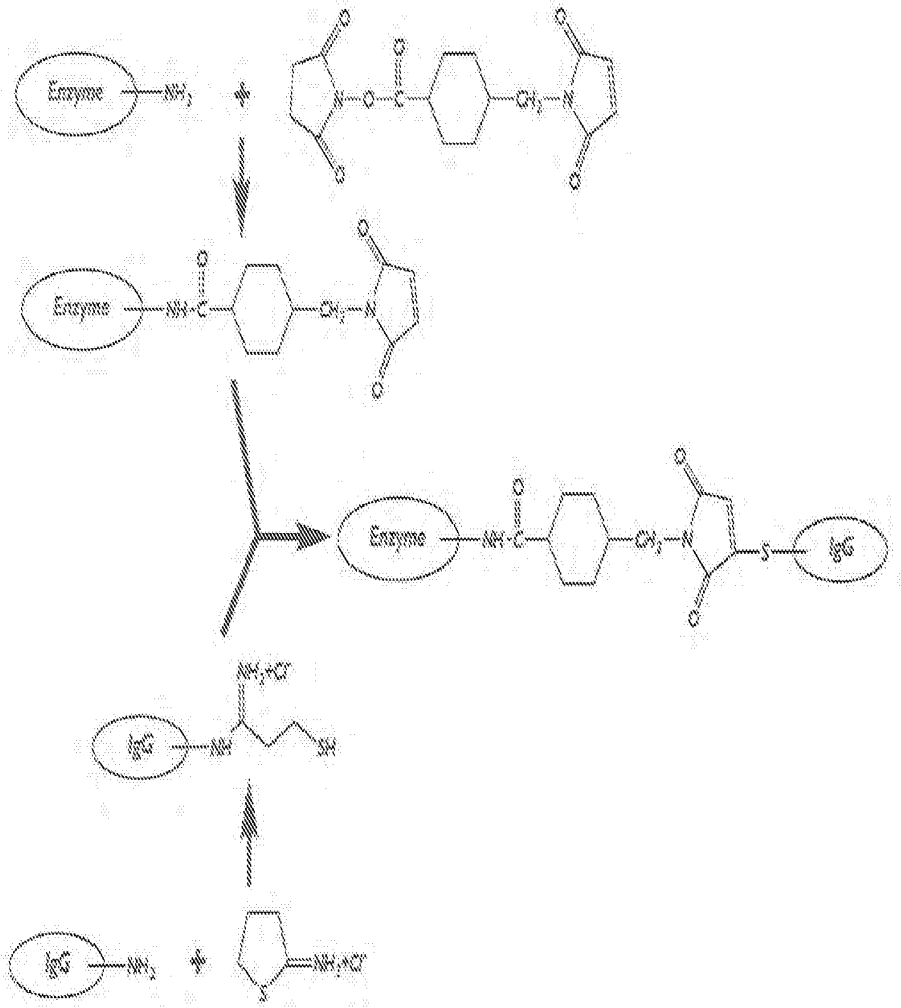


Figure 2.15 Example reaction scheme for attachment of an enzyme to antibody using SMCC attachment to enzyme and 2-iminothiolane activation of IgG

titration of lysine groups to determine the level of substitution is an insensitive procedure, the presence of one thiol group per antibody can be easily measured using reagents such as dithionitrobenzoic acid (DTNB) or dithiodipyridine (DTDP).

Both homobifunctional and heterobifunctional reagents are readily available (Figure 2.14). Homobifunctional cross-linkers which are either lysine-reactive or thiol-reactive can be used for IgG conjugation, although in some cases their use can result in unacceptable aggregation. The use of heterobifunctional linkers often allows more control of the conjugation reaction. Heterobifunctional reagents with one thiol-reactive and one amine-reactive group are particularly useful for the production of stable conjugates with minimum aggregation, such as antibody–enzyme conjugates. A typical reaction strategy is shown in Figure 2.15. The introduction of such a stable linkage is desirable in many cases though for some applications, for example in the construction of immunotoxins (see Chapter 4), a reducible linkage has been shown to be useful. In these cases linkers such as SPDP can be used to form a disulphide linkage after reaction of SPDP with each reagent individually, or alternatively heterobifunctional reagents with a disulphide group between the two functional groups can be used. In a similar way, sites for attachment of other functionalities can be designed into the linkers used. For example, sites for radiolabelling either with iodine or by chelation of radioactive metals can be incorporated.

Site-specific attachment

As a consequence of the loss of antigen-binding avidity often seen on conjugation, several methods for attachment of reagents at specific sites away from the antigen-binding site have been investigated (Table 2.5). As mentioned above, the generation of free cysteines by selective reduction of the hinge region has been used for both IgG and antibody fragments, for example to attach fluorescent groups (Packard *et al.*, 1986) or for site-specific radiolabelling (King *et al.*, 1994). The Fc region carbohydrate also provides a natural specific attachment site for IgG molecules. Carbohydrate is usually modified by periodate oxidation to generate reactive aldehydes which can then be used to attach reactive amine containing compounds by Schiff base formation. As the aldehydes can react with amine groups, reactions are carried out at low pH so that the lysine residues are protonated and unreactive. Hydrazide groups are most suitable for attachment to the aldehydes generated since they are reactive at low pH to form a hydrazone linkage. The linkage can then be further stabilised by reduction with sodium cyanoborohydride to form a hydrazine linkage (Figure 2.16). Comparisons of conjugates made by attachment to

Table 2.5 Approaches to site-specific labelling of antibodies and antibody fragments

Strategy	Reference
Modification of hinge cysteine residues	Packard *et al.*, 1986
Introduction of surface cysteines in constant region domains by protein engineering	Lyons *et al.*, 1990
Modification of Fc carbohydrate	Rodwell *et al.*, 1986
Introduction of glycosylation site to light chain by protein engineering	Leung *et al.*, 1995
Introduction of extra lysine residues by protein engineering	Hemmiki *et al.*, 1995
Addition to C-terminus of antibody fragments by reverse proteolysis	Fisch *et al.*, 1992

Samsung Bioepis Exhibit No. 1002
Page 5329

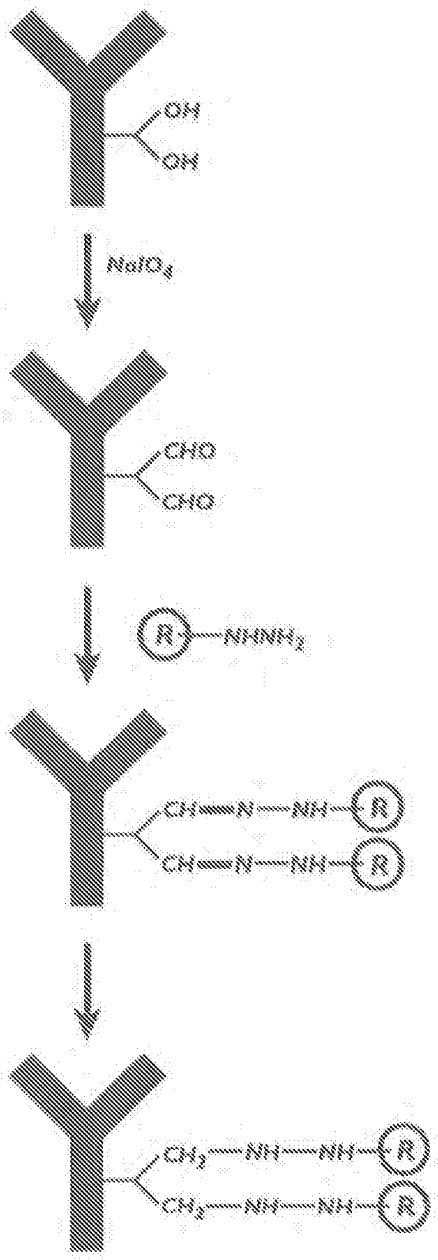


Figure 2.16 Reaction scheme for site-specific attachment to antibody carbohydrate via periodate oxidation

Samsung Bioepis Exhibit No. 1002
Page 5330

Fc carbohydrate with conventional conjugates with ligands randomly attached to lysine residues have demonstrated significantly improved antigen-binding properties (Rodwell *et al.*, 1986). The disadvantages of this approach are the relatively harsh conditions required, which can damage and aggregate some antibody molecules. Methionine residues present in some antibody variable regions may be particularly susceptible to oxidation by periodate, which can lead to loss of antigen-binding avidity. In some cases histidine or tryptophan residues might also be affected.

Antibody engineering can be used to introduce specific attachment sites into antibody molecules, and this can be incorporated as part of the design of an engineered molecule. Extra cysteine residues can be introduced onto the surface of antibody constant domains to provide a specific attachment site without the need to disrupt native disulphide bonds. Introduction of specific cysteine sites in the C_{H1} domain of the IgG heavy chain has been shown to result in sites to which ligands can be attached without any loss of antigen binding (Lyons *et al.*, 1990). Alternatively, extra carbohydrate sites can be engineered into the molecule to allow attachment via periodate oxidation. Some antibody light chains have an unusual natural glycosylation site, and thus the light chain has been used as a site to introduce a glycosylation site into antibodies which do not normally have carbohydrate attached to the light chain (Leung *et al.*, 1995). A third engineering strategy is to introduce extra lysine residues into the surface of the constant region domains (Hennuniki *et al.*, 1995). Although this approach does not introduce a unique labelling site, lysine-reactive reagents are more likely to modify the antibody at the increased concentration of lysine residues in the constant region, resulting in the retention of more antigen-binding reactivity.

A more specialised approach is the use of reverse proteolysis to attach reagents specifically at the C-terminus of Fab′ heavy chains (Fisch *et al.*, 1992). After production of a $F(ab')_2$ fragment by the protease lysyl endopeptidase, experimental conditions can be altered such that the same protease working in reverse is capable of the specific attachment of carbohydrazide groups to the C-terminus of the $F(ab')_2$ heavy chains. These carbohydrazide groups could then be used as an attachment point for a radiolabelled chelator reacting via an aldehyde group to form a hydrazone linkage. A similar approach has also been used to attach an aldehyde-modified enzyme to the C-terminus of $F(ab')_2$ (Werlen *et al.*, 1994).

Fusion proteins

An attractive alternative to chemical conjugation for antibody–protein conjugates is the direct expression of fusion proteins. This approach can be viewed as another method of site-specific attachment, as the protein to be fused is attached away from the antigen-binding site and no further modification is necessary. The antibody or antibody fragment is fused to the protein at the gene level, usually via attachment of the sequence to either full length or a truncated heavy chain. Expression of functional fusion proteins was first shown by fusion of a Fab fragment to Staphylococcal nuclease (Neuberger *et al.*, 1984). Since then the production of active antibody and antibody fragment fusion proteins with a variety of different enzymes, cytokines, and other proteins has been achieved (Table 2.6). Fusion proteins overcome problems with loss of antigen-binding ability on chemical conjugation and are also well-defined, homogeneous reagents. Disadvantages are that the flexibility of using linkers with varying chemical stability is lost, and in some cases fusion proteins have proved difficult to express in a functional form.

Samsung Bioepis Exhibit No. 1002
Page 5331

Table 2.6 Some examples of antibody fusion proteins

Antibody form	Fusion	Reference
Enzyme fusions		
$F(ab')_2$	Staphylococcal nuclease	Neuberger *et al.*, 1984
scFv, dsFv	β-lactamase	Goshorn *et al.*, 1993 Rodrigues *et al.*, 1995
Fab′	β-glucuronidase	Bosslet *et al.*, 1992
scFv, $F(ab')_2$	Alkaline phosphatase	Wels *et al.*, 1992 Ducancel *et al.*, 1993
scFv	Urokinase	Heivoet *et al.*, 1991
Toxin fusions		
scFv, dsFv	Pseudomonas exotoxin	Brinkmann *et al.*, 1991 Reiter *et al.*, 1996
scFv, Fab, $F(ab')_2$	Gelonin	Better *et al.*, 1995
Fab′, scFv	Angiogenin	Rybak *et al.*, 1992 Newton *et al.*, 1996
scFv	Eosinophil-derived neurotoxin	Newton *et al.*, 1994
Cytokines and growth factors		
scFv, $F(ab')_2$	IL-2	Fell *et al.*, 1991 Dorai *et al.*, 1994
IgG, Fab	IL-2, GM-CSF TNFα, TNFβ	Gillies *et al.*, 1992 Gillies *et al.*, 1993
Fab, $F(ab')_2$, IgG	IGF-1, IGF-2	Shin *et al.*, 1994
Other proteins		
$F(ab')_2$	Aequorin	Casadei *et al.*, 1990
Fab, $F(ab')_2$, IgG	Avidin	Shin *et al.*, 1997
Fab	Biotin carboxyl carrier protein	Weiss *et al.*, 1994
Fab, $F(ab')_2$, IgG	Transferrin	Shin *et al.*, 1995
Fab	Staphylococcal enterotoxin A	Dohlstein *et al.*, 1994
scFv	Protein A fragment	Tai *et al.*, 1990
$F(ab')_2$	Metallothionein	Das *et al.*, 1992

The choice of antibody form for fusion purposes depends on the other properties of the molecule required, e.g. overall size, pharmacokinetics and valency. As shown in Table 2.6, fusion proteins with single-chain Fv, disulphide-linked Fv, Fab, $F(ab')_2$ and IgG have all been constructed. However, the choice of construct can have unexpected effects on the assembly and activity of the fusion protein. A series of fusions of interleukin-2 (IL-2) to the heavy chain of the chimeric anti-ganglioside antibody ch14.18 have been made with addition directly after the Fab such that the entire Fc is replaced by IL-2, after C_{H2} such that the C_{H2} domain is replaced with IL-2, and fusion to the end of the entire heavy chain (Gillies *et al.*, 1992). The Fab fusion was inactive in antigen binding and the fusion

Samsung Bioepis Exhibit No. 1002
Page 5332

to C_H2, although capable of binding antigen, did not assemble correctly to the divalent species even though a full-length hinge region was present. The fusion to the end of the entire heavy chain resulted in enhanced antigen binding of the antibody and the molecule was shown to be fully assembled. The IL-2 fused to the antibody remained active although the antibody effector functions were slightly reduced.

In some case spacer peptides have been included between the antibody and fused protein which may assist folding or activity of the fused protein. For example, a linker between an scFv toward the transferrin receptor and the RNAse angiogenin was shown to be required for optimal activity of both the scFv and the enzyme (Newton *et al.*, 1996). Also, spacers can be designed to allow proteolytic release of the fused protein when internalised into cells. Other choices in the nature of the construct also need to be made, as the fusion can be made at the C-terminus or N-terminus of the antibody chain and to heavy or light chain. In a study of antibody fragment fusions to the plant toxin gelonin, fusions to Fab, $F(ab')_2$ and scFv were made at either the N- or C-terminus (Better *et al.*, 1995). Of these immunotoxins, the divalent $F(ab')_2$ variants were the most potent, as might be expected due to divalent antigen binding and possibly more effective internalisation into target cells. scFv and Fab conjugates with gelonin fused at the N-terminus or C-terminus of the light or heavy chain were all approximately equipotent. Linkers were examined with sites for trypsin-like proteases or with sites for the lysosomal enzymes cathepsin B and D. These are expected to be cleaved intracellularly and may assist gelonin release and hence cytotoxicity. However, in this study there was little difference between the cytotoxicity of the constructs with and without the cleavable linkers, suggesting that the fusion proteins themselves may be active without the need for release of free gelonin.

The types of protein used in fusion proteins includes those used to induce dimerisation (Section 2.4.2), peptide or protein tags used to simplify antibody fragment purification (see Chapter 5), enzymes for both therapeutic and diagnostic purposes, cytokines and growth factors, and many others as shown in Table 2.6. Fusions can also be used to couple some non-proteinaceous materials to antibodies via specific binding proteins. Fusion of the metal-binding protein metallothionein to antibody fragments can be used to allow subsequent binding of the radionuclide ^{99m}Tc (Das *et al.*, 1992). A small peptide which can chelate ^{99m}Tc has also been used effectively (George *et al.*, 1995). Another approach to radiolabelling is to attach a peptide sequence which can be enzymically labelled with ^{32}P (Neri *et al.*, 1996). Non-proteinaceous materials which are naturally bound by other proteins can be attached by fusion of a suitable 'acceptor' sequence to the antibody fragment followed by expression in *E. coli*. In this way lipid-tagged scFv fragments have been produced by fusion of the amino terminal part of the *E. coli* major lipoprotein, which is sufficient for attachment of lipid during expression (Laukkanen *et al.*, 1993). Biotin can be attached via the biotin-carboxyl carrier protein (BCCP) subunit of *E. coli* acetyl-CoA carboxylase (Weiss *et al.*, 1994). In this case a Fab-BCCP fusion was expressed in *E. coli* and biotin was attached during secretion of the fusion protein.

2.7 Engineering pharmacokinetics and biodistribution

For therapeutic applications of MAbs, the pharmacokinetics and biodistribution of the agent to be used in humans is of great importance. The degree of binding to the target antigen *in vivo* is dependent not only on binding characteristics of the antibody such as affinity and avidity which can be measured *in vitro*, but also on a variety of other factors such as the accessibility of the antigen, the concentration, and the time of exposure of the

Samsung Bioepis Exhibit No. 1002
Page 5333

antibody to the target antigen. Access to antigens in the vascular compartment is relatively straightforward when antibodies are injected intravenously, but access to other body tissues is usually more difficult. Such extravascular targets require the antibody to pass through the endothelial cell layer lining the blood capillaries to reach interstitial fluid compartments. The permeability of blood vessels to proteins varies according to the size of the protein, with smaller proteins able to reach interstitial spaces more quickly. Therefore, large proteins such as intact antibody molecules penetrate into tissues relatively slowly, while antibody fragments penetrate tissues more quickly. However, the amount of antibody to reach the target tissue is also dependent on the concentration in circulation and the rate of clearance from the blood, and therefore the study of antibody pharmacokinetics has an important role to play.

In both human and animal systems, antibody clearance from the blood follows a classical two-compartment kinetic model. Initially, a short distribution phase, or alpha phase, takes place during which the antibody is distributed throughout body tissues, i.e. equilibrates between intravascular and extravascular compartments. This is followed by an elimination or clearance phase (beta phase) in which the antibody is metabolised and excreted. The rates of these two phases are commonly calculated and quoted as half-life values ($t_1\alpha$ and $t_1\beta$). Alternatively, a single-compartment kinetic model may be used, in which case a single half-life can be calculated.

2.7.1 Pharmacokinetics of IgG

Human IgG molecules have a long circulating half-life in humans, with $t_1\alpha$ of 18–22 hours and $t_1\beta$ of 21–23 days for human IgG1, 2 or 4 (reviewed by Mariani and Strober, 1990). Human IgG3 has a shorter half-life with a $t_1\beta$ of approximately 9 days. This may be due to the extended hinge region of IgG3 which makes the molecule more susceptible to proteolytic cleavage. Human IgM is cleared more quickly, with a half-life of approximately 5 days. This very large protein remains largely in the vascular compartment and thus pharmacokinetic data are normally described using a one-compartment kinetic model. The pharmacokinetics of human IgG are unusual in that the half-life varies with concentration. At very high IgG levels, such as those seen in myeloma patients which can approach 100 mg per ml of serum, the half-life is decreased to similar values to IgM, while in patients with hypogammaglobulinemia with reduced plasma IgG levels a prolonged half-life is observed. It has been postulated that a receptor-mediated event is thus responsible for maintaining the long circulating half-life for IgG, through IgG binding, preventing degradation and resulting in recirculation to the plasma (Brambell *et al.*, 1964). This hypothesis has been supported by subsequent experimental investigations. It is possible to calculate the amount of IgG which is protected from degradation by this receptor-mediated process in humans at full saturation. This is approximately 150 mg per kg body weight per day (Mariani and Strober, 1990).

It has been known for some time that this receptor-mediated recycling is mediated by the Fc region of the antibody, as isolated Fc has a long half-life, whereas Fab and $F(ab')_2$ are cleared relatively rapidly. Also, co-administering Fc with IgG can result in reduced IgG half-life in a similar manner to increasing the IgG dose. Recently the amino acids in the Fc fragment important for maintaining IgG in circulation in mice have been elucidated by site-directed mutagenesis studies (Kim *et al.*, 1994; Ghetie *et al.*, 1996). The receptor binding site is located at the interface region between the C_H2 and C_H3 domains and overlaps with the binding site for Staphylococcal protein A, commonly used for purification of IgG (see Chapter 5). Thus protein A complexes are rapidly cleared due to

Samsung Bioepis Exhibit No. 1002
Page 5334

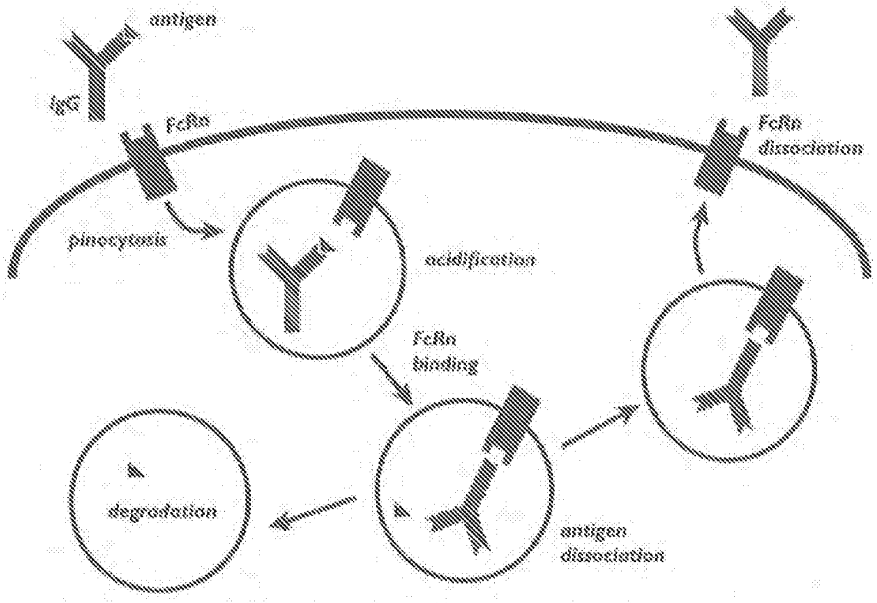


Figure 2.17 Mechanism of FcRn-mediated salvage of IgG from catabolic degradation: the low pH of the endosome results in tight binding of IgG to the FcRn receptor, leading to protection from degradation and return to the cell surface where the higher pH allows re-release of the IgG into circulation

competition with the receptor-mediated process, and isolated Fc fragments have the same $t_{\frac{1}{2}}\beta$ as the whole IgG molecule. The cellular receptor responsible for maintaining half-life has been identified as the neonatal Fc receptor, FcRn. This is the same receptor which is responsible for transfer of IgG from the mother to the neonate which provides immunity for the first few weeks after birth. Mice deficient in this receptor are unable to acquire IgG from the mother, have reduced plasma IgG levels and clear administered IgG or Fc with an abnormally short half-life (Ghetie *et al.*, 1996). The FcRn-mediated pathway for maintaining high IgG levels is thought to operate through its pH dependence (Figure 2.17). IgG is strongly bound to FcRn at low pH and dissociates at higher pH. IgG is internalised into cells and salvaged from the endosome during acidification due to binding to FcRn at low pH. Low pH may also strip any bound antigen from the antibody which would then be degraded in the lysosome. Antibody is then recycled to the cell surface where the higher pH of the extracellular fluid promotes dissociation from FcRn and return of the IgG to the circulation.

Many murine MAbs have been examined in human studies in a variety of diagnostic and therapeutic situations. The half-lives of such murine antibodies vary both from antibody to antibody and from patient to patient, and depend on a number of factors including isotype and the presence of circulating antigen. In general, $t_{\frac{1}{2}}\beta$ values for murine antibodies in humans have been in the range of 15–48 hours on the first administration. Such studies are complicated by human immune responses to murine antibodies (HAMA, see Section 2.3) which result in rapid clearance of murine antibodies on repeat injection. There is now a growing collection of data on the pharmacokinetics of chimeric and humanised antibodies in man, some of which is summarised in Table 2.7. These data are

Samsung Bioepis Exhibit No. 1002
Page 5335

Table 2.7 Pharmacokinetics of some chimeric and humanised antibodies in humans

Antibody	Indication	$t_{1/2\alpha}$	$t_{1/2\beta}$	Reference
c17-1A (γ1)	Colorectal carcinoma	18 ± 2 hours	181 ± 16 hours	LoBuglio *et al.*, 1989 Meredith *et al.*, 1991
cB72.3 (γ4)	Colorectal carcinoma	18 ± 7 hours	224 ± 66 hours	Khazaeli *et al.*, 1991 Meredith *et al.*, 1992a
c14.18 (γ1)	Melanoma	24 ± 1 hours	181 ± 73 hours	Saleh *et al.*, 1992
cL6 (γ1)*	Carcinoma		84–109 hours	Goodman *et al.*, 1992
C2B8 (γ1)*	B-cell lymphoma		186 (38–232 hours)	Maloney *et al.*, 1994
cAnti-CEA (γ4)*	Colorectal carcinoma	7 (1.4–18 hours)	91 (30–202 hours)	Buchegger *et al.*, 1995
hu3PLAP (γ1)*	Carcinoma		73.1 ± 30.2 hours	Hird *et al.*, 1991
Humanised anti-Tac(γ1)*	Graft v. host disease		80† hours (44–360)	Anasetti *et al.*, 1994
Humanised M195 (γ1)*	Myeloid leukemia	0.3 ± 0.4 hours	38 ± 9 hours	Caron *et al.*, 1994
CDP571 (γ4)	Healthy volunteers	19.6 hours (1–49)	225† hours (87–537)	Stephens *et al.*, 1995
BMN-14 (γ1)*	CEA producing cancer	8.3 ± 6.2 hours	56 ± 32 hours	Sharkey *et al.*, 1995
rhuMAb HER2 (γ1)	Breast cancer		199 ± 120 hours	Baselga *et al.*, 1996
hCTM01 (γ4)*	Ovarian cancer	4.2 ± 6.1 hours	64.7 ± 20.2 hours	van Hof *et al.*, 1996

*In these cases circulating antigen, or large pools of readily accessible antigen, are known to be present which may reduce antibody half-life in some cases.
†Increase in t_β with increasing dose.

Samsung Bioepis Exhibit No. 1002
Page 5336

Table 2.8 Approaches used to manipulate antibody pharmacokinetics

Use of particular isotypes
Clearing regimes: anti-antibody *in vivo*
avidin–biotin system
extracorporeal immunoadsorption
Chemical modification: galactosylation
Engineering antibody domain deletions
Antibody fragments

difficult to compare due to different antibodies being used in a variety of different clinical conditions with, in some cases, the presence of pools of circulating antigen, or because some of the antibodies used target readily available antigen on blood cells. However, it is apparent that the $t_1\beta$ values for chimeric and humanised antibodies are consistently higher than those for murine antibodies. In some instances the same antibody has been administered to man in both murine and chimeric forms. In all of these cases the antibody half-life is significantly longer for the chimeric antibody, for example the $t_1\beta$ of 17-1A was increased from 16 hours for the murine antibody to 101 hours for the chimeric IgG1 version, and that of B72.3 from 24–48 hours for the murine antibody to 224 hours for the chimeric IgG4 (LoBuglio *et al.*, 1989; Khazaeli *et al.*, 1991). In the one report to date of the pharmacokinetics of a humanised antibody in healthy volunteers, the anti-TNF antibody CDP571 had a half-life which approached the 21 day half-life of normal human IgG4 (Stephens *et al.*, 1995), suggesting that in healthy subjects at least, the normal mechanisms for regulation of IgG half-life described above also apply to humanised antibodies.

The long half-life of humanised antibodies is ideal for some applications, for example in providing protection against infection, or providing long-term neutralisation of a cytokine. However, antibodies have also been widely investigated for their ability to deliver diagnostic or therapeutic agents such as drugs, radionuclides or protein toxins to sites such as tumour cells in attempts to detect and/or destroy them. In these cases a long circulating half-life can result in unacceptable toxicity. For example, a circulating antibody–radionuclide conjugate will irradiate other tissues and limit the amount of radioactivity that can be delivered to a tumour. The ideal molecule would target the toxic agent to the tumour cells and clear rapidly from the rest of the body. Therefore there has been much interest both in strategies to remove antibody from circulation more rapidly and in designing molecules with shorter *in vivo* half-lives. Some approaches to this problem are summarised in Table 2.8.

The choice of a particular isotype for an engineered antibody can influence the pharmacokinetic properties of the antibody. The γ1 and γ4 isotypes of chimeric B72.3 have been compared directly for their pharmacokinetics in cynomolgous monkeys (Hotzell *et al.*, 1991). The $t_1\beta$ was 90 hours for the γ1 and 262 hours for the γ4 isotype. Although direct comparisons have not been done in humans, the same trend has been observed (see Table 2.7), with γ1 antibodies usually clearing faster than γ4. However, the clearance of γ1 antibodies is still slow, and other strategies are required to reduce the half-life further.

2.7.2 *Pharmacokinetics of antibody fragments*

As the catabolic site for IgG lies within the Fc domain, there is no specific mechanism for maintaining the serum level of $F(ab')_2$ or smaller antigen-binding fragments and these are

Samsung Bioepis Exhibit No. 1002
Page 5337

cleared from circulation more rapidly than IgG. One of the most useful approaches to developing molecules with short *in vivo* half-life has thus been the use of antibody fragments. Small antibody fragments are filtered through the kidney, from the glomerular capillaries to the renal tubules, and thus rapidly excreted. The nominal molecular weight cut-off for kidney filtration is believed to be approx. 60–70 kDa, and thus Fab and Fv fragments are rapidly cleared through this route. During this process antibody fragments are often re-absorbed by the kidney tubules and metabolised. As well as shorter $t_1\beta$ values, antibody fragments also distribute to, and penetrate, tissues more rapidly, which is reflected in a shorter α phase. For example, the ability to penetrate into tumour tissue has been shown to be size-related, with smaller fragments demonstrating faster penetration (Yokota *et al.*, 1992).

There have been many studies comparing the pharmacokinetics of IgG with $F(ab')_2$ and Fab fragments in animal models. In the majority of studies Fab clears faster than $F(ab')_2$, which is considerably faster than IgG. For example, analysis of the murine antibody MOPC21 revealed the residence time in the body for IgG, $F(ab')_2$ and Fab to be 8.5, 0.5 and 0.2 days respectively (Covell *et al.*, 1986). A similar pattern was observed with B72.3 Fab, $F(ab')_2$ and IgG labelled with either of two different radioisotopes (Brown *et al.*, 1987). In several human studies $F(ab')_2$ has been shown to be cleared more rapidly than IgG. For example, Buist *et al.* (1993) found a plasma elimination half-life of 70 hours for chimeric MOv18 and 20 hours for $F(ab')_2$. However, this is not the case for all studies. In a trial of ^{131}I-radiolabelled anti-CEA antibody a similar half-life was observed for IgG and $F(ab')_2$ (Lane *et al.*, 1994).

$F(ab')_2$ molecules from different IgG isotypes have different pharmacokinetic properties. A comparison of chimeric $F(ab')_2$ of the human IgG isotypes γ1, 2 and 4 revealed that the γ2 version was retained in circulation longer than the other isotypes (Buchegger *et al.*, 1992). This pattern may reflect the number of disulphide bonds in the hinge region, 4 for γ2 , 2 for γ1 and γ4, as $F(ab')_2$ with more disulphide bonds between the two heavy chains may take longer to be metabolised, either reductively or proteolytically, *in vivo*. As $F(ab')_2$ is above the molecular weight cut-off for rapid kidney clearance, metabolism to smaller fragments probably plays an important role in $F(ab')_2$ pharmacokinetics. Recombinant $F(ab')_2$ of the same isotype with different numbers of disulphide bonds inserted in the hinge region also demonstrated that more disulphide bonds results in a longer half-life (Rodrigues *et al.*, 1993). Similarly, $F(ab')_2$ with a chemically cross-linked hinge region has been shown to have a longer residence time in the blood than $F(ab')_2$ linked by a single disulphide bond (Rodrigues *et al.*, 1993; King *et al.*, 1994).

Fv and scFv fragments have also been examined in experimental systems and shown to clear very rapidly (Milenic *et al.*, 1991; King *et al.*, 1992b). A comparison of scFv with other forms of the antibody CC49 showed very rapid plasma and whole body clearance for the scFv in both mouse and monkey studies, with 80% of scFv lost from the plasma in 15 minutes in mice and 95% removed from the plasma of monkeys by 30 minutes (Milenic *et al.*, 1991). Calculation of $t_1\alpha$ and $t_1\beta$ values can be misleading due to not taking account of the duration of each phase which may differ between antibody fragment forms. However, results for comparisons of some fragment forms of two antibodies, one murine and one chimeric, are demonstrated in Table 2.9. Differences in overall values probably reflect differences in the method of calculation used in each case, but it is easily seen that the overall trends are maintained between fragments. The pharmacokinetics of divalent scFv fragments prepared by a number of techniques including disulphide linkage, chemical cross-linking and the use of dimerisation domains have also been examined (Adams *et al.*, 1993; King *et al.*, 1994; Hausschild *et al.*, 1995). In

Samsung Bioepis Exhibit No. 1002
Page 5338

Table 2.9 Pharmacokinetics of antibody fragments in mice: data for CC49 from Milenic *et al.* (1991) and for cB72.3 from King *et al.* (1994); all values in hours

	CC49		cB72.3	
	$t_{1/2}\alpha$	$t_{1/2}\beta$	$t_{1/2}\alpha$	$t_{1/2}\beta$
IgG	0.65	113	11.1	56.4
$F(ab')_2$	0.43	12	0.87	26.1
Cross-linked $F(ab')_2$			3.14	21.2
Fab'	0.15	1.3		
scFv	0.06	1.5	0.23	4.0

all cases the half-life of the dimeric species is increased only slightly, the di-scFv still being rapidly removed from circulation. The preparation of tri-scFv results in a further small increase in half-life, though these multivalent molecules are still cleared rapidly (King *et al.*, 1994).

Further control of half-life of antibody fragments can be exerted by PEGylation as described in Section 2.3.3. PEGylation of many different proteins, including antibody fragments, has resulted in conjugates with increased half-life. Comparison of the *in vivo* properties of IgG, $F(ab')_2$ and Fab' fragments with and without PEG attached revealed that PEGylated IgG was relatively unaffected, whereas the half-life of $F(ab')_2$ and Fab' was increased by PEGylation (Pedley *et al.*, 1994). Application of this technology to smaller Fv-based fragments allows the preparation of a series of molecules with a range of different half-lives which can then be selected for a particular application.

Selective engineering of the IgG molecule can also be carried out to affect half-life. Removal of antibody carbohydrate in the C_{H2} domain can be achieved by substitution of the Asn residue at position 297 which is normally glycosylated. Such aglycosyl antibodies may have a reduced half-life, although this appears to be dependent on isotype. An aglycosylated mouse:human chimeric IgG4 and chimeric IgG3 antibody cleared faster than the native IgG, whereas aglycosyl chimeric IgG1 was relatively unaffected (Tao and Morrison, 1989; Rhind *et al.*, 1990). Aglycosyl mouse IgG2b also clears more rapidly than the glycosylated antibody (Wawrzynczak *et al.*, 1992). This may reflect differences in the role of the antibody carbohydrate in maintaining the conformation of the two C_{H2} domains, which in turn would affect FcRn interaction. Alternatively, differing conformational effects between isotypes may result in increased susceptibility to proteolysis for those isotypes which clear rapidly in aglycosyl form. More subtle variation in the carbohydrate structure can also affect half-life. A chimeric IgG1 antibody produced in a mutant CHO cell line, which is incapable of processing a high-mannose intermediate through the terminal steps of the glycosylation pathway, has a shorter half-life than the same antibody produced in a wild-type CHO line (Wright and Morrison, 1994). This is probably a result of enhanced clearance through mannose receptors in the liver, as proteolytic stability of the antibody was unaffected.

The serum persistence of Fc has been increased by introducing mutations into the FcRn binding region (Ghetie *et al.*, 1997b). Random mutagenesis of the sequence around the FcRn binding site, followed by selection using phage display, allowed variants with higher affinity for FcRn to be isolated with serum half-life increased from 120 to 150 hours. Specific engineering to disrupt FcRn binding in an IgG has not yet been reported, although mutations in the C_{H2} and C_{H3} domains which have this effect in Fc fragments

Samsung Bioepis Exhibit No. 1002
Page 5339

have been described (Kim *et al.*, 1994). Mutation of these key residues may allow the production of antibodies with a range of serum half-lives. Removal of the entire C_H2 domain of the IgG heavy chain has been reported (Mueller *et al.*, 1990). The resulting truncated heavy chain still allows assembly of a tetrameric structure with full antigen-binding ability. The pharmacokinetics of such C_H2 deleted variants are similar to those of stabilised $F(ab')_2$ fragments with a $t_1\alpha$ of 1.5 hours and a $t_1\beta$ of 12 hours reported in mice (Mueller *et al.*, 1990). This again demonstrates disruption of the FcRn receptor binding site.

As described in Section 2.5, bispecific diabodies can be produced with one specificity for target antigen and one for serum immunoglobulin. This results in not only the ability to retarget effector functions, but also a significant increase in half-life compared to a control diabody (Holliger *et al.*, 1997). A β-phase half-life of 10 hours was reported for serum Ig targeted diabody, compared to less than 1 hour for the control diabody. This was still significantly less than the IgG alone, possibly as a result of dissociation of the diabody from the IgG during the low pH values experienced during FcRn-mediated recycling (Figure 2.17).

2.7.3 *Clearance*

A further approach to remove antibody from circulation has been to allow antibody to bind to its target for a period of time and then attempt to remove the residual antibody. Such 'clearance' strategies have been investigated particularly with radiolabelled anti-tumour antibodies. Clearance can be achieved using a second antibody with specificity for the anti-tumour antibody. After the desired time interval, injection of the second antibody results in binding to the radiolabelled antitumour antibody to form immune complexes. These are then rapidly removed by clearance through the liver and to a lesser extent other clearance organs. Initial work used liposomally entrapped second antibody to achieve this, although equivalent results were later generated with second antibody alone (Begent *et al.*, 1982; Pedley *et al.*, 1989). Effective clearance of the radiolabelled antibody has been observed using such systems with enhanced tumour : blood ratios of radioactivity allowing improved imaging of tumours, and resulting in less toxicity associated with blood-borne activity (Blumenthal *et al.*, 1989). A variation of this approach uses the high-affinity interaction between avidin and biotin to remove antibody from circulation. The initial radiolabelled antibody is conjugated to biotin before use and then can be cleared through administration of avidin or streptavidin (Paganelli *et al.*, 1991). A comparison of these two approaches found that superior clearance could be achieved with second antibody, with a larger and faster reduction in blood levels achieved (Marshall *et al.*, 1994).

Problems in using this approach include the relative complexity and expense of preparing and using a clearing agent. Also, the clearance system may result in the sudden deposition of large amounts of immune complex and consequently radioactivity into the liver, or other clearance organ, which may result in toxic effects. This can be overcome using a related method known as extracorporeal immunoadsorption. In this procedure blood is pumped outside the body through an extracorporeal shunt in which the radiolabelled antibody is removed by binding to an affinity column before being returned to the patient (Johnson *et al.*, 1991b). The affinity column can be prepared from either antigen or second antibody attached to a solid support. Clinical trials with this approach have proved that most of the circulating activity can be removed from the patient efficiently,

Samsung Bioepis Exhibit No. 1002
Page 5340

although the complexity and expense of such a procedure has meant that it has not been widely used.

2.7.4 *Chemical modification*

Chemical modification of antibody has also been used to alter half-life of antibody. Attachment of galactose, galactosylation, allows recognition of the conjugate by the asialoglycoprotein receptor in the liver resulting in very rapid clearance. This procedure was developed for use with antibodies administered locally, for example intra-peritoneally for ovarian cancer, such that when antibody leaked out from the peritoneal cavity to the blood, the circulating activity was rapidly removed (Mattes, 1987). However, penetration of antibody from the peritoneal cavity to ovarian tumours is not very effective and hence galactosylated antibody has also been evaluated for intravenous cancer therapy (Ong *et al.*, 1991). In this case it was necessary to inhibit the action of the asialoglycoprotein receptor for 2–3 days by injection of large amounts of asialo-bovine submaxillary mucin which initially saturates the receptor. Inhibition for 2–3 days allowed the galactosylated antibody to bind to experimental tumours in nude mice, after which time the inhibitor was cleared and antibody removed from the circulation. In common with the *in vivo* clearance mechanisms described above, this procedure can result in the rapid deposition of antibody in the liver, and thus is restricted to use with therapeutic agents which will not be toxic to liver cells. Radioiodinated antibody appears to be rapidly metabolised by the liver with the excretion of radio-iodine, and thus may be useful for therapy with such galactosylated conjugates (Ong *et al.*, 1991). But perhaps the most useful application of galactosylated antibody is as a second antibody clearing agent. Use of galactosylated second antibody increases the rate of removal of the first antibody from the circulation and has been applied to the removal of antibody–enzyme conjugates from circulation prior to prodrug therapy (Sharma *et al.*, 1990; see Chapter 4).

2.7.5 *Fc region to extend half-life*

The finding that antibody half-life is dependent on the Fc region as described above, and that isolated Fc regions have the same extended t_{β} as IgG molecules, has led to the use of the Fc region to extend the half-life of other proteins. CD4 is a receptor for the HIV virus, and has been of interest for AIDS therapy. However, soluble CD4 has a very short plasma half-life and was therefore difficult to examine *in vivo*. Fusion proteins between CD4 and IgG Fc, termed immunoadhesins, have been prepared which use the IgG Fc region to increase dramatically the half-life of the soluble CD4 protein (Capon *et al.*, 1989). Since then a similar approach has been used to produce Fc fusion proteins with a range of different proteins in attempts to improve half-life (Chamow and Ashkenazi, 1996). An additional property of many immunoadhesins is the retention of some Fc effector functions, and a range of applications are open to such molecules both as research reagents and as potential human therapeutics. The C_H2 domain alone can also be used to extend half-life, though the increase in t_{β} is less than can be achieved with whole Fc fusions. However, the use of this single domain allows the design of molecules with an internal C_H2 domain. For example, a CD4–C_H2–PE40 toxin molecule has been produced with extended plasma half-life (Batra *et al.*, 1993).

Samsung Bioepis Exhibit No. 1002
Page 5341

3

Monoclonal Antibodies in Research and Diagnostic Applications

3.1 Introduction

Antibodies have proved to be invaluable reagents for the detection and quantitation of many types of substances both *in vitro* and *in vivo* and have, therefore, found wide application in both the research laboratory and the diagnostics industry. Many of the techniques used in these two situations are similar, and thus it is helpful to review them together. In a chapter of this length it is impossible to cover all the ways that MAbs have been used in these areas; thus the emphasis in this chapter is on applications which are particularly widely used and those which are likely to benefit most from the ability to design engineered or modified antibodies.

3.2 Immunoassays in diagnostics and research

Immunoassays are carried out to detect and quantitate the presence of a particular antigen or antibody in a test fluid. The development of immunoassays began with polyclonal antisera and thus precedes the introduction of MAbs. However, today MAbs are widely used, and many assays routinely used in, for example, hospital clinical biochemistry laboratories which rely on them. In fact MAbs have found widespread use in laboratories of all types, in applications from drug discovery to detecting drugs of abuse in athletes and horses. In contrast to polyclonal antisera, production of MAbs allows a potentially unlimited supply of identical reagent which can be selected from a number of different clones to have the optimal characteristics for the intended assay. For some assay formats such as radioimmunoassay, however, polyclonal antisera remain the best reagents, as they are frequently of higher apparent affinity than MAbs. Therefore, it is useful to examine the types of immunoassays to appreciate the role of monoclonal antibody reagents. Some of the properties of MAbs of interest for immunoassay development are given in Table 3.1.

Samsung Bioepis Exhibit No. 1002
Page 5342

Table 3.1 Properties of monoclonal antibodies important for immunoassay design

Unlimited quantity of identical antibody
Antibodies to distinct epitopes can be selected from panels of clones
Easily purified to reduce background binding
Readily digested to produce fragments with equivalent specificity
Can be produced to impure antigen
Often of lower apparent affinity than polyclonal antisera

3.2.1 Radioimmunoassay

Radioimmunoassay (RIA) methodology was first developed in the 1960s and represented the first sensitive method for immunoassay (Yallow and Berson, 1960). RIAs are competitive assays based on the competition of radiolabelled antigen with antigen present in the sample to be assayed for binding to antibody (Figure 3.1). The antibody complex is then separated from the free antigen and the amount of bound material determined by counting the radioactivity. Separation can be achieved by a number of methods, although it is most convenient to use antibody immobilised onto a solid phase directly, or to

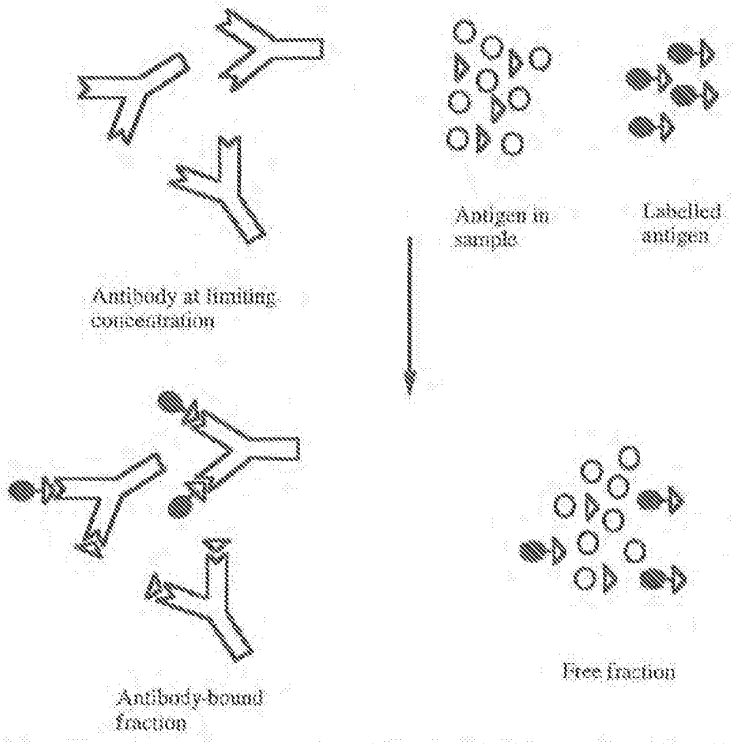


Figure 3.1 Format of radioimmunoassay: antibody at limiting concentration is added to sample containing antigen and radiolabelled antigen; the antibody-bound fraction is then separated and either free or antibody-bound counts (or both) are measured

Samsung Bioepis Exhibit No. 1002
Page 5343

remove the antibody–antigen complex by a second immobilised antibody which recognises the first antibody used. Antibody can be immobilised to many types of solid supports, e.g. microparticles such as cellulose, sepharose and paramagnetic particles, or to large solid phase plastic surfaces such as beads, tubes or microwells. The RIA assay format requires excess antigen over antibody and for high sensitivity small amounts of high affinity antibody are required. Polyclonal antisera often have very high apparent affinity or avidity, probably due to a small proportion of the total antibody present having high affinity and to cooperativity between antibody molecules. The mixture of antibodies present in a polyclonal antiserum may allow attachment of several antibody molecules to the antigen at different sites and, as antibody molecules are bivalent, this can lead to the formation of multimeric complexes, which lead to a low off-rate and higher apparent affinity. Therefore, polyclonal sera have remained the ideal reagents for RIA, and although MAbs can be used their performance is often not optimal. However, RIA techniques are relatively long and tedious to carry out and in many cases have been superseded by MAb techniques based on other assay formats.

3.2.2 *Immunoradiometric assay*

The immunoradiometric assay (IRMA) uses an excess of labelled antibody rather than antigen to quantitate the antigen present. This means that sensitivity is not dependent on antibody affinity. The IRMA was initially developed as a single-site assay in which excess labelled antibody was added to the sample containing antigen to form immune complexes (Miles and Hales, 1968). The remaining antibody was then removed by incubation with solid-phase antigen and after separation of the solid and liquid phases, counting of either phase could be carried out to determine the amount of antigen present (Figure 3.2). This form of IRMA is not widely used due to the requirement for large amounts of both labelled antibody and immobilised antigen. A variation of the IRMA, the two-site IRMA, has become a much more useful assay format. In this assay antigens are quantitated using two antibodies which bind to different epitopes (Woodhead *et al.*, 1974). Antibody is immobilised onto a solid phase and incubated with sample containing antigen. After binding of the antigen to the solid-phase antibody, excess labelled second antibody is added which binds to a second epitope on the antigen. The excess labelled antibody is then washed away and the amount of bound antibody quantitated by counting (Figure 3.3). Requirements for this type of assay are an antigen which is large enough to contain two epitopes, and supplies of two different specific antibodies. This type of assay format is very difficult to achieve with polyclonals, and requires extensive purification of the antisera to isolate preparations binding to individual epitopes. MAbs are ideally suited, however, as different clones can be tested until two non-competing antibodies are identified. These assays are much more rapid, sensitive and robust than competitive binding assays such as RIA and are now widely used.

Iodine 125 (^{125}I), a high-energy gamma emitter, is usually used as the isotope for antibody labelling in IRMA applications. It allows the development of sensitive assays due to the ability to achieve high specific activity labelled antibody preparations. ^{125}I is attached to antibodies via two main methodologies, either by attachment to tyrosine residues or by attachment to lysine residues. Attachment to tyrosine is achieved by oxidation of sodium iodide ($Na^{125}I$) to form molecular iodine which reacts rapidly with tyrosine side chains (Figure 3.4). The reaction is then stopped by the addition of a reducing agent (e.g. sodium metabisulphite) to stop generation of reactive iodine, or by

Samsung Bioepis Exhibit No. 1002
Page 5344

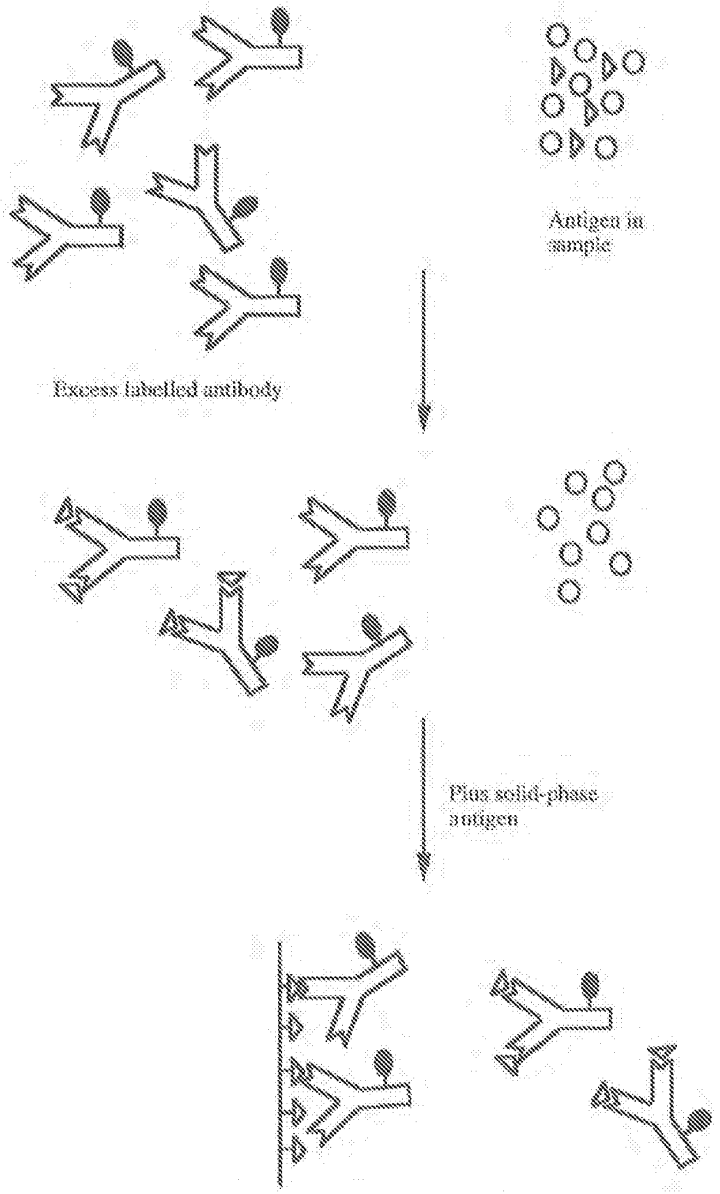


Figure 3.2 Format of immunoradiometric assay: excess labelled antibody binds antigen in the sample and is then added to solid-phase antigen; determination of bound or free counts allows quantitation of the amount of antigen present in the sample

adding excess free tyrosine to mop up all of the reactive iodine. High specific activities can be generated using this methodology, although care must be taken that oxidative damage to the antibody is minimised. Commonly used oxidants are chloramine T or solid-phase reagents such as iodogen coated onto a plastic bead or tube. The use of solid-phase

Samsung Bioepis Exhibit No. 1002
Page 5345

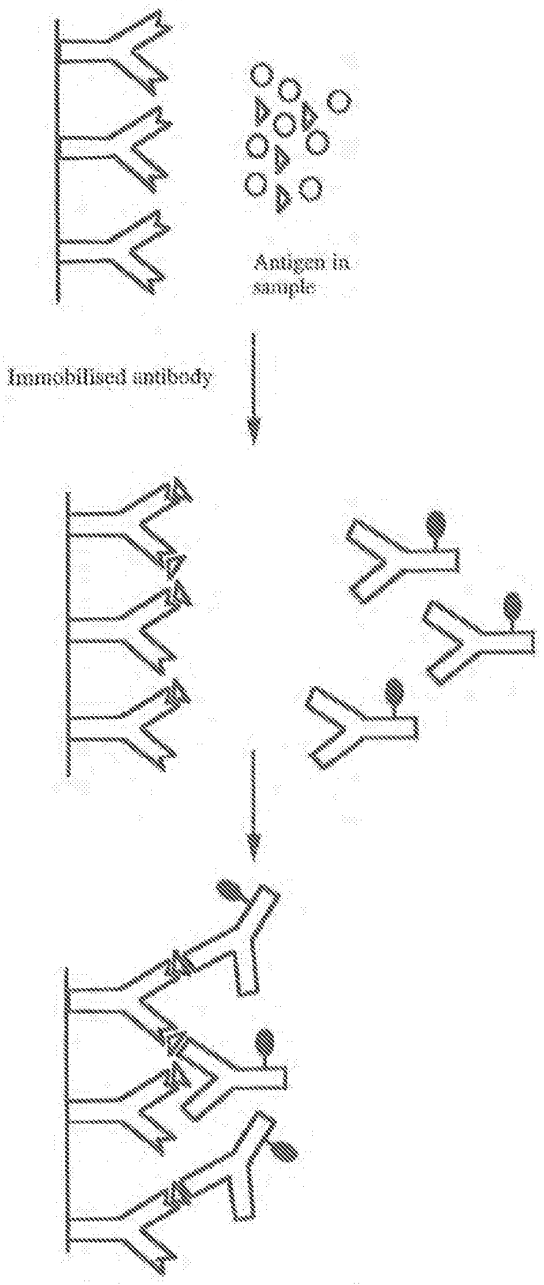


Figure 3.3 Format of two-site immunoradiometric assay: immobilised antibody captures antigen in the sample; a second antibody reactive with a second epitope on the antigen is then used in labelled form to bind to antigen and reveal the presence of antigen

Samsung Bioepis Exhibit No. 1002
Page 5346

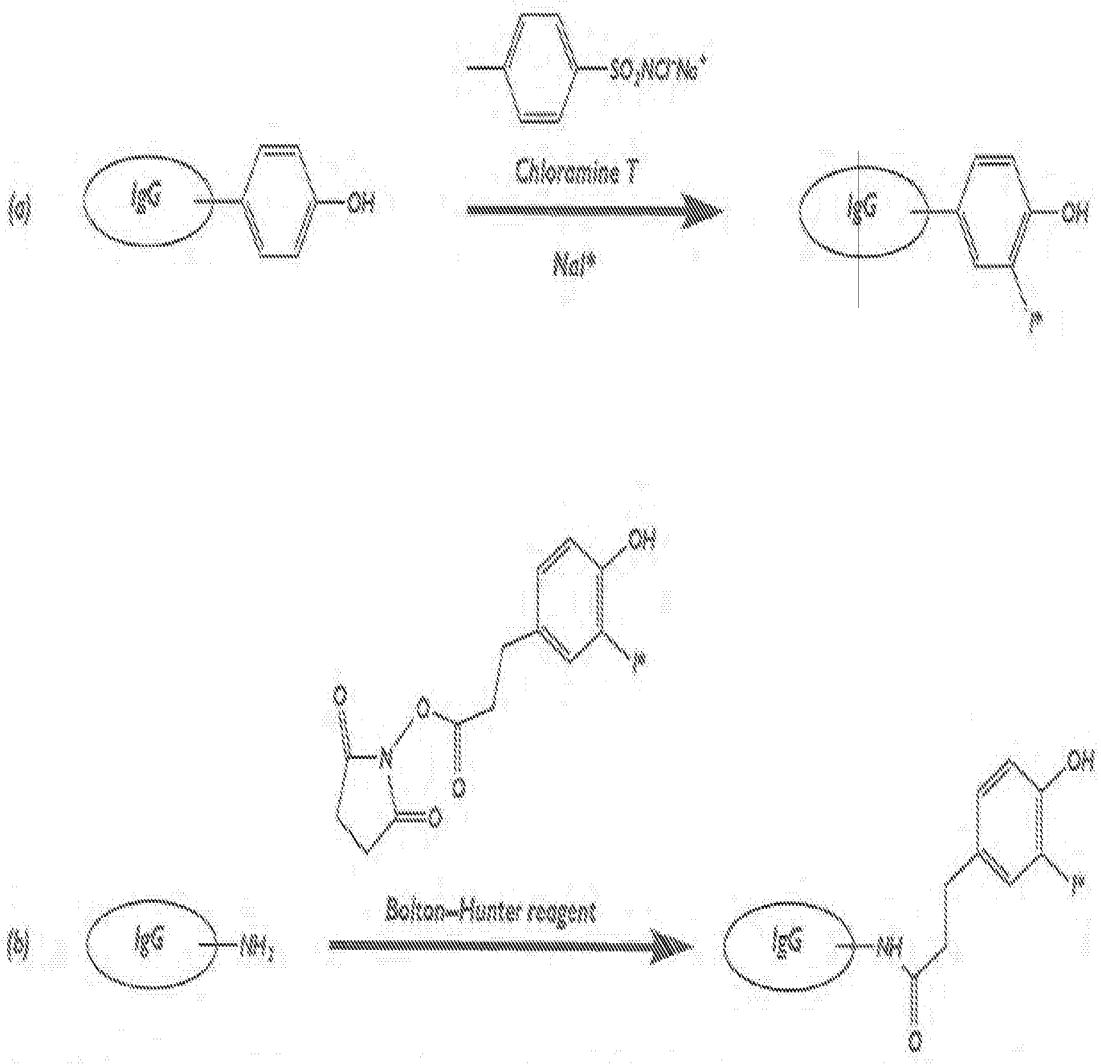


Figure 3.4 Radioiodination of MAb by (a) the use of chloramine T and sodium iodide and (b) Bolton–Hunter reagent (Bolton and Hunter, 1973)

reagents allows rapid removal of oxidant from the system and thus can result in less damage to the antibody. Attachment to lysine residues is accomplished via indirect labelling using amino reactive compounds pre-labelled with ^{125}I. This approach was first described using the ^{125}I-labelled *N*-hydroxysuccinimide ester of 3-(4-hydroxyphenyl) propanoic acid (Bolton and Hunter, 1973), subsequently known as Bolton–Hunter reagent. This and other similar reagents are commercially available in radiolabelled form and allow simple labelling of antibodies under mild conditions (Figure 3.4). Specific activities achieved with Bolton–Hunter reagent are usually lower than with oxidative methods as the incorporation efficiency is lower, but for antibodies sensitive to oxidative damage and in cases where relatively low specific activity is required, this method is particularly useful.

The limitation in sensitivity of the two-site IRMA is the specific activity of the labelled antibody, the affinity of the first antibody and the level of non-specific binding. MAbs can be readily purified, and antigen-binding fragments such as F(ab')$_2$ easily produced, which can reduce levels of non-specific binding to surfaces. Low specific activity is often due to antibody damage during radiolabelling. The availability of large amounts of MAb allows the screening of several radiolabelling methods and the development of a suitable method to minimise such damage.

3.2.3 *Non-isotopic immunoassays*

A major disadvantage with the use of the two-site IRMA is the need to use radiolabelled antibody. ^{125}I has a half-life of 60 days and thus new labelled preparations need to be continually produced. Also, the use of radiolabelled materials requires stringent precautions in the laboratory to prevent radioactive contamination. Several alternative labels have been investigated, which are replacing radioisotopes. These include enzymes, fluorescent and chemiluminescent labels, and indirect methods (Table 3.2).

Suitable enzymes can be attached to antibodies without loss of activity, and can convert substrate to a coloured product which is then quantitated spectrophotometrically. Enzyme detection has become the standard method for most laboratory immunoassays and two-site (or sandwich) ELISAs (enzyme-linked immunosorbent assays) are very

Table 3.2 Commonly used non-isotopic labels for immunoassays

Enzymes	Horseradish peroxidase
	Alkaline phosphatase
	Urease
	β-galactosidase
	Xanthine oxidase
Fluorescent labels	Fluorescein
	Phycoerythrin
	Europium chelates and other lanthanide chelates
Chemiluminescent	Luminol/isoluminol
	Acridinium esters
Indirect methods	Biotin
	Streptavidin/avidin
	Protein A, protein G

Samsung Bioepis Exhibit No. 1002
Page 5348

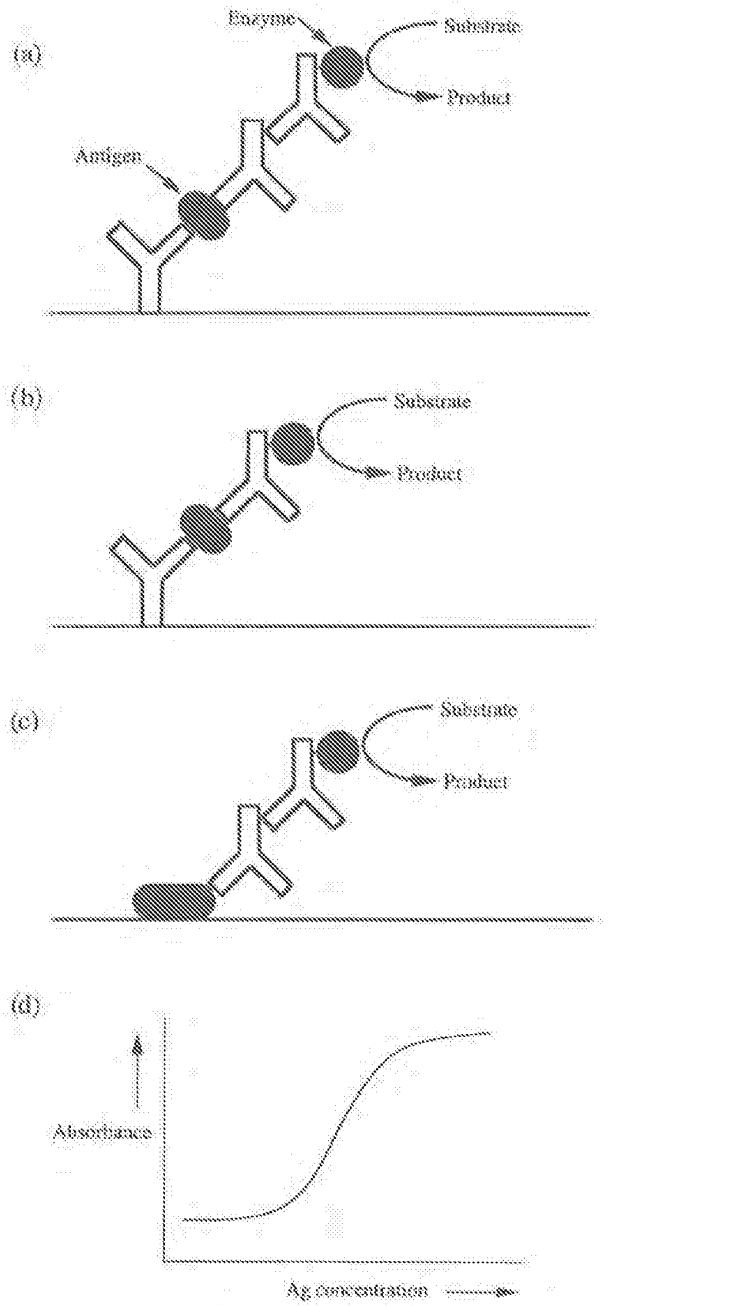


Figure 3.5 Common ELISA formats: (a) two-site ELISA for antigen quantitation using enzyme conjugated anti-antibody to detect; (b) two-site ELISA for antigen quantitation using directly conjugated second antibody; (c) determination of antigen-specific antibody; (d) expected form of standard curve for such direct binding ELISA formats

Samsung Bioepis Exhibit No. 1002
Page 5349

widely used. These are similar in format to immunoradiometric assays, although often an extra antibody layer is included to help amplify the signal generated by the enzyme (Figure 3.5). The use of such formats on microtitre plates allows assays to be carried out simply and quickly. Commonly used enzymes such as alkaline phosphatase and horseradish peroxidase can convert substrate to product with a high turnover number to allow the generation of a large amount of coloured product per enzyme molecule bound, resulting in amplification of the signal.

ELISAs are commonly used for measuring antibody as well as antigen. In this case ELISAs with immobilised antigen can be used to measure the amount of antigen-specific antibody by detecting with an anti-antibody reagent after binding. Alternatively, the amount of total antibody in a sample can be quantitated in a two-site ELISA using, for example, immobilised antibody to the light chain and detecting with enzyme conjugated to antibody directed to the heavy chain or vice versa. ELISAs can also be used in a number of competitive formats, for example in the detection of soluble antigens (Figure 3.6), and can also be carried out with whole cells as the antigen (Feit *et al*., 1983). ELISAs are usually carried out on plastic microtitre plates on which the 'capture' reagent is immobilised. These plates are usually made of polystyrene which is optically clear and are commonly used in the standard 96-well format. Such plates are particularly advantageous as they allow dilutions of standards and samples to be run under identical conditions on the same plate, which is ideal for an accurate result. Antibody or antigen is normally immobilised to the plate by passive adsorption through hydrophobic interactions, although it has been suggested that covalent binding may improve assay performance (Douglas and Monteith, 1994).

Antibody–enzyme conjugates are often produced by chemical coupling (see Section 2.6.2). This is usually a satisfactory procedure although there are often variations from batch to batch and in some cases it can lead to loss of some of the antigen-binding activity of the antibody or to loss of enzyme activity. Site-specific conjugation to Fc region carbohydrate has been investigated, and alkaline phosphatase conjugates prepared in this way (Section 2.6.2) have been shown to retain full binding activity (Hussain and Bienarz, 1994). Recently, attempts have been made to generate suitable reagents by expression of antibody–enzyme fusion proteins. Alkaline phosphatase fusions to both scFv and $F(ab')_2$ fragments have been prepared, expressed in *E. coli* and shown to be fully functional for both antigen binding and enzyme activity (Wels *et al*., 1992; Ducancel *et al*., 1993). For application in immunoassays a general reagent consisting of anti-human IgG scFv or $F(ab')_2$ fused to alkaline phosphatase has been produced and shown to be capable of detecting human IgG to hepatitis B antigen in an ELISA (Carrier *et al*., 1995). An additional advantage of this approach is that the use of antibody fragments has been shown to reduce background binding in ELISAs compared to intact IgG molecules. The ease of recombinant techniques for the direct expression of antibody fragment–enzyme fusion proteins suggests that this route of production may become increasingly important in immunoassay development.

3.2.4 *Improving sensitivity*

Several approaches have been investigated to improve the sensitivity of immunoassays, to develop ultrasensitive assays, by manipulation of the detection system (Kricka, 1993). Increases in enzyme amplification of the signal generated have been described in which the product of the antibody–enzyme conjugate reaction is used to set up a cycle in which

Samsung Bioepis Exhibit No. 1002
Page 5350

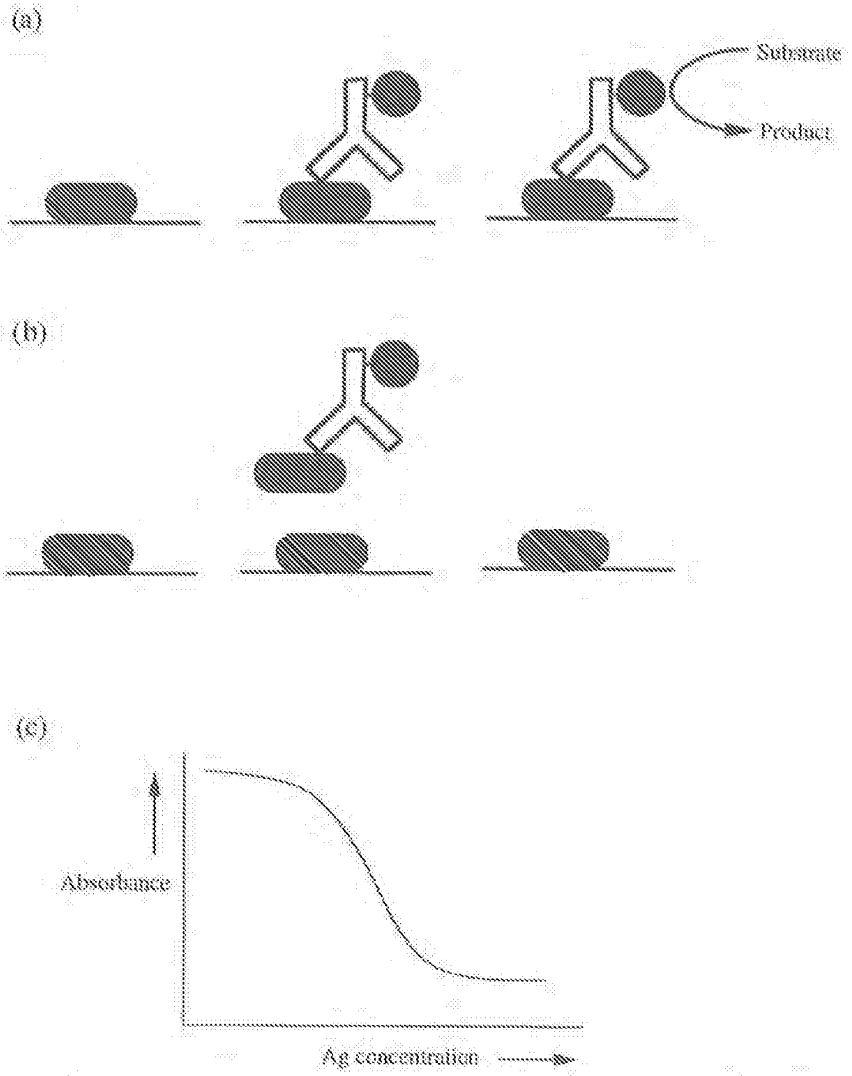


Figure 3.6 Competitive ELISA format assay: (a) in the absence of antigen in the sample, conjugated antibody binds to immobilised antigen and gives rise to the production of coloured product; (b) in the presence of antigen in the sample, conjugated antibody is prevented from binding to immobilised antigen leading to the absence of signal; (c) expected form of standard curve from such a competitive ELISA

a second enzyme system generates large amounts of coloured product as shown in Figure 3.7 (Stanley *et al.*, 1985). A high degree of signal amplification results as each NAD molecule generated can be responsible for the production of many hundred formazan molecules, and this has led to the development of several immunoassays with reported sensitivities over 100-fold higher than conventional alkaline phosphatase substrates such

Samsung Bioepis Exhibit No. 1002
Page 5351

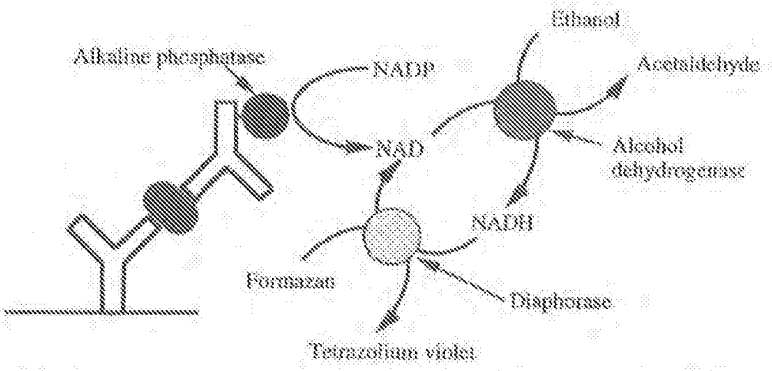


Figure 3.7 Example of an amplification mechanism for enhancing signal in ELISA.

as *p*-nitrophenyl phosphate (Clark and Price, 1986). In many cases such highly sensitive assays may have problems with the level of non-specific background and thus careful optimisation of the assay is usually required.

Enzyme labels can also be made more sensitive by the use of substrates, resulting in the generation of fluorescent or chemiluminescent substances, which can be detected with greater sensitivity than colorimetric reactions. Substrates resulting in both fluorescent and chemiluminescent assays are available for the most commonly used enzymes, horseradish peroxidase and alkaline phosphatase (e.g. Albrecht *et al.*, 1994; Akhavan-Tafti *et al.*, 1995). Enzyme amplification cycles have also been combined with fluorescent readouts to allow determination of very small amounts of alkaline phosphatase, down to one thousandth of an attomole (one zeptomole or 350 molecules) when applied to an immunoassay of proinsulin (Cook and Self, 1993).

Fluorescent labels can also be directly attached to antibody and used as a detection system for immunoassays. The major problem with the use of fluorescent labels, however, is the background level of fluorescence generated by many biological substances and by plastics used for immobilisation. Hence conventional fluorescent compounds used in immunocytochemistry (see Section 3.4) such as fluorescein are of relatively little use. This can be overcome by the use of time-resolved fluorescent techniques using lanthanide chelates as the fluorescent reagents (Diamandis, 1988). In this technique pulses of light are used to excite the fluorescent material present and then the emitted light is measured after a short time interval of a few hundred microseconds. As background fluorescence is due to very rapid events, with fluorescence lifetimes of 100 nanoseconds or less, the interference can be removed by using fluorescent compounds which emit light for longer periods (1000 microseconds or more) and measuring emitted light after the background fluorescence has died away. Measurement of light emitted by such systems requires instruments which can pulse and measure fluorescence many times per second to build up a strong signal, and several such instruments have been commercialised. Chelates of lanthanides such as europium or terbium are suitable reagents as they have ideal properties of long fluorescence lifetimes, large Stokes shifts (the difference between the wavelength of light used for excitation and that emitted) and narrow emission wavelength bands. Many sensitive assays have been developed using this technology and they are widely used in clinical diagnostics. Attempts have also been made to produce recombinant molecules capable of binding lanthanides directly. A fusion protein between a

Samsung Bioepis Exhibit No. 1002
Page 5352

single-chain Fv and an engineered lanthanide binding protein has been produced and shown to bind terbium (MacKenzie *et al.*, 1995).

Chemiluminescent reactions have also been explored as a sensitive means of detection. Chemiluminescence results from a reaction in which the product is in an electronically excited state. Electrons in the excited state then relax to their ground state with the emission of light. Most useful chemiluminescent labels for direct attachment to antibodies have been the acridinium esters, which are capable of coupling to antibodies or antigens without a large loss in their quantum yield (Weeks *et al.*, 1983). They are usually coupled to antibody or antigen as *N*-hydroxysuccinimide esters which allows high coupling efficiencies, and chemiluminescence is initiated simply by the addition of hydrogen peroxide. An alternative luminescent reagent for attachment to antibody is the calcium-activated photoprotein aequorin, initially isolated from the jellyfish *Aequorea victoria*. Aequorin conjugated antibody has been used for the development of several sensitive immunoassays for hormones such as thyrotrophin, chorionic gonadotrophin, lutrophin and follitrophin (Rigl *et al.*, 1995). As a protein, aequorin can also be attached to antibody by recombinant means, and the construction and expression of a functional Fab'–aequorin fusion protein has been described (Casadei *et al.*, 1990).

The use of an antibody-linked peroxidase system to generate a chemiluminescent acridinium ester has been reported to result in longer duration of light than that generated from directly conjugated acridinium esters (Akhavan-Tafti *et al.*, 1995). Peroxidase also catalyses the oxidation of luminol by hydrogen peroxide which can be used to generate a strong signal in the presence of a suitable enhancer such as 4-iodophenol, 1,6-dibromo-2-naphthol or 6-hydroxybenzothiazole (Thorpe and Kricka, 1986). Such enhanced chemiluminescent assays have been shown to be useful in many situations, for example in the measurement of thyroxine (Christofides and Sheehan, 1995). Other enzymes can also be used, and xanthine oxidase has been shown to be useful in a luminol-based immunoassay system for interleukin-5 (Rongen *et al.*, 1997). Several substrates for alkaline phosphatase conjugates have been developed which give rise to chemiluminescent compounds after enzyme action (Bronstein *et al.*, 1989). The use of the substrate adamantyl 1,2-dioxetane aryl phosphate (AMPPD) has been shown to result in particularly low detection levels, down to one zeptomole, when applied to immunoassay of thyrotrophin (Bronstein *et al.*, 1989).

A simple method to increase sensitivity of immunoassays might be simply to put more of the label on to each detecting antibody molecule. This is limited by loss of immunoreactivity of the antibody when too many labelling molecules are attached. One way to increase this is to attach a carrier molecule, which can be loaded up with labels. Poly-lysine has been used in this way for the attachment of multiple fluorescent groups (Exley and Ekeke, 1981). However, the most useful strategy to date has been the use of the biotin:avidin or biotin:streptavidin interaction. Each streptavidin or avidin molecule can bind four biotin groups with high affinity. The detecting antibody can be labelled with several biotin molecules until the limit for retention of antibody-binding activity is reached. When bound in an assay, biotinylated antibody can then be revealed with streptavidin that is labelled with multiple numbers of signal generating molecules such as enzymes or fluorescent or chemiluminescent reagents, hence leading to a considerable amplification in signal. For example, thyroglobulin molecules labelled with up to 480 europium chelate groups can be attached to streptavidin and used to detect biotinylated antibody resulting in an amplification of 4500- to 6750-fold (Diamandis, 1991). Biotinylated antibody can also be produced by recombinant means by use of Fab fusion proteins to the biotin–carboxyl carrier protein (BCCP) subunit of *E. coli* acetyl-CoA carboxylase (Weiss

Samsung Bioepis Exhibit No. 1002
Page 5353

et al., 1994). However, production of fully biotinylated material is difficult due to the requirement for sufficient biotin ligase enzyme to allow *in vivo* incorporation of biotin during expression of the Fab–BCCP fusion protein. Only approximately one sixth of the fusion protein produced was able to react with streptavidin (Weiss *et al.*, 1994).

Another approach to amplify signal is to attach the detecting antibody to a liposome, into which have been entrapped many detectable molecules, such as enzymes, fluorescent or chemiluminescent reagents. Liposomes are closed spherical structures in which a phospholipid bilayer encloses an aqueous compartment in which it is possible to entrap 10^5–10^6 water-soluble molecules in a stable manner. When used in an immunoassay, the liposome can be lysed at the end of the assay and the contents quantitated. Immunoliposomes can be produced by coupling antibody to lipid via a heterobifunctional cross-linker, either randomly or site-specifically via antibody carbohydrate (Torchilin, 1994; Ansell *et al.*, 1996). However, immunoliposomes often show a lower association rate than free antibody and may also increase non-specific binding levels. Nevertheless, direct comparisons of immunoassays for thyrotrophin with antibody conventionally labelled with an acridinium ester (10 labels per antibody) with an immunoliposome containing approx. 250 000 labels per liposome resulted in an improvement in signal to background ratio of approx. 3-fold for the immunoliposome assay (Law *et al.*, 1989). Lipid-tagged antibodies have also been produced by expression of scFv fragments fused to part of the *E. coli* major lipoprotein which is tagged with lipid when expressed in *E. coli*. The resulting lipid-tagged antibody fragments can be incorporated into liposomes with high efficiency and have been used to generate europium chelate loaded liposomes for a use in fluoroimmunoassay (Laukkanen *et al.*, 1995). A higher signal was generated using liposomally entrapped europium chelate compared to conventionally labelled scFv, resulting in a more sensitive assay.

The polymerase chain reaction (PCR – see Chapter 1) has also been adapted for use as a sensitive detection system for immunoassay, termed immuno-PCR (Sano *et al.*, 1992). Antibody is coupled to DNA through the avidin–biotin system. In the first system described, streptavidin is first bound to immobilised antibody through a protein A–streptavidin conjugate. This is then used to capture biotinylated DNA which is then detected and amplified through PCR with the resulting products analysed by electrophoresis. The resulting assay has been shown to be extremely sensitive, capable of detecting as few as 600 molecules in an immunoassay of bovine serum albumin (Sano *et al.*, 1992). PCR has also been applied to the simultaneous determination of multiple analytes (Hendrickson *et al.*, 1995). In this case human thyroid stimulating hormone, human chorionic gonadotropin and *E. coli* β-galactosidase were measured by a combination of three specific MAbs, each conjugated to a unique oligonucleotide which were designed to result in different size PCR products from the same primer sequence. The different size products could be resolved by electrophoresis, resulting in an assay which was 2–3 orders of magnitude more sensitive than a comparable ELISA.

3.2.5 *Assay formats*

The majority of immunoassay formats rely on the separation of bound and free antibody before detection. Homogeneous assays in which there is no need to separate bound and free antibody have been investigated in attempts to produce simplified assays. Several formats have been successful as competitive assays which are easy to use and automate,

Samsung Bioepis Exhibit No. 1002
Page 5354

although sensitivity is often relatively low. The enzyme multiplied immunoassay technique, known as EMIT, uses glucose-6-phosphate dehydrogenase–antigen conjugates which catalyse the conversion of substrate to coloured product. When bound by antibody, enzyme activity is inhibited and therefore the amount of coloured product is proportional to the amount of competing antigen present (Armbruster *et al.*, 1993). Later versions of this system use a recombinant form of the enzyme which is more suitable for automation (Vogl *et al.*, 1996). A similar system, cloned enzyme donor immunoassay, known as CEDIA, also operates as a competitive assay. In this system two inactive fragments of β-galactosidase, enzyme donor and enzyme acceptor, are produced by recombinant means which when mixed together associate to produce active enzyme. When the enzyme donor is conjugated to antigen, antibody binding prevents association and formation of the active enzyme. Any free antigen competes for binding to the antibody, hence allowing enzyme activity to be generated by association of the antigen–enzyme donor with the acceptor (Henderson *et al.*, 1986). Such homogeneous systems have been developed for immunoassay of many small molecular weight substances such as cortisol, ferritin, digoxin, folate, vitamin B12 and drugs of abuse (Van der Weide *et al.*, 1992; Armbruster *et al.*, 1995). Other homogeneous assay formats have also been developed with fluorescent endpoints, including fluorescence polarisation (Colbert *et al.*, 1985), fluorescence energy transfer (Calvin *et al.*, 1986) and fluorescence quenching (Barnard *et al.*, 1989). Many other formats have been investigated, including some based on light scattering which may be simple to automate (Armbruster *et al.*, 1993). Overall the advantages of homogeneous assays are in speed and convenience. However, the sensitivity of homogeneous immunoassays remains limited, largely because the use of a separation step in other assay formats allows washing, which reduces or removes interfering substances and non-specific binding events.

For most large diagnostic laboratories there is now a requirement for a high degree of automation in performing immunoassays, and many automated assays are in use and continue to be developed. Both heterogeneous and homogeneous assay formats have been automated using a wide variety of detection systems, including enzyme reactions, fluorescence, chemiluminescence and light-scattering techniques (Chan, 1996). Attempts are being made to develop all-in-one formats suitable for automation (Lovgren *et al.*, 1996). Whether any of these systems will eventually dominate the future market for automated assays or whether the current range of immunoassay techniques will continue to be extended remains to be seen.

While large diagnostics centres require specialised instrumentation, there has also been much interest in developing 'point of need' assays which can be performed simply in the clinic, doctor's office or home to give a simple + or − readout. Applications include home pregnancy testing (measurement of chorionic gonadotrophin, hCG) and fertility testing (lutrophin measurement). Several useful formats have been developed and commercialised based on immunochromatographic test strips and membranes. For example, the hCG test strip, known as Clearblue One Step (Unipath) has been developed for home use. In this test coloured submicron latex particles are used to indicate a result by formation of a blue line in the large window and the small window (positive) or the small window alone (negative). A sample of urine is captured on a porous applicator which is then held in contact with the test membrane which has three zones of antibody (Davidson, 1992). Urine is used as the mobile phase and as it passes along the membrane it reaches the first zone consisting of blue microparticles with a Mab to the alpha subunit of hCG attached. The urine picks up the particles and carries them to the second zone, under the large window, which has a MAb to the beta subunit of hCG immobilised on the membrane.

Samsung Bioepis Exhibit No. 1002
Page 5355

If hCG is present the coloured particles will bind resulting in a blue line under the large window. The urine continues to migrate along the membrane and reaches the third zone, under the small window, when excess or unreacted particles will be trapped resulting in a blue line whether hCG is present or not, allowing a control on the assay format. This assay can be carried out in three minutes with an overall performance (sensitivity and specificity) of 99% (Davidson, 1992). Many variants of such tests are now available which have successfully extended the applications of MAbs in the home for such applications as pregnancy testing (hCG assay), ovulation prediction (luteinising hormone, follicle stimulating hormone) and contraception (luteinising hormone and oestrone-3-glucuronide). Similar tests are under development or in use for applications such as identification of infectious diseases, drugs of abuse screening and monitoring disease markers.

3.2.6 *Advantages of monoclonal antibodies in immunoassay*

Although many immunoassays are still carried out with polyclonal antibody reagents, MAbs have clearly demonstrated an important role as described above, and have allowed a whole new generation of assays to be developed of importance in the research laboratory, the diagnostics industry and even the home. In many cases the combination of monoclonal and polyclonal reagents may allow powerful assays to be developed, and the design of a particular assay can take account of the properties of the individual reagents available.

By selection of an appropriate MAb, the interference of closely related substances in an assay can often be removed so that specificity is increased. For example, a MAb has been raised to the C-terminal part of human brain acetylcholinesterase which can distinguish between brain acetylcholinesterase and that present in red blood cells (Boschetti *et al.*, 1996). Brain acetylcholinesterase leaks into the amniotic fluid in prenatal neural tube defects as well as neurological disorders. Samples of amniotic fluid from amniocentesis are often contaminated with blood and thus an assay which can distinguish between the two forms could be important in allowing accurate diagnosis of such serious conditions, reducing any false positive results. Closely related compounds may also be problematic in therapeutic drug measurements. Monoclonal reagents have shown superior specificity in the measurement of digoxin (Datta *et al.*, 1996), and can be used to discriminate between a drug itself and its metabolites, as shown in the case of cyclosporin monitoring to allow adjustment of individual dosing regimens (Quesniaux, 1991). Another common form of interference in immunoassays is the presence of autoantibodies to the sample antigen. The use of MAbs in such cases also allows reduced interference, as demonstrated in the case of an IRMA for the measurement of serum thyroglobulin (Marquet *et al.*, 1996).

Assay sensitivity may also be increased with MAbs. Simple substitution of a polyclonal capture antibody with a monoclonal has resulted in increased sensitivity in a fluorescent assay for salmon calcitonin (Rong *et al.*, 1997). Similarly, sensitivity for the human cancer marker, prostate specific antigen, has been increased in another immunofluorometric assay which may be of utility in the diagnosis and staging of prostate and breast cancers (Ferguson *et al.*, 1996).

One problem with the use of MAbs has been in the detection of small molecular weight materials, such as many drugs, which are often not large enough to contain two distinct epitopes for use in two-site assays. Thus the measurement of such substances has relied largely on competitive techniques, using detection either with radioisotopes

Samsung Bioepis Exhibit No. 1002
Page 5356

(RIA) or with non-isotopic techniques such as those described above. Non-competitive approaches are now being developed. Two-site ELISAs have been developed based on the interaction of a small molecular weight analyte, such as digoxin, with an antibody which can then be detected by an antibody which recognises the new epitope formed by the bound analyte in the immune complex (Self *et al.*, 1994). Alternatively, assays have been developed using one second antibody which sees the antibody–analyte immune complex and one which recognises the primary antibody only when the analyte is not bound (Mares *et al.*, 1995).

MAbs have found applications in the measurement of a wide range of substances including hormones, metabolites, disease markers, therapeutic drugs, drugs of abuse and food and environmental contaminants such as mycotoxins, microorganisms, herbicides and pesticides. Antibody engineering technology is also beginning to have an impact on the design of immunoassays. Direct expression of fusion proteins may result in improved, homogeneous reagents in both enzyme-based and fluorescent assays. Antibody specificities can be directly expressed as fragments which may exhibit fewer problems of non-specific binding, and the power of phage selection of antibodies (see Chapter 1) allows rapid selection of useful binding specificities. Phage libraries may also allow the generation of useful antibodies which are difficult or impossible to obtain by other means, such as for antigens which are highly conserved between species, and it has been demonstrated that phage-bound antibody fragments may even be used directly in immunoassays (Navarra-Teulon *et al.*, 1995). The applications of bispecific antibodies are also likely to increase as their production becomes simpler (see Section 2.5). For example, bispecific antibodies can be produced with specificities for two epitopes on the same target which may offer advantages in specificity and affinity of binding and extend applications in immunoassay development (Cook and Wood, 1994).

Bispecific antibodies are also finding application in simple immunoassay formats. Rapid diagnosis of pulmonary embolism (PE) in the clinic may be crucial to further treatment and it has been suggested that high levels of the fibrin degradation product, D-dimer, is associated with PE (Bridey *et al.*, 1989). A rapid assay has been developed as an exclusionary test for PE which can allow exclusion of patients from potentially hazardous anti-coagulant therapy (Ginsberg *et al.*, 1995). This assay uses a bispecific antibody in which one arm recognises D-dimer and one red blood cells. The antibody causes agglutination of the patient's own red blood cells in the test tube, hence allowing a simple positive or negative test to be performed within a few minutes of taking a blood sample.

Entirely novel assay formats are also possible based on recombinant antibody fragment production. It has been shown that the interaction between the two variable domains of an antibody (V_H and V_L) is considerably strengthened in the presence of antigen (King *et al.*, 1993). This interaction has been used to develop an open sandwich ELISA method for the detection of antigen in which V_L immobilised on an ELISA plate is used to bind antigen in the presence of phage displayed V_H. The resulting complex is then detected by use of a peroxidase labelled anti-phage antibody (Ueda *et al.*, 1996). If such antigen-promoted association of V_H and V_L is generally applicable then an exciting new class of immunoassays could result for both large and small antigens.

3.3 Immunosensors

An alternative to conventional immunoassays is the use of a biosensor based on antibody:antigen interaction, termed an immunosensor. Immunosensors are useful analytical

Samsung Bioepis Exhibit No. 1002
Page 5357

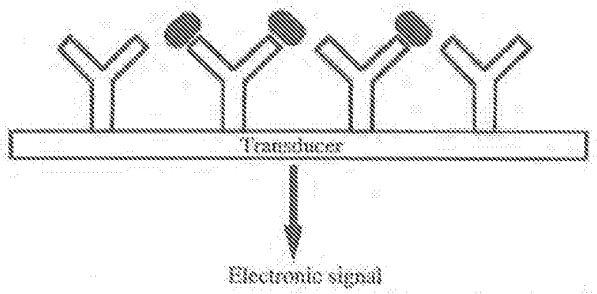


Figure 3.8 Diagram of a general immunosensor: antigen binding is detected by generation of signal which is converted to electronic format by a transducer

tools for monitoring antibody:antigen reactions in real time, often without the need for labelling either of the components. As such there has been great interest in developing immunosensors for analytical applications in the clinic, as well as for environmental monitoring and for food analysis. Immunosensors have been produced which are capable of detecting medical diagnostic markers such as hormones, drugs (therapeutic and abused), microorganisms and environmental pollutants such as pesticides. Under appropriate conditions immunosensors can also be used to analyse the kinetics of antibody:antigen binding.

An immunosensor is a solid-state device in which the antibody:antigen reaction is detected via a transducer which provides a signal that the binding reaction has taken place (Figure 3.8). Three main types of transducer have been used in biosensor technology; these exploit changes in mass (piezoelectric or acoustic wave), electrochemical properties (potentiometric, conductimetric or amperometric) or optical properties (fluorescent, reflective, luminescent, surface plasmon resonance or waveguide properties).

3.3.1 *Mass-detecting immunosensors*

Piezoelectric materials, such as quartz crystals, can be made to resonate by application of an alternating electric field with the frequency of the resulting oscillation being determined by the mass of the crystal. In piezoelectric biosensors, quartz crystals are coated with antibody. Binding of antigen results in a change in mass which is monitored through the change in frequency of the crystal oscillation. One problem with the use of such immunosensors is non-specific binding of sample components to the crystal, but sensitive devices have been produced for such applications as insulin measurement (Raman-Suri *et al.*, 1995), methamphetamine detection in human urine (Miura *et al.*, 1993), herbicide detection in drinking water (Yokoyama *et al.*, 1995) and detection of viruses and bacteria (Konig and Gratzel, 1993).

A further variant of the piezoelectric sensor uses surface acoustic waves to monitor binding to the crystal surface. In this application the oscillation of the crystal is at higher frequency and an acoustic wave is generated by application of an alternating voltage across interfaced electrodes, known as an interdigital transducer. A second interdigital transducer detects the acoustic signal a few millimetres away. Binding of sample to the crystal slows the acoustic wave, with the change in velocity being proportional to the analyte concentration. Such devices have the potential for higher sensitivity but may suffer more interference from factors such as temperature, pressure and conductivity, all of which may affect the properties of the acoustic wave. An acoustic wave sensor for

Samsung Bioepis Exhibit No. 1002
Page 5358

measurement of the pesticide atrazine enabled sensitive detection in a competitive assay, with the ability to re-use the device 48 times with only a 30% loss in response (Tom-May *et al.*, 1995).

3.3.2 *Electrochemical immunosensors*

Potentiometric immunosensors are based on the change in potential that results when antibody is immobilised on an electrode and its antigen binds to it. Antibodies, like all proteins, are polyelectrolytes and in many cases binding of antigen will alter the charge. Therefore the potential difference between an electrode with immobilised antibody and a reference electrode will depend on the concentration of antigen present. However, direct detection results in only small changes in potential (1–5 mV) and low signal : noise ratios because the the charge density on the antibody is low compared with background ions. Also, signal is dependent on variables such as pH and ionic strength of the sample and sensitive to interference from other ions. Attempts to improve such sensors have included the development of antibody field-effect transistor devices (immunoFET). ImmunoFET operates through measurement of conductivity through a channel region between source and drain electrodes. The conductivity is controlled by the strength of electric field generated by the gate, providing an amplification effect. Antibody is immobilised on a membrane which must be thin enough to allow redistribution of the small charge changes which occur on antigen binding. However, reliable immunoFET sensors have not been developed due, at least in part, to problems in manufacturing suitable membranes.

Conductimetric sensors monitor variations in the conductivity between two electrodes by measuring variations in the current across the electrodes. Many reactions lead to variations in ion concentration through either the consumption or production of ionic species, and thus such sensors have been developed with many enzyme systems. Application of conductimetric sensor technology to immunosensors has been limited to date.

Amperometric sensors measure the current generated when electroactive species are reduced or oxidised at the electrode. Antibodies are not directly electroactive and thus enzyme labels are used to generate electroactive species which can then be measured. Most often such sensors use oxygen or hydrogen peroxide electrodes, with the current produced being directly proportional to the amount of oxygen or hydrogen peroxide reduced or oxidised. Catalase was the first label used to construct an immunosensor in this way. MAb to human chorionic gonadotropin (hCG) was immobilised on an oxygen electrode to which hCG and catalase-labelled hCG could compete for binding (Aizawa *et al.*, 1979). Subsequently alternative labels, such as alkaline phosphatase, have been used in the detection of several antigens (Treloar *et al.*, 1994). Substrates for alkaline phosphatase which can be used to generate electroactive compounds include *p*-aminophenol phosphate or *N*-ferrocenyl-4-aminophenol phosphate. Alternative labels have also been developed for such applications as measurement of apolipoprotein E in serum (Meusel *et al.*, 1995) and detection of herbicides such as 2,4-dichlorophenoxyacetic acid using a disposable immunosensor (Kalab and Skladal, 1995). The development of amperometric immunosensors has also been extended into *in vivo* use for the measurement of corticosteroids (Cook, 1997). This system used an immunosensor with antibodies immobilised on the electrode surface surrounded by a dialysis membrane which allows corticosteroids to equilibrate across. When the corticosteroid to be measured has equilibrated, a solution of corticosteroid conjugated to horseradish peroxidase is introduced into the internal cavity of the electrode, which then competes for antibody binding. The electrode surface

Samsung Bioepis Exhibit No. 1002
Page 5359

can then be washed and peroxidase substrate added which is detected amperometrically. The probe can be regenerated with 1 mM HCl, allowing re-use every three minutes. The small size of this probe allowed implantation into the circulatory system of animals for monitoring hormone levels in real time, with the probe viable for several hundred measurements over 48 hours. The development of such systems may allow real-time, *in vivo* monitoring of a range of substances in the future.

3.3.3 *Optical immunosensors*

Optical immunosensors can be used with or without labels. Some sensors use labels such as enzymes or fluorescent or luminescent reagents to generate the optical signal, whereas in other cases binding is detected through the use of techniques such as total internal reflection, surface plasmon resonance or dielectric waveguides.

Labelled antibody immunosensors based on fluorescence or luminescence are attractive due to the relatively simple instrument design possible. Also, the use of optical fibre technology has enabled many devices to be miniaturised. These essentially operate via the generation or quenching of light at the surface of the optical fibre. Chemiluminescent assays have been developed based on the generation of light from the peroxidase catalysed oxidation of luminol, and fluorescent devices have been developed using different fluorescent labels, for applications such as the measurement of the cancer marker, prostate specific antigen in whole blood (Daniels *et al.*, 1995).

The most widely used optical immunosensors do not require labelled reagents. Surface plasmon resonance (SPR) uses light directed towards a layer of low refractive index from one of higher refractive index at an angle such that total internal reflection occurs. A metal film coated with a dextran layer is used to immobilise the antibody (or antigen). When light hits the metal film, surface plasmons in the film are excited and an evanescent wave is generated, a process known as SPR, which results in a decrease in the intensity of reflected light (Figure 3.9). When antigen binds to the immobilised antibody, the resulting change in refractive index causes a change in the angle at which the drop in reflected light intensity occurs. Continuous monitoring of the angle and intensity of reflected light therefore allows real-time analysis of binding events. A commercial system based on this principle, known as the BIAcore™, is now widely used for the analysis of biological interactions (Malmqvist, 1993). In this instrument SPR technology has been combined with a microfluidics system such that continuous monitoring is possible (Figure 3.9). This not only enables quantitative measurements to be made but also enables detailed kinetic analysis of binding interactions to be performed in real time. This instrument has therefore found many applications in the analysis of antibody:antigen interactions. BIAcore™ analysis is now used to determine the binding affinities of MAbs, and is widely used to monitor the outcome of antibody engineering experiments, for example in the selection of phage displayed antibodies, in the characterisation of antibody fragments and in epitope mapping (Malmborg and Borrebaeck, 1995).

Another commercially available type of optical biosensor, the IAsys™, is based on a combination of SPR technology with waveguide technology. This device has found applications in the area of bioprocess monitoring. The production of an Fv fragment could be monitored through fermentation and purification processes (Holwill *et al.*, 1996). Measurements were performed in five seconds, with an overall assay time of two minutes. This constant monitoring of the process allowed in-process decisions to be made to improve the process efficiency. A comparative study which used both BIAcore™ and the IAsys™

Samsung Bioepis Exhibit No. 1002
Page 5360

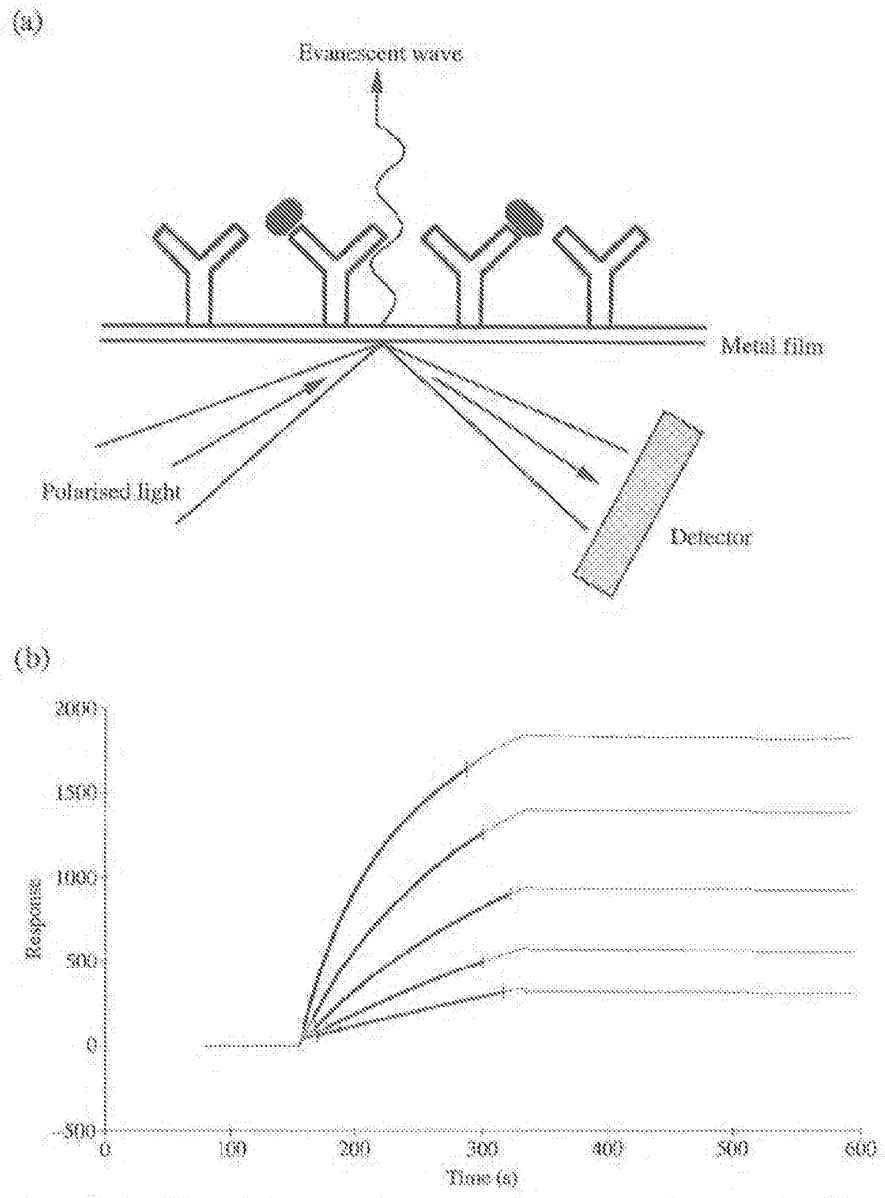


Figure 3.9 (a) Schematic diagram of BIAcore optical biosensor. Polarised light is reflected in the gold film of the sensor chip and detected on a diode array. Surface plasmon resonance is observed as a decrease in light intensity for a specific angle of incidence. The angle changes as the refractive index in the vicinity of the surface changes due to binding of large molecules on the immobilised ligand on the sensor chip. (b) Example of BIAcore sensorgrams for the binding, and slow dissociation, of IgG to an antigen-coated chip at five different concentrations. Measurement of initial on-rate and off-rate can be made to allow estimation of binding affinity.

Samsung Bioepis Exhibit No. 1002
Page 5361

system to monitor the kinetics of binding of lysozyme to anti-lysozyme antibody found good agreement between results obtained from the two systems (Yeung *et al.*, 1995).

3.4 Immunocytochemistry

Immunocytochemistry allows the detection and location of antigens within cells and tissues by the application of labelled antibody (either directly labelled or via a labelled second antibody reagent) followed by microscopy. Suitable labels to allow both light microscopy and electron microscopy applications are available. In the research laboratory immunocytochemistry is used to visualise antigens and their subcellular locations in tissues. Double-labelling techniques, in which antibodies to two different antigens are applied with different labels, allow comparison of the relative distributions of two antigens in the same tissue samples. This is compared with conventional histological stains and markers to build up a detailed picture of antigenic distribution. Immunocytochemistry is also widely used in diagnostic pathology, particularly in the identification of types of tumour cells, infectious organisms or inflammatory cells.

Detailed methodologies for immunocytochemistry procedures for light and electron microscopy have been described (Beesley, 1993; Polak and Priestley, 1992), and therefore the technique is summarised only briefly here. Immunocytochemistry requires firstly tissue preparation followed by antibody binding and then detection. Two major types of tissue sections are used in immunocytochemical studies: fixed and frozen. Frozen sections are more difficult to prepare but the technique is relatively gentle and leaves more of the immunological features of the tissue intact. Fixed tissue sections are prepared, often in formalin-based fixative, and embedded in paraffin prior to cutting slices. Such fixed sections are simple to prepare, retain cellular morphology and can be stored for long periods but the process of preparation is more likely to destroy antigenic epitopes within the tissue. Nevertheless such fixed sections are widely used in diagnostic pathology due to their ease of preparation, and the existence of extensive slide libraries which can be used for comparative purposes. Cell suspensions from cell culture experiments, and cell smears from, for example, needle aspirates or tissue scrapings, can also be used for immunocytochemistry after suitable fixing procedures. Such slides obviously do not preserve the morphology of the intact tissue but are useful in many subcellular studies, or in the identification of infectious organisms in tissue samples.

MAbs offer improved specificity compared to many polyclonal reagents also used in immunocytochemistry. However, the loss of a MAb epitope on tissue fixing is more likely than the loss of all the epitopes recognised by a polyclonal antiserum. Therefore, polyclonal reagents may be preferred for the detection of some antigens, particularly when using fixed tissue sections. Non-specific binding is generally reduced by use of monoclonal reagents. In some tissues non-specific binding via Fc receptors may also be a significant problem which can be overcome by use of antibody fragments such as Fab and $F(ab')_2$. In addition, antibody fragments may ease the penetration of the reagent into tissues. Smaller fragments such as engineered Fv fragments may also be beneficial and have also been developed for use in immunocytochemistry applications (Kleymann *et al.*, 1995).

Antibodies can be directly labelled for immunocytochemistry, but if several antigens are to be detected, this requires preparation of several individual labelled reagents. The use of labelled second antibody allows the detection of several antibodies with a single reagent. For example, sections can be probed with mouse MAbs and then detected with

Samsung Bioepis Exhibit No. 1002
Page 5362

Table 3.3 Examples of antibody labels for immunocytochemistry

Enzymes	Substrates	Colour
Horseradish peroxidase	Diaminobenzidine	Brown
	Tetramethylbenzidine	Blue
	Aminoethylcarbazole	Red
	4-chloro-1-napthol	Blue-black
Alkaline phosphatase	Napthol AS phosphate + fast red, blue or violet	Red, blue or violet
	Bromochloroindolyl phosphate + nitroblue tetrazolium	Blue
Fluorescent labels	Fluorescein	Green
	Rhodamine	Red
	Phycoerythrin	Orange
	Texas Red	Red
	Cyanin 3 or 5	Red or orange
Metal labels	Colloidal gold 1–40 nm	Electron-dense
	Gold + silver enhancement	Dark brown or black

a labelled anti-mouse Ig reagent. Second antibody methods also allow some amplification on detection, and avoid the problem of developing labelling methods to minimise loss of antigen-binding properties with many different antibodies. Alternatively, biotinylated antibodies which can be detected with a labelled streptavidin conjugate are widely used.

The major types of label used in immunocytochemistry are enzymes used to generate insoluble coloured products, fluorescent labels and colloidal gold particles (Table 3.3). Both fluorescent and enzyme substrate labels are available in different colours such that two or more antigens may be visualised simultaneously. For example, fluorescein and phycoerythrin can be used to detect two different antigens under a fluorescent microscope with differential colour fluorescence, green and orange respectively. Horseradish peroxidase is the most widely used enzyme label for animal tissues, though not for plant tissues due to the presence of endogenous peroxidase activity which can lead to problems with non-specific staining. Conversely plant tissues can be stained with alkaline phosphatase conjugates, although endogenous activity makes this technique unsuitable for many animal tissues. In practice, however, both enzymes are suitable for most types of tissue, as endogenous activity is easily inhibited. Colloidal gold is suitable as a label for use in both light and electron microscopy. In light microscopy, the signal from gold-labelled antibodies is seen as a pinkish colour. This is usually intensified by the use of silver enhancement in which the gold catalyses the reduction of silver ions to metallic silver which results in a stable signal. Under the electron microscope, colloidal gold is seen as dense black round particles that can easily be distinguished from cellular structures. Different size particles are available, for example, 5 nm and 30 nm, which can be used to allow the detection and relative quantification of two antigens simultaneously.

Many of the problems observed with immunocytochemistry are similar to those seen with immunoassay development. Non-specific binding is also a problem which can be overcome in many cases by purification of suitable reagents, or the use of antibody fragments or alternative labelling techniques.

Samsung Bioepis Exhibit No. 1002
Page 5363

One of the major uses of immunocytochemistry is the identification and classification of human tumour types (Giovagnoli and Vecchione, 1996). Information gained in this way can be of direct benefit in patient care. MAbs can allow the identification of the primary site of metastatic carcinoma, give information on the malignancy of the tumour and in some instances be an aid to determining prognosis. For example, the breast cancer marker $p185^{HER2}$ has been associated with increased probability of relapse and poor outcome (Slamon *et al*., 1987). Immunocytochemistry to detect expression of this marker in breast cancer tissue is thus useful in determining likely patient prognosis. Similarly, applications of MAbs in the detection and identification of microorganisms are important clinically such as in the demonstration of human cytomegalovirus infection (Jahn and Plachter, 1993). There is also a role for immunocytochemistry in other disease states in the detection of cellular infiltrates and the determination of their activation state, the localisation of adhesion molecules and identification of locally produced cytokines. In pulmonary diseases such as allergic asthma and sarcoidosis, the analysis of cytokines from cells present in bronchoalveolar lavage fluid using immunocytochemistry can give an insight into the disease process (Krouwels *et al*., 1997).

3.5 Flow cytometry and cell sorting (FACS)

Flow cytometry and fluorescence activated cell sorting techniques (FACS) are widely used in cell biology research for both analytical and preparative purposes. Fluorescently labelled antibodies capable of binding to the cells of interest are used to tag these cells, such that the cells become fluorescent. FACS equipment resolves mixtures of cells based on this fluorescence. Within the instrument a stream of single cells passes through a laser light beam and fluorescent cells can be individually detected. The distribution of antigens within the population of cells can therefore be readily studied. Preparative isolation of populations of cells becomes possible as the instrument can selectively impart an electrostatic charge to labelled cells and not others. The cells then pass through an electric field when the cells carrying a charge are deflected into a different container from the uncharged cells. As such this technique can be considered as an immunopurification technique for whole cells, which has been used for both research and clinical applications. Applications include the separation of cell populations for functional studies and the isolation of rare transfected cells to simplify cloning.

FACS techniques can also be performed with two different fluorescent labels, allowing the simultaneous detection of two cellular antigens. Such analyses are widely used in characterising populations of cells, for example in the characterisation of hematopoietic stem cells used for autologous transplantation, using antibodies to CD34 and CD19 (Fritsch *et al*., 1995). The use of FACS for preparing a purified population of CD34 positive bone marrow cells for use in transplantation studies has also been described (Rondelli *et al*., 1996). The application of flow cytometry to detect cellular antigens is not restricted to cell surface markers: intracellular substances can also be detected. The use of flow cytometry in the detection of intracellular interleukin-4 and -5 and interferon-γ has been compared with immunocytochemistry (Krouwels *et al*., 1997). In this comparison flow cytometry was shown to be more suitable for use with double staining techniques for detection of more than one antigen and allowed isolation of defined cell populations. However, the detection of these cytokines was apparently less sensitive than with immunocytochemistry.

Samsung Bioepis Exhibit No. 1002
Page 5364

3.6 Western blotting (immunoblotting)

The technique of western blotting, also known as immunoblotting, is used to detect protein antigens after polyacrylamide gel electrophoresis (PAGE). PAGE is used to separate proteins in an electric current, and in its most widely used form is carried out in the presence of the detergent sodium dodecyl sulphate (SDS). SDS denatures proteins and binds to them such that a uniform negative charge is imparted to each protein. Therefore, when proteins are run through a gel using an electric current in the presence of SDS (SDS-PAGE) they can be separated on the basis of molecular weight. The range of molecular weights which can be separated depends on the pore size in the gel used and this is normally controlled by the concentration of acrylamide and cross-linker used in formation of the polyacrylamide gel. Proteins separated on SDS-PAGE are then visualised using a general protein stain such as coomassie blue or, more sensitively, silver stain. However, general protein stains stain every protein, and it is often desirable to be able to identify specific proteins. This can be achieved by western blotting. After SDS-PAGE the proteins present in the gel are transferred laterally (blotted) onto a membrane of nitrocellulose or an alternative polymer such as polyvinylidene difluoride (PVDF). The protein (or proteins) of interest can then be detected using the desired antibody which is either conjugated to a suitable label or detected by a secondary labelled antibody reagent. Labels commonly used include the radioisotope iodine-125 followed by exposure to X-ray film, colloidal gold with silver enhancement (see Section 3.4), and the enzymes horseradish peroxidase and alkaline phosphatase either with insoluble coloured substrates or, in the case of peroxidase, to generate a chemiluminescent readout by oxidation of luminol in the presence of hydrogen peroxide. Chemiluminescent detection by HRP-conjugated antibodies is usually enhanced with chemical enhancers and is a particularly sensitive detection method. An additional advantage is that after detection the signal can be removed by stripping off the bound antibody with protein denaturants as first developed for use with radiolabelled antibodies (Kaufmann *et al.*, 1987). This allows the blot to be reprobed with another antibody and re-detected, thus allowing several different antigens to be examined sequentially on the same blot. Western blotting is not confined to SDS-PAGE but can also be used to identify proteins separated by electrophoresis under non-denaturing conditions or by isoelectric focusing.

An example of a western blot is shown in Figure 3.10. Both polyclonal and monoclonal antibodies are used in western blotting. MAbs offer excellent specificity, but as detection is carried out on denatured proteins, reactivity of the MAb with the native conformation of the protein does not necessarily allow the antibody to be used in western blotting. This is because the antibody may recognise an epitope not made up of a stretch of linear polypeptide sequence but of parts of the protein which are spatially close in the protein's native conformation due to folding. Polyclonal antisera which recognise many different epitopes are more likely to contain antibodies which recognise some linear polypeptide sequences, and thus are often more successful in western blotting applications. However, screening can be carried out to identify those MAbs which are suitable for blotting.

The applications of western blotting in the research laboratory are wide and include the detection and analysis of natural proteins of low abundance, the analysis of recombinant protein expression and the detection of contaminant proteins. The combination of immunological reactivity with size analysis gained from western blotting allows analysis of protein processing and post-translational modifications. The use of MAbs allows precise information to be gained which would not be possible otherwise. This is demonstrated by a recent study to determine the role of matrix metalloprotease enzymes in human arthritis

Samsung Bioepis Exhibit No. 1002
Page 5365

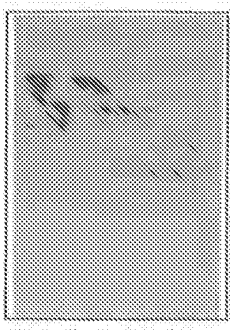


Figure 3.10 Example of a western blot. SDS-PAGE was carried out using a 4–20% polyacrylamide gel under non-reducing conditions. The blotted gel was probed with a murine monoclonal antibody to IgG Fd (Fab heavy chain) followed by a polyclonal rabbit anti-mouse IgG–horseradish peroxidase conjugate. The blot was then developed using enhanced chemiluminescence. Lane 1, recombinant human IgG4 (hIgG4); lane 2, $F(ab')_2$ derived from hIgG4; lane 3, hIgG1; lane 4, hIgG4 and $F(ab')_2$ mixture; lane 5, γ1 hFab' and $F(ab')_2$ expressed in mammalian cells; lane 6, γ1 hFab' and $F(ab')_2$ expressed in *E. coli*; lane 7, γ4 hFab'(a) expressed in mammalian cells; lane 8, γ4 hFab'(b) expressed in *E. coli*.

(Fossang *et al.*, 1996). Aggrecan is the major proteoglycan in cartilage and the molecule that allows the tissue to bear weight by deforming under compression. A MAb recognising the cleaved products of metalloprotease action on aggrecan allowed the detection of cleaved products in synovial fluid by western blot which could be quantitated with an ELISA using the same antibody. This analysis allows the protease activity involved to be characterised, leading to further insight into the degradative mechanisms involved in the arthritic process.

Western blotting can also be used in routine diagnostic procedures, for example in the confirmation of HIV infection (Guttler, 1996). In this case anti-HIV antibodies are detected initially by ELISA tests, and reactive results are confirmed by western blotting which can allow differentiation of HIV types and subtypes. Western blotting has also been used to determine alcohol abuse by measurement of carbohydrate-deficient transferrins (Anton and Bean, 1994). Carbohydrate-deficient transferrins are produced in greater quantities than usual during periods of heavy alcohol intake due to decreased glycosylation of transferrin before release into the blood. Western blotting of serum samples allows separation and detection of the carbohydrate-deficient transferrin from the bulk of normal transferrin. In this case isoelectric focusing is used as the separation technique, where the difference in charge of the carbohydrate-deficient transferrin provides the basis for good separation from a large amount of normal transferrin.

Recombinant fusion proteins are also beginning to play a role in western blotting applications. Fusions of an scFv recognising the plant protein phytochrome have been made to both alkaline phosphatase and the IgG binding protein staphylococcal protein A (Gandecha *et al.*, 1994). Western blotting of extracts from oat seedlings containing phytochrome demonstrated that both of these fusion proteins could be successfully used for western blotting. The alkaline phosphatase conjugate was visualised directly using the chromogenic substrates BCIP/NBT (bromochloroindolyl phosphate + nitroblue

Samsung Bioepis Exhibit No. 1002
Page 5366

tetrazolium), whereas the protein A conjugate was used in a two-step method, detected by an IgG–alkaline phosphatase conjugate. The fusion proteins were expressed in *E. coli*, and detection on the western blots was possible using the fusion proteins directly from *E. coli* lysates without the need to purify the reagents. The functionality of such conjugates suggests that the role of recombinant fusion proteins in the immunoassay field in general will increase and allow the use of simple single-step procedures, which may be advantageous in reducing non-specific binding problems.

3.7 Immunopurification

Immunopurification uses the specificity of the antibody to bind to a substance of interest and isolate it in a purified form. Antibody is usually coupled to a solid phase, such as beaded agarose, and packed into a chromatography column for use in the procedure known as immunoaffinity chromatography. This is a type of affinity chromatography in which antibodies are used as the binding moiety to retain the protein, or other substance of interest, while contaminating material flows through the column. The bound protein is then eluted by a change in buffer conditions to those which promote dissociation of the antibody–antigen complex (Figure 3.11). Immunoaffinity chromatography is used for the simple, rapid purification of proteins in the research laboratory both for preparation of the protein itself and as a quantitative measure, i.e. assay. In addition, immunoaffinity chromatography is used commercially for the purification of a number of pharmaceutical proteins, particularly high value proteins which are difficult to purify by other means. Because the interaction between antibody and antigen is very specific, a high degree of purification is achieved in a single step. Therefore, the use of immunoaffinity chromatography negates the requirement to develop protein-specific, multi-step purification protocols and allows rapid isolation of protein for research purposes.

Elution from the immunoaffinity chromatography column is achieved by disruption of the antibody–antigen interaction using mild denaturation such that both antigen and antibody are not irreversibly denatured. Commonly used eluents include extremes of pH and chaotropic agents such as thiocyanate, which often allow active material to be obtained. However, if the affinity of the antibody for antigen is very high it may be difficult to elute bound antigen without irreversible denaturation. Relatively low affinity MAbs are therefore ideal for successful immunopurification. Antibodies with affinity constants in the range 10^{-6}–10^{-8} M have been suggested to be most suitable (Phillips, 1989). However, the best way to select an antibody for immunopurification is by testing a panel of antibodies, to see which allow elution of the antigen under mild conditions. This can be achieved conveniently using ELISA or BIAcore format assays in which several eluent solutions are also screened, or antibody coupled to solid-phase beads can be used in small-scale experiments. When suitable antibodies have been identified, the antibody is coupled to a solid phase, usually a cross-linked beaded agarose material such as Sepharose, suitable for use in immunoaffinity chromatography. Many such matrices are commercially available in pre-activated form, which allows simple attachment of the antibody through amine groups with matrices derivatised with linkers such as *N*-hydroxysuccinimide or cyanogen bromide (see Section 2.6.2). Matrices are also available which allow site-specific attachment of antibodies, such that more of the antigen-binding ability is retained.

Random coupling through lysine residues often results in only 10–15% of the antibody retaining its antigen-binding activity due to a combination of chemical modification of

Samsung Bioepis Exhibit No. 1002
Page 5367

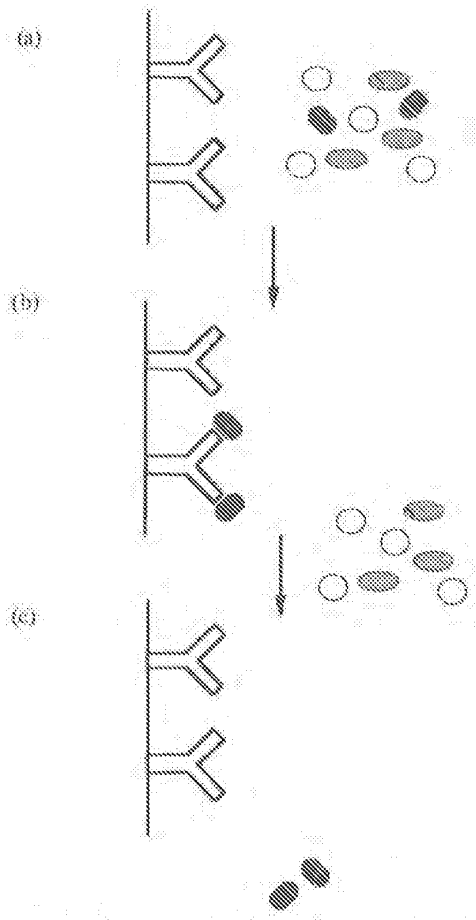


Figure 3.11 Immunoaffinity chromatography: immobilised antibody is used to selectively bind the ligand of interest (a); after washing to remove non-bound material (b), bound ligand is eluted in purified form (c), regenerating the immobilised antibody for re-use

important amino acid residues in the binding site of the antibody, and lack of orientation of antibody on the solid phase, such that many binding sites may be occluded (Fowell and Chase, 1986). Similar methodology is used for site-specific attachment as for site-specific protein–protein conjugates (see Section 2.6.2). Site-specific attachment can be achieved through attachment to Fc carbohydrate by periodate oxidation followed by reaction with hydrazide activated matrix (O'Shanessy, 1990). By attaching the antibody via the Fc this method orients the antibody such that the antibody-binding sites are less likely to be occluded by the matrix as well as avoiding modification of important amino acids in the CDR regions. Similarly, Fc region binding proteins have been used to orient the antibody correctly such that the antigen-binding site is still available. Staphylococcal protein A and Streptococcal protein G are widely used in antibody purification as they bind IgG specifically through Fc region binding sites between the C_{H2} and C_{H3} domains (see Chapter 5). Protein A and protein G linked to solid phases are therefore widely available reagents.

Samsung Bioepis Exhibit No. 1002
Page 5368

Binding of antibody for immunopurification to the protein A matrix followed by chemical cross-linking can therefore be used to immobilise antibody in oriented fashion, retaining a larger proportion of active antigen-binding sites (Sisson and Castor, 1990). Fab′ fragments can also be used for site-specific attachment to matrices through the hinge region free thiol groups. Maleimide or iodoacetyl activated matrices can be used to generate Fab′ solid phases in which most of the antigen-binding ability of the Fab′ is retained (Prisyazhnoy *et al.*, 1988). Immobilised Fab′ fragments may have additional advantages in that the lower avidity of the monovalent Fab′ fragment for antigen may allow elution of bound antigen under less harsh conditions than for intact IgG, particularly when low immobilised density on the solid phase is used.

Immunoaffinity chromatography is widely used for the purification of both natural and recombinant proteins for research purposes, particularly when the source of the protein contains only small amounts among many other contaminating proteins. With recombinant proteins it is not always necessary to have a MAb to the protein, as specific 'tags' can be added to the gene before expression such that the resulting fusion protein can be easily purified. The commercially available FLAG system is one such 'tag' (Hopp *et al.*, 1988). The FLAG epitope consists of the 8 amino acid sequence Asp-Tyr-Lys-$(Asp)_3$-Lys, and vectors are available which allow fusion of the gene for this sequence to the recombinant gene of interest. The resulting fusion protein can then be purified using an immobilised MAb which recognises a calcium complex of the FLAG peptide. The MAb used is conformation-specific and in the absence of calcium the antibody dissociates from the peptide tag. This system therefore allows for very mild elution of the tagged recombinant protein using EDTA to remove calcium or, alternatively, a competitive peptide can be used (Hopp *et al.*, 1996). The FLAG system has proved of wide applicability and has been used to purify a wide range of protein targets including interleukin-3 (Park *et al.*, 1989), β2-adrenergic receptors (Guan *et al.*, 1992), tumor necrosis factor (Su *et al.*, 1992), transcription factors (Chiang and Roeder, 1993), antibody fragments (Knappik and Pluckthun, 1994) and interleukin-5 receptors (Brown *et al.*, 1995).

Immunoaffinity chromatography is also increasingly used as a form of immunoassay. Immobilised antibody is used to capture the analyte of interest, which is then eluted and quantitated by UV absorbance or other means. The development of useful assays has been facilitated by the development of new solid-phase materials which can be operated at high flow rates allowing very fast assays to be performed and automated (Afeyan *et al.*, 1992). Assays are not restricted to proteins, and for other analytes immunoaffinity chromatography is often coupled with detection techniques such as high-performance liquid chromatography (HPLC), gas chromatography (GC) or mass spectrometry (MS). For example, the β2-agonists salbutamol and clenbuterol have been determined in tissue samples (Pou *et al.*, 1994), and both aflatoxin contamination in cheeses and cannabis metabolites in saliva have been determined by combining immunoaffinity chromatography with HPLC (Dragacci *et al.*, 1995; Kircher and Parlar, 1996).

The high affinity of the antibody:antigen interaction also allows immunoaffinity chromatography to be used for the specific removal of trace contaminants which may be present at low levels. This includes the removal of protein contaminants from pharmaceutical proteins for *in vivo* use, and applications such as the removal of trace bacterial contamination from foodstuffs. Molloy *et al.* (1995) have demonstrated that an immobilised scFv which recognises a lipoprotein surface component of *Pseudomonas aeruginosa* can remove cells of this bacterium from mixed cultures and from milk. This study demonstrated that immobilised antibody fragments could be effective and potentially

Samsung Bioepis Exhibit No. 1002
Page 5369

economic in this type of application and suggests that scale-up of this technology may be possible to allow the use of immunoaffinity columns for removal of toxic materials from the environment.

The use of immunoaffinity columns for specific removal of materials may also find clinical application. Several groups have attempted to develop systems in which plasma is removed from the patient, passed through an immunoaffinity column and then returned to the patient. Sato *et al.* (1989) have suggested that specific removal of IgE is useful in the treatment of patients with allergy or other conditions in which the level of IgE is abnormally high. Another application is the use of immunoaffinity techniques for the removal of radiolabelled antibodies from the blood following radioimmunotherapy of tumours (Norrgren *et al.*, 1991). Radioimmunotherapy is described in Section 4.2.5. Briefly, antibody to a tumour-associated antigen is used to target a radioisotope to tumour cells in an attempt to kill them. However, to deliver a toxic dose to the tumour requires injection of a large amount of radiolabelled antibody which is often toxic to normal tissues such as bone marrow. Therefore attempts have been made to allow antibody to localise to tumour cells and then remove excess antibody from the blood using immunoaffinity techniques, hence reducing normal tissue toxicity.

Many recombinant proteins are now used as pharmaceuticals. Immunopurification would be an attractive method for purification of many of these pharmaceutical proteins. However, the production of MAbs in large amounts is relatively expensive compared to other chromatographic ligands, and therefore the use of immunopurification techniques in the production of proteins for pharmaceutical use has been limited. Also, when intended for use in the manufacture of a pharmaceutical protein the MAb must be produced to a standard which is as high as that demanded of the pharmaceutical protein itself. Nevertheless, immunopurification has been used for some high-value proteins which are difficult to prepare by other means, for example interferons, factor VII, factor VIII and factor IX (Ballon and Roy, 1990; Kim *et al.*, 1992).

Antibody fragments which can be produced in bacterial expression systems may be manufactured much more economically than intact IgG (see Chapter 5) and thus there has been interest in the development of immunoaffinity columns using Fv and scFv fragments. Engineered antibody fragments can also be produced with sites for specific attachment to the solid phase designed such that minimal antigen binding activity is lost on immobilisation. For example a hinge region containing a single thiol group can be engineered onto the C-terminus of an scFv fragment to allow site-specific conjugation (King *et al.*, 1994). Both Fv and scFv fragments have been immobilised and used for immunoaffinity chromatography (Berry *et al.*, 1991; Spitznagel and Clark, 1993; Molloy *et al.*, 1995). scFv may be a better reagent to use as the covalent linkage between V_H and V_L results in less leakage from the column during elution (Berry and Pierce, 1993). In a comparison of IgG, Fab' and Fv fragments it was found that immobilisation of Fv resulted in the highest binding capacity, presumably due to the small size of the fragment (Spitznagel and Clark, 1993). However, antibody fragments with exposed hydrophobic patches on the surface of the protein must not be used, as these may result in high levels of non-specific binding to the column. Use of a single V_H domain which could bind antigen (dAb) was not successful as an immunopurification reagent for this reason (Berry and Davies, 1992).

The development of these low-cost affinity materials will no doubt open up a range of applications for immunopurification which have been limited to date by cost. This could include the design of reagents for the simple purification of pharmaceutical proteins, and

Samsung Bioepis Exhibit No. 1002
Page 5370

applications such as removal of environmental contaminants. A further example of this is the generation of an scFv to the herbicide paraquat specifically for use as an immunopurification reagent (Graham *et al.*, 1995). The use of phage technology will allow the selection of the most suitable antibody for a particular application, which can then be reconstructed in the most suitable format for immobilisation.

3.8 Antibodies in structural biology

MAb fragments have also found a role in the determination of protein structures by X-ray crystallography. Many proteins of biological interest are unable to form the high-quality crystals required for structural determination due to heterogeneity, insolubility, flexibility or polydispersity in solution. Antibody fragments such as Fab and Fv are soluble, bind tightly to antigen and can thus effectively transform aggregated protein into a soluble monodisperse sample suitable for crystallisation (Kovari *et al.*, 1995). The HIV capsid protein p24 was able to be crystallised only as a Fab–p24 complex which prevented oligomerisation of the p24 protein (Prongay *et al.*, 1990). In the case of HIV reverse transcriptase, a Fab fragment was used to decrease the mobility of a region of the protein allowing rapid determination of the structure (Jacobo-Molina *et al.*, 1993). Fv fragments are also particularly useful and have been used to allow determination of the membrane protein bacterial cytochrome c oxidase by enlargement of the polar surface to allow formation of crystal lattice (Ostermeier *et al.*, 1995). It is usually necessary to screen a large number of antibody fragments to find a suitable one for crystallisation which not only stabilises the protein of interest but also allows suitable crystal contacts to form. Ideal antibody fragments will not alter the conformation of the protein of interest, although it should be remembered that it is often the case that the conformation of the antibody itself may change on binding antigen (see Chapter 1). Selection of appropriate antibody fragments from phage display may allow rapid identification of suitable reagents.

3.9 *In vivo* diagnostics

The use of antibodies for imaging disease has been developed to the point where there are now several products marketed for clinical use (Table 3.4) and many more under development. Antibodies to suitable antigens can be used to confirm the presence and extent of disease in the body, identify its location and monitor the progress of any therapy. To achieve this the antibody is labelled with a suitable radioisotope, usually a short half-life gamma emitter, and the emissions from the isotope detected with a gamma camera. Antibodies can also be labelled with positron emitting isotopes for use in positron emission tomography scanning. This technique is variously known as radioimmunodetection (RAID) or radioimmunoscintigraphy (RIS). Advances in instrumentation, developing such techniques as single photon emission computerised tomography (SPECT), have contributed to the popularity of development of RIS by allowing improved accuracy and detection of lesions not seen using other techniques. An alternative form of radioimmunodetection is used to aid surgical procedures, known as radioimmunoguided surgery (RIGS). In this technique radiolabelled antibody localised to the site of disease is detected by a hand-held monitor to allow simple identification and resection of diseased tissue during surgery.

Samsung Bioepis Exhibit No. 1002
Page 5371

Table 3.4 Some commercially marketed monoclonal antibody imaging agents

Imaging agent	Antibody	Isotope	Disease	Company
OncoScint CR/OV	B72.3 IgG (anti-TAG72)	^{111}In	Colorectal and ovarian cancer	Cytogen
CEA-Scan	Immu-4 Fab' (anti-CEA)	^{99m}Tc	Colorectal cancer	Immunomedics
LeukoScan	anti-NCA-90 Fab'	^{99m}Tc	Infection/inflammation	Immunomedics
Myoscint	anti-myosin Fab	^{111}In	Heart disease	Centocor
ProstaScint	7E11-C5.3 IgG	^{111}In	Prostate cancer	Cytogen
Verluma	NR-LU-10 Fab'	^{99m}Tc	Small-cell lung cancer	NeoRx

3.9.1 Radioimmunodetection of human tumours

A major impetus to the development of RAID has been the desire to develop techniques to allow detection of human tumours and determine the extent and location of disease, particularly monitoring metastatic spread. In many cases accurate, early diagnosis using RAID can be a contributor to effective treatment, with the major use of RAID being to monitor disease in patients following treatment by surgery, radiotherapy or chemotherapy (Larson, 1995). Many tumour-associated antigens are now known and characterised, and antibodies to these allow targeting to different tumour types (Table 3.5). However, obtaining high-quality tumour images from radiolabelled antibodies is not straightforward and the immunoconjugate to be used requires careful optimisation of not only the antibody specificity and the radioisotope, but also the form of the antibody used and the method of attachment of the isotope to the antibody. Some of the many factors affecting RAID are given in Table 3.6.

Tumour-associated antigens

Useful tumour-associated antigens are absent on normal tissues and present at high levels on tumour cells, preferably homogeneously on all cells of the tumour. Antigen should also not be shed from the tumour into the blood. In reality there are no perfect, tumour-specific antigens and the choice of a suitable target requires compromise over one or more of these characteristics. Nevertheless, high-quality tumour imaging can be achieved through optimisation of the immunoconjugate and the use of alternative strategies to overcome the individual problems of particular targets. For example, antibodies to antigens which are present on normal tissues, but inaccessible to the administered antibody, have been used successfully in imaging studies, and the presence of circulating antigen can often be overcome through increasing the dose of antibody administered to saturate circulating antigen, or the use of an unlabelled antibody pre-dose (van Hof *et al.*, 1996).

Samsung Bioepis Exhibit No. 1002
Page 5372

Table 3.5 Some commonly used tumour-associated antigens, with examples of antibodies raised against them

Antigen	Tumour type	Representative antibody
Tumour-associated glycoprotein72 (TAG72), 72 kDa glycoprotein	Pancarcinoma	B72.3, CC49
Carcinoembryonic antigen (CEA), 180 kDa glycoprotein	Pancarcinoma	NP-4, A5B7
Polymorphic epithelial mucin (PEM), >100 kDa glycoprotein	Ovarian, breast, lung	HMFG1
Epithelial membrane antigen (EMA), 40 kDa glycoprotein	Colorectal (and other epithelial tumours)	17-1A
Epidermal growth factor receptor (EGFR), 175 kDa glycoprotein	Breast, lung	425
$p185^{HER2}$/ c-*erb*-B2 (185 kDa glycoprotein)	Breast, lung	4D5
Prostate-specific membrane antigen (PSMA), 100 kDa glycoprotein	Prostate	7E11-C5.3
CD33 67 kDa glycoprotein	Myeloid leukemia	P67.6, M195
CD20 35 kDa glycoprotein	Lymphoma	C2B8
GD2 ganglioside	Melanoma, neuroblastoma	14-18

The homogeneity of tumour-associated antigen expression is less important for RAID than for targeted therapy (see Chapter 4), as good imaging can be achieved as long as enough labelled antibody can reach the tumour site to generate a high tumour : background ratio. Hence tumours with as few as 15% of the cells expressing the relevant antigen have

Table 3.6 Factors affecting radioimmunodetection of cancer

Tumour-associated antigen	Specificity (presence in normal tissues) Antigen density and homogeneity in tumour Presence of shed antigen in blood
Antibody	Specificity Affinity and avidity Molecular size – penetration into tumour – pharmacokinetics Internalisation Immunogenicity
Radioisotope	Method of attachment to antibody (stability of conjugate and retention of antigen binding) Half-life Suitability of emission energy for imaging
Tumour	Location in the body Size Vascularisation and vascular permeability

Samsung Bioepis Exhibit No. 1002
Page 5373

been successfully imaged (Doerr *et al.*, 1991). When developing an antibody for RAID it is desirable that it can be used for several different types of tumour. Such pan-reactive antibodies have been identified using widely distributed tumour-associated antigens such as carcinoembryonic antigen (CEA) which is present on cells of colorectal, breast, ovarian and lung tumours, and polymorphic epithelial mucin which is present on ovarian, lung and breast tumour cells (Table 3.5).

Form of antibody

The generation of a high tumour : background ratio is also the key to the selection of the best form of antibody to use for tumour imaging. Intact antibody circulates in the blood for long periods and builds up relatively high levels of activity at the tumour site. However, the long residence time of labelled antibody in the blood means that relatively poor tumour : blood ratios are generated at early time points, and tumour imaging is only possible days later when sufficient antibody has cleared from the blood. Antibody fragments clear much more rapidly (see Section 2.7) and although lower levels of activity are accumulated at the tumour site, higher tumour : blood ratios are generated at early time points, allowing imaging to take place on the same day. Many studies have been carried out with $F(ab')_2$ and Fab' fragments generated by digestion from IgG and, more recently, the generation of recombinant fragments of anti-tumour antibodies has allowed smaller fragments such as Fv and scFv to be tested. Studies of Fab' and $F(ab')_2$ compared to IgG demonstrate that faster imaging is possible with antibody fragments leading to the detection of small tumour deposits with higher sensitivity (Lane *et al.*, 1994; Behr *et al.*, 1995). In comparative studies of IgG, $F(ab')_2$, Fab', Fv and scFv, higher ratios were generated with smaller fragments, the best being the Fv and scFv fragments (Milenic *et al.*, 1991; King *et al.*, 1992b). There is also strong evidence that smaller fragments penetrate further into the tumour mass, again with scFv more effective than Fab' and $F(ab')_2$ (Yokota *et al.*, 1992). Clinical imaging with scFv has also been found to be very effective. Using an anti-CEA scFv derived from phage display, Begent *et al.* (1996) have demonstrated that high-quality tumour images could be obtained with improved sensitivity over conventional imaging using X-ray computerised tomography (CT). Several liver metastases of colorectal tumours not seen at all by conventional CT were detected using scFv imaging. However, good tumour imaging is also dependent on the biology of the system under investigation. Hence although for most tumours antibody fragments are preferable, for some tumours which are less accessible or less well vascularised, early imaging with antibody fragments may be difficult and better results may be observed by imaging later with an intact IgG (Behr *et al.*, 1995).

Further attempts to optimise the form of antibody for RAID have led to the testing of divalent and trivalent Fab' and scFv fragments. Fab' and scFv fragments are monovalent with respect to antigen binding which leads to low avidity of binding (see Chapter 1). Production of multivalent forms of scFv may enable the benefits of small size to be retained while enabling better binding ability leading to increased tumour uptake. scFv's expressed with a hinge region attached have been used to prepare di-scFv's which have shown increased tumour uptake leading to superior tumour : blood ratios (Adams *et al.*, 1993; King *et al.*, 1994). Tri-scFv's have improved ratios further due to increased antigen-binding ability with only a small increase in blood retention (King *et al.*, 1994). Cross-linked Fab' fragments also show improved tumour targeting which may lead to improved agents for both RAID and targeted therapy (see Chapter 4).

Samsung Bioepis Exhibit No. 1002
Page 5374

Table 3.7 Commonly used radioisotopes for development of RAID

Radionuclide	Emission	Half-life (hours)	Energy (keV)	Comments
^{131}I	γ + β	193	364	Reactor produced, also used as therapeutic isotope (β emission)
^{123}I	γ	13	159	Cyclotron produced
^{111}In	γ	68	171	Cyclotron produced, matched to ^{90}Y for therapy, used clinically
^{99m}Tc	γ	6	141	Generator produced, most suited to available cameras, readily available and widely used clinically
^{67}Ga	γ	80	184	Cyclotron produced, in clinical use as citrate
^{64}Cu	β+	13	511	For use in PET scanning

Radioisotopes

Suitable radioisotopes for RAID are usually short half-life gamma emitters, although positron emitting isotopes are also under development for positron emission tomography (PET) applications (Table 3.7). For use in RAID, radioisotopes must be of sufficient energy to reach the detector outside the body whilst having a low linear energy transfer (LET, the energy deposited by the isotope over its pathway) resulting in the minimum damage to cells within the body. Optimal tumour imaging requires a good match between the energy of the gamma emission and the gamma camera detector. Most current gamma cameras are best used with gamma emissions in the range of 100–200 keV. Isotopes also need to be available in carrier-free form and must have suitable chemical properties to allow attachment to antibody. They must not have hazardous daughter isotopes, and should be of suitable half-life to allow preparation of the radiolabelled antibody, localisation and clearance of blood activity. As such the half-life of the isotope used needs to be matched to the biological half-life of the form of antibody used. For example, indium-111 is well suited to RAID with intact IgG, while technetium-99m is well suited to rapidly clearing antibody fragments such as Fab′ and scFv.

Iodine isotopes have been widely investigated due to their ready availability and well-developed methods for radioiodination of antibodies at either tyrosine or lysine residues (see Section 3.2.2). ^{131}I is not an ideal isotope for RAID, and is used primarily in experimental studies of tumour targeting for therapy. However, the ability to image tumours using the same labelled immunoconjugate used for therapy is valuable as it allows tumour dose estimates to be determined (DeNardo *et al.*, 1996a). ^{123}I is a potentially useful diagnostic nuclide although it is relatively poorly available and expensive. Its short half-life means it is best suited to imaging with antibody fragments and has been used successfully to image colorectal tumours with anti-CEA F(ab′)$_2$, Fab and scFv (Goldenberg *et al.*, 1990; Begent *et al.*, 1996). One problem common to all radioiodinated antibodies is their relative instability *in vivo*. Antibodies are deiodinated *in vivo* which leads to iodine leaking out from the tumour and often to the accumulation of free iodine in the thyroid and stomach. The accumulation of free iodine can be blocked by pre-treatment

Samsung Bioepis Exhibit No. 1002
Page 5375

with cold iodine, although deiodination cannot be prevented. Internalising antibodies may also be rapidly metabolised leading to the expulsion from the cell of small molecular weight iodinated metabolites (Press *et al*., 1996).

Technetium-99m is probably the isotope of choice for most RAID applications. It is cheap, very readily available from generators in all hospital nuclear medicine departments, leads to low radiation exposure to the patient and has ideal physical properties for detection by gamma cameras. ^{99m}Tc can be attached to antibody either directly or via a chelating agent. ^{99m}Tc is prepared from a generator in the +7 state as pertechnetate and requires reduction, usually achieved with Sn^{2+} or ascorbate, to the +5 state for antibody labelling. Thiol groups are particularly good ligands for binding ^{99m}Tc and direct labelling of IgG is achieved through reduction of disulphide bonds in the antibody molecule, presumably in the hinge region, which are then used to bind ^{99m}Tc. Sn^{2+} can be used to reduce both pertechnetate and the antibody itself, and often an 'intermediate chelator' is used such as glucarate. Several kit formulations have been developed to allow ^{99m}Tc labelling via such direct labelling methods (e.g. Pak *et al*., 1992, Alauddin *et al*., 1992). Similarly, Fab' fragments can be labelled via free thiol groups in the hinge region, and scFv fragments have been engineered specifically to contain C-terminal cysteine residues to allow ^{99m}Tc labelling (George *et al*., 1995; Verhaar *et al*., 1996).

Bifunctional chelating agents have been developed for radiolabelling with different metallic radionuclides. These are termed bifunctional due to having a reactive group for antibody attachment and a chelating group for binding the radiometal. Thus the bifunctional reagent can be used to form an antibody–chelator conjugate which can then bind radiometal under mild conditions. Alternatively, the chelator can be pre-labelled with radiometal before attachment to the antibody, although this method has the disadvantage that more handling of radiolabelled materials is required and manipulations need to be carried out rapidly to avoid extensive radioactive decay. Several chelators for ^{99m}Tc labelling have been developed, many of which use thiol ligands. Diamide dimercaptide ligands, also known as N_2S_2 ligands, form a stable tetradentate complex with technetium (Figure 3.12), although problems with non-specific binding of technetium to antibody protein have resulted in a pre-labelling method being most successful (Fritzberg *et al*., 1988). Nevertheless methods have been developed to reduce this to a kit form for simple radiolabelling of antibody fragments (Kasina *et al*., 1991). N_3S ligands (Figure 3.12), alternative N_2S_2 chelates based on bis-aminoethanethiols, and other chelating groups such as hydrazino nicotinamides have also been developed which allow formation of the antibody–chelator conjugate before labelling (Weber *et al*., 1990; Eisenhut *et al*., 1996; Ulmer *et al*., 1997). Macrocyclic ligands based on cyclam which form very stable technetium complexes have also been examined (Morphy *et al*., 1988). Recombinant proteins with specific groups capable of binding technetium are also under development, for example fusion of the metal-binding protein metallothionein to antibody fragments can be used to allow subsequent binding of ^{99m}Tc (Das *et al*., 1992).

Comparisons of direct labelling with chelation have demonstrated that the use of defined chelation gives a more stable complex and results in higher retention of technetium in tumour tissue and less non-specific uptake in normal tissues, resulting in improved tumour images (Hnatowich *et al*., 1993; Ulmer *et al*., 1997). Nevertheless, clinical imaging of tumours has been successful with both types of procedure (Behr *et al*., 1995; Eary *et al*., 1989), and commercially available preparations include technetium-based tumour imaging agents using Fab' fragments labelled with both methodologies. Immunomedics' CEA-scan™ for imaging colorectal tumours uses a direct labelling procedure, whereas Verluma™ developed by NeoRx for imaging lung tumours is based on chelation methodology.

Samsung Bioepis Exhibit No. 1002
Page 5376

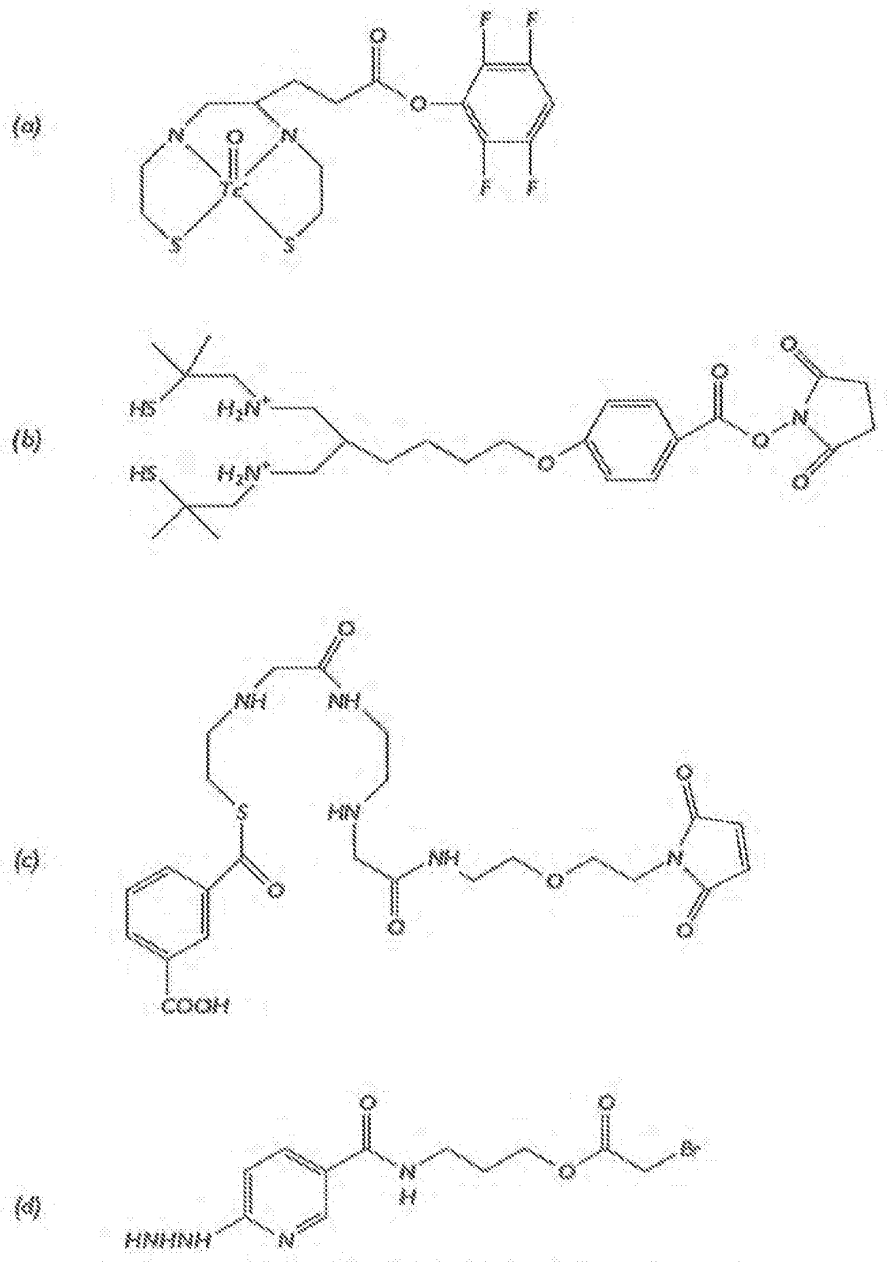


Figure 3.12 Examples of ligands for attachment to MAb which can be used to chelate technetium-99m: (a) active ester of ^{99m}Tc-4,5-bis-(thioacetamide)pentanoate for attachment to amine groups (Fritzberg *et al.*, 1988); (b) 6-(4′-(4″-carboxyphenoxy)butyl) -2-10-dimercapto-2,10-dimethyl-4,8-diazaundecane for attachment to amine groups (Eisenhut *et al.*, 1996); (c) *N*-(5-maleimido-3-oxapentyl)[2-[(3-carboxybenzoyl)-thio]acetyl]glycylglycylglycinamide (N_3S ligand) for attachment to thiol groups (Weber *et al.*, 1990); (d) bromoacetyl hydrazinonicotinamide hydrobromide for attachment to thiol groups (Ultee *et al.*, 1977)

Samsung Bioepis Exhibit No. 1002
Page 5377

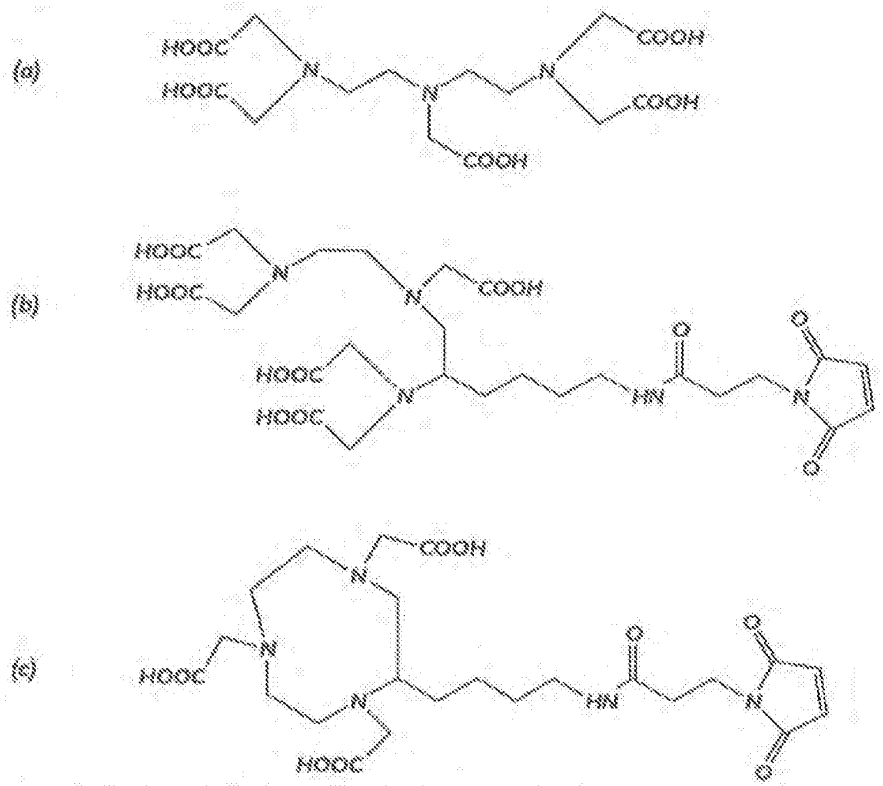


Figure 3.13 Examples of ligands for attachment of indium-111 to MAb. (a) DTPA; (b) derivatised DTPA (Harrison *et al.*, 1991); (c) 9N3 macrocycle (Turner *et al.*, 1994)

Indium-111 is also widely used for RAID applications and requires the use of a bifunctional chelator to allow labelling. Early studies used the cyclic anhydride of diethylenetriaminepentaacetic acid (DTPA) for attachment to antibody and subsequent chelation of ^{111}In (Hnatowich *et al.*, 1983). Although efficient radiolabelling can be achieved, the stability of the ^{111}In–DTPA complex is relatively low as one of the chelation arms is used for attachment to the antibody. *In vivo* dissociation of the complex can take place, leading to the formation of ^{111}In–transferrin which is subsequently deposited in the liver (Schumacher *et al.*, 1990). Subsequently, new derivatives of DTPA were produced such that a separate antibody attachment site could be used, preserving all eight coordination sites for ^{111}In (Figure 3.13), and resulting in more stable immunoconjugates (Brechbiel *et al.*, 1986). However, the most stable complexes of ^{111}In known are formed with macrocyclic ligands, and maleimide derivatives for attachment to antibodies have been developed which retain all of their stability toward binding ^{111}In (Figure 3.13; Craig *et al.*, 1989). Comparative biodistributions of the 9N3 macrocyclic ligand with DTPA and a bifunctional DTPA derivative revealed that the macrocyclic ligand resulted in improved tumour localisation with higher levels of activity in tumour and less in normal tissues (Turner *et al.*, 1994). The macrocyclic ligand DOTA (12N4), best known for labelling with ^{90}Y for radioimmunotherapy (see Section 4.2.5), can also be used to form a stable

Samsung Bioepis Exhibit No. 1002
Page 5378

complex with ^{111}In. Comparative biodistributions demonstrate little difference between the 9N3 and 12N4 macrocycles, although labelling of 12N4 with ^{111}In is more difficult (Turner *et al.*, 1994). Labelling procedures for 9N3 macrocyle immunoconjugates have been developed to allow reproducible ^{111}In incorporation efficiencies of >95% (Haines, A.M.R. and King, D.J., unpublished data), and clinical studies with such conjugates show good tumour imaging characteristics (van Hof *et al.*, 1996).

Fewer studies have been performed with alternative radionuclides, although gallium-67 is a potentially useful reagent which forms stable complexes with macrocyclic ligands (Craig *et al.*, 1989). Positron emission tomography (PET) imaging can be used with suitable radionuclides such as copper-64, bromine-76 and zirconium-89, allowing high resolution imaging to be carried out. Suitable macrocyclic ligands for stable attachment of ^{64}Cu have also been developed (Moi *et al.*, 1985). However, the application of antibodies to PET has been slow to develop due at least partly to the expensive equipment required for imaging studies to be performed. Experimental PET imaging studies have demonstrated good tumour imaging in animal models with a variety of isotopes (Anderson *et al.*, 1992; Lovqvist *et al.*, 1997; Meijs *et al.*, 1997). In clinical studies of colorectal carcinoma, ^{64}Cu-labelled antibody demonstrated impressive radioimmunodetection of small tumours in the abdomen and pelvis (Philpott *et al.*, 1995).

Two- and three-step targeting approaches

An alternative approach to improving tumour : blood ratios for RIS is the use of two-step targeting strategies. The simplest form of two-step tumour imaging is to use a second antibody reagent to clear blood background activity, hence improving the signal : noise ratio and the quality of the image. Immune complexes formed by a second antibody are rapidly removed from the circulation by the reticuloendothelial system, particularly in the liver. This can be viewed as an alternative to the use of rapidly clearing antibody fragments. Antibodies to the antibody itself or the radiolabelled chelator can be used (Reardan *et al.*, 1985; Pedley *et al.*, 1989). The use of antibodies to the chelator ensures that any radiolabelled metabolites in the circulation are also rapidly removed. Biotinylated antibodies have also been investigated and shown to be rapidly cleared by the administration of streptavidin (Marshall *et al.*, 1994).

In alternative approaches the administration of antibody and radiolabel are separated. Antibody is allowed to localise to tumour and sufficient time is allowed for antibody clearance from the blood and non-target tissues. Radioisotope is then injected separately in a form which can be readily captured by the tumour-bound antibody (Figure 3.14). Bispecific antibodies have been developed with specificity for tumour and a radiometal chelator such as DTPA (Goodwin *et al.*, 1988; LeDoussal *et al.*, 1989). After localisation of the bispecific antibody, and clearance from the blood, radiolabelled metal chelate is added which is then bound by the antibody localised at the tumour site and rapidly cleared from the rest of the body through renal excretion. Early studies revealed that divalent metal chelators which were able to bind two antibody binding sites simultaneously resulted in enhanced affinity for the tumour site and improved imaging (Goodwin *et al.*, 1988; LeDoussal *et al.*, 1989). A successful strategy may be the use of a di-Fab or tri-Fab construct, with one or two Fab arms binding to the tumour antigen and one available for binding the radiolabelled chelator. Bivalent chelator may then cross-link two antibodies at the tumour surface, resulting in its increased affinity. Such bispecific antibodies have been successfully used to image medullary thyroid carcinoma, colorectal

Samsung Bioepis Exhibit No. 1002
Page 5379

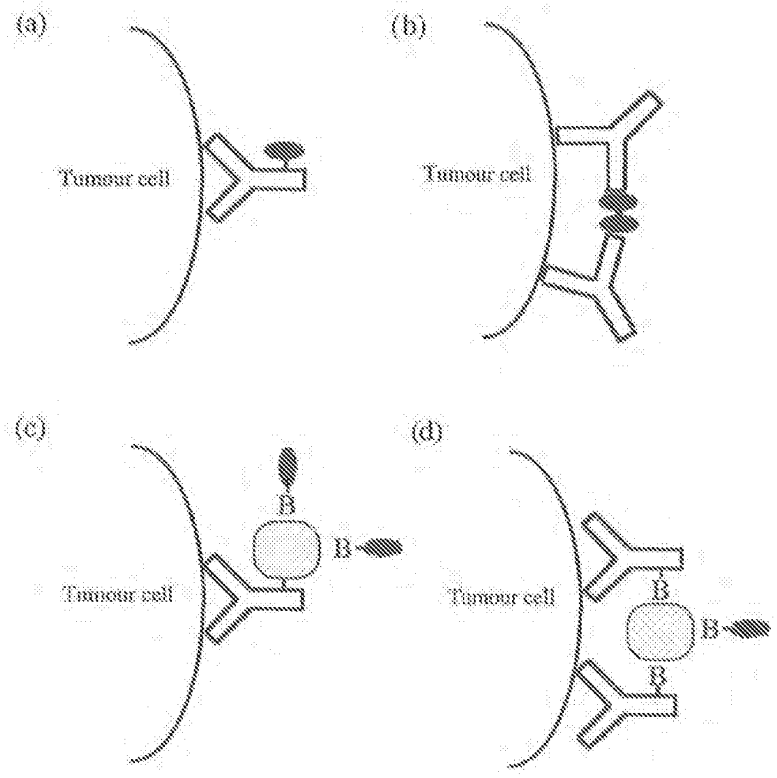


Figure 3.14 Strategies for tumour imaging: (a) one-step, directly labelled antibody; (b) two-step, bispecific anti-tumour antibody and anti-labelled hapten; (c) two-step, anti-tumour antibody–avidin conjugate and labelled biotin; (d) three-step, anti-tumour antibody–biotin conjugate, avidin, then labelled biotin (see text for details)

tumours and non-small cell lung tumours in patients (Peltier *et al.*, 1993; LeDoussal *et al.*, 1993; Vuillez *et al.*, 1997).

Alternative strategies have also been developed using the high affinity of the avidin:biotin system to capture radiolabelled small molecules from the blood as a two- or three-step imaging system. In the two-step version, antibody–avidin conjugate is injected and allowed to localise to tumour and clear from the blood. This is followed by injection of a low molecular weight biotinylated radiolabelled ligand which is captured by the tumour-bound avidin complex (Hnatowich *et al.*, 1987). Further development of this system has resulted in a three-step system in which biotinylated MAbs are injected and allowed to bind to the tumour site. This is followed by injection of avidin (or streptavidin) which binds to the tumour-bound antibody and has the additional advantage of forming a complex with antibody remaining in the blood which is rapidly cleared. Radiolabelled biotin is then added which is bound at the tumour site and cleared from the rest of the body (Paganelli *et al.*, 1991). Such three-step targeting allows the use of cocktails of biotinylated antibodies if desired which improves sensitivity through simultaneous detection of several tumour antigens, and has been used to detect small tumour deposits in patients, not visible by other techniques (Magnani *et al.*, 1996). The disadvantages are

Samsung Bioepis Exhibit No. 1002
Page 5380

their relative complexity, and the immunogenicity of some of the components used, such as streptavidin. Whether such methods will find long-term application remains to be seen.

3.9.2 Radioimmunoguided surgery

Radioimmunoguided surgery (RIGS) may prove to be a useful aid to surgery for the removal of tumour tissue. Patients are pre-administered radiolabelled antibody, and a hand-held gamma detecting probe is used intraoperatively to locate occult tumour which may otherwise escape detection. Some studies have suggested that the sensitivity obtained with RIGS may be better than with radioimmunoscintigraphy (Hinkle *et al.*, 1991), and RIGS has been shown to improve assessment of tumour spread and improve surgical intervention (Arnold *et al.*, 1992). For example, in patients with primary colorectal tumours RIGS with the anti-TAG72 antibody CC49 allowed detection of 86% of tumours, whereas in patients undergoing second-look surgery for recurrent disease 97% of tumour deposits were detected (Arnold *et al.*, 1992), the sensitivity of detection apparently being linked to antigen expression. Most studies have used antibody labelled with the weak gamma emitter ^{125}I, although ^{99m}Tc-labelled antibody has also been successfully tested (Ind *et al.*, 1994). Many of the parameters involved in effective RIGS are the same as those described above. For example, improvements are offered by the use of rapidly clearing scFv fragments allowing RIGS to be performed more rapidly after administration of the labelled targeting molecule (Nieroda *et al.*, 1995). Additionally, limitations due to antigen expression can be addressed by appropriate selection of antibodies or, in the case of CC49, by up-regulation of TAG72 antigen expression with γ-interferon treatment (Nieroda *et al.*, 1995).

3.9.3 Non-tumour radioimmunodetection

MAb-based radioimmunodetection has also been applied in several other disease situations, where precise imaging may offer useful clinical information. These include detection of myocardial necrosis, imaging of blood clots and the detection of infection and inflammation.

Myocardial necrosis takes place in a range of ischaemic, inflammatory and toxic heart diseases and results in breakdown of cell membranes, exposing cardiac myosin which is then accessible to a MAb administered systemically. Fab to the heavy chain of cardiac myosin labelled with ^{111}In has been used to image damage as a result of myocardial infarction (Khaw *et al.*, 1987), myocarditis and cardiac transplant rejection (Carrio *et al.*, 1988). It may also be beneficial in the identification of cardiac involvement in the autoimmune disease systemic lupus erythematosus (Merguet *et al.*, 1995), and in the assessment of cardiac toxicity following cancer chemotherapy with doxorubicin (Carrio *et al.*, 1995). Localisation of labelled antibody to the site of damage is usually very rapid, even when blood flow is reduced as a result of that damage, and the quality of the images obtained is dependent on rapid clearance of blood activity. Therefore most studies have used Fab fragments with rapid clearance times, usually labelled with ^{111}In via the chelator DTPA. However, with ^{111}In-Fab it normally takes 24–48 hours for blood pool activity in the heart to decrease sufficiently to allow good interpretation of images, which has limited the use of imaging in conditions such as myocardial infarction where rapid imaging is required. New agents are now being designed to allow more rapid imaging. In an

Samsung Bioepis Exhibit No. 1002
Page 5381

animal model system, an anti-myosin scFv has been shown to retain the same accumulation at sites of infarction as the Fab fragment, although clearing more rapidly from the blood (Nedelman *et al.*, 1993). In addition the scFv was labelled with ^{99m}Tc via a bifunctional chelator, which allows earlier imaging than with ^{111}In as discussed above (Section 3.9.1). Another approach has been to chemically modify anti-myosin Fab′ so as to impart a negative charge (Khaw *et al.*, 1991). Positively charged Fab′ may interact non-specifically through ionic interactions with negatively charged cell surfaces. Chemical modification of the Fab through attachment of a negatively charged polymer decreased background activity and allowed earlier imaging.

There has been much interest in the development of anti-fibrin antibodies for imaging blood clots, in both deep vein thrombosis and arterial thrombosis. Anti-fibrin antibodies have been identified which bind to fibrin but not circulating fibrinogen and thus can be used to visualise thrombosis. Both ^{111}In and ^{99m}Tc have been used in clinical studies, with high specificity and sensitivity of thrombus detection in deep vein thrombosis (DeFaucal *et al.*, 1991; Schiable *et al.*, 1992). Results in imaging arterial disease have been less impressive and ^{99m}Tc-antifibrin Fab′ was less effective in thrombus detection than labelled platelets (Stratton *et al.*, 1994). Venous thrombi comprise mostly thrombin and red blood cells whereas acute arterial thrombi contain more platelets with smaller amounts of fibrin and red blood cells. Hence a smaller antigen pool is present in arterial disease, which may be responsible for poorer imaging results. Anti-platelet MAbs have also been investigated as agents for detection of thrombi (Oster *et al.*, 1985), and may be potentially useful in the detection of active arterial thrombi, although further clinical evaluation is required. Again attempts to use more rapidly targeting and clearing molecules have been made. An scFv has been expressed with specificity for fibrin fragment D-dimer (Laroche *et al.*, 1991), and a peptide designed from an antibody CDR loop to mimic an anti-platelet antibody binding site has been produced (Knight *et al.*, 1994). Although some specificity was seen for ^{99m}Tc-labelled peptide, the levels accumulated at the thrombus were too low for useful imaging.

RAID is also under development in several other disease states to enable localisation of disease and to monitor therapy. Antibodies specific for polymorphonuclear leukocyte (PMN) surface antigens have been developed for imaging inflammatory processes at sites of infection. A ^{99m}Tc-Fab′ to the cell surface antigen NCA-90 has shown rapid targeting in clinical studies detecting soft-tissue infections and osteomyelitis, and is the basis of the agent LeukoScan™ (Becker *et al.*, 1994). Similarly, ^{99m}Tc-labelled anti-CD15 antibody has been used to image inflammatory disease, labelled either directly or via a DTPA-based chelator with no significant difference in image quality (Thakur *et al.*, 1996). Labelled polyclonal human IgG is also capable of imaging sites of inflammation, probably due to increased vascular permeability at the site or to binding to Fc receptors up-regulated at the site (Rubin *et al.*, 1989). The use of specific reagents may, however, increase confidence in interpretation of the scans as non-specific reagents may lead to scans which are difficult to interpret with often low or diffuse uptake (Thakur *et al.*, 1991). An interesting follow-up to non-specific imaging uses a two-step strategy (Kranenburg *et al.*, 1997). An unlabelled, monoclonal anti-DTPA antibody is used to localise to the site of infection non-specifically. After localisation and clearance from the blood has taken place, DTPA labelled with ^{99m}Tc is administered which binds to the sequestered antibody with the remainder rapidly cleared from the circulation, leading to low background levels and clearer imaging.

An alternative to imaging via antibodies to PMNs is to image the inflamed endothelium. Certain adhesion molecules are up-regulated during inflammation as part of the mechanism

Samsung Bioepis Exhibit No. 1002
Page 5382

of PMN recruitment to the site. Up-regulation of the selectins allows rolling of the PMN along the endothelial cells and up-regulation of intercellular adhesion molecule-1 (ICAM-1) allows the PMN to become firmly attached to the endothelial cell and then to migrate into the interstitium. Imaging sites of inflammation by antibodies to these endothelial cell adhesion molecules may therefore give additional selectivity. ^{111}In-labelled antibodies to both E-selectin and ICAM-1 have been investigated in animal models with promising results (Keelan *et al.*, 1994; Sasso *et al.*, 1996).

The abundance of CD4 on inflammatory cells present in arthritic joints has led to interest in targeting CD4 to image arthritic joints. Antibodies to CD4 have been used to assess targeting in animal models of arthritis. Design of the reagent is crucial for success, as intact IgG demonstrated no benefit over a non-specific antibody, whereas a ^{99m}Tc-labelled Fab' led to improved imaging (Kinne *et al.*, 1995). Anti-CD4 reagents may also be useful in monitoring the distribution of CD4 positive lymphocytes, which could be useful in assessing patients with HIV infection (Rubin *et al.*, 1996). RAID has also been investigated for detection of atherosclerotic plaques using an antibody towards proliferating smooth muscle cells (Narula *et al.*, 1995) and lesions in Alzheimer's disease using a cationised antibody towards beta A4 protein (Bickel *et al.*, 1994). Cationisation through chemical modification of the antibody with charged groups is believed to aid transport of antibodies through the blood:brain barrier, which is obviously helpful to image brain lesions in Alzheimer's disease.

Samsung Bioepis Exhibit No. 1002
Page 5383

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International Application Number:	
Confirmation Number:	7606
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First Named Inventor/Applicant Name:	Leonard BELL
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First Named Inventor	Leonard BELL
Art Unit	1644
Examiner Name	J. L. Rogers
Attorney Docket Number	AXJ-114BUSCN5

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	2	1755674	EP	B1	2014-11-19	Alexion Pharmaceuticals, Inc		

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	Art Unit	1644
	Examiner Name	J. L. Rogers
	Attorney Docket Number	AXJ-114BUSCN5

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	10	3056218	EP	A1	2016-08-17	Alexion Pharmaceuticals, Inc		☐
	11	2380907	EU	B1	2016-11-30	Alexion Pharmaceuticals, Inc		☐
	12	2004/050017	WO	A2	2004-06-17	Alexion Pharmaceuticals, Inc		☐
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	Application Number	15642096
	Filing Date	2017-07-05
	First Named Inventor	Leonard BELL
	Art Unit	1644
	Examiner Name	J. L. Rogers
	Attorney Docket Number	AXJ-114BUSCN5

	14	2004/108158	WO	A1	2004-12-16	Alexion Pharmaceuticals, Inc		☐
	15	2005/092073	WO	A2	2005-10-06	Protein Design Labs, Inc		☐
	16	2006003179	WO	A2	2006-01-12	Novo Nordisk As		☐

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	1	ADDERSON, E., et al., "Immunoglobulin Light Chain Variable Region Gene Sequences for Human Antibodies to Haemophilus influenza Type b Capsular Polysaccharide Are Dominated by a Limited Number of Vk and VI Segments and V J Combinations," J. Clin. Invest. vol. 89: 729-738 (1992).	
	2	ALBERTS, B. et al., "Intracellular Compartments and Protein Sorting," in Molecular Biology of the Cell, Chapter 12:55 pages (1994).	
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	18	Declaration of Dr. Bruce Andrien with Exhibits, dated May 23, 2017, 36 pages.	
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	Attorney Docket Number	AXJ-114BUSCN5

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	Filing Date	2017-07-05
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	Art Unit	1644
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	Attorney Docket Number	AXJ-114BUSCN5

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A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Jill Gorny Sloper, Esq./	Date (YYYY-MM-DD)	2019-04-29
Name/Print	Jill Gorny Sloper, Esq.	Registration Number	60,760

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Electronic Signature for Jill Gorny Sloper, Esq.: /Jill Gorny Sloper, Esq./

Docket No.: AXJ-114BUSCN5
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Leonard Bell *et al.*

Application No.: 15/642,096

Confirmation No.: 7606

Filed: July 5, 2017

Art Unit: 1644

For: TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT

Examiner: J. L. Rogers

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

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Respectfully submitted,

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EFS ID:	35848470
Application Number:	15642096
International Application Number:	
Confirmation Number:	7606
Title of Invention:	TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS BY AN INHIBITOR OF COMPLEMENT
First Named Inventor/Applicant Name:	Leonard BELL
Customer Number:	133485
Filer:	Jill R. Gorny Sloper/Anita Costa
Filer Authorized By:	Jill R. Gorny Sloper
Attorney Docket Number:	AXJ-114BUSCN5
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Review

Stability engineering of the human antibody repertoire

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ABSTRACT

Human monoclonal antibodies often display limited thermodynamic and colloidal stabilities. This behavior hinders their production, and places limitations on the development of novel formulation conditions and therapeutic applications. Antibodies are highly diverse molecules, with much of the sequence variation observed within variable domain families and, in particular, their complementarity determining regions. This has complicated the development of comprehensive strategies for the stability engineering of the human antibody repertoire. Here we provide an overview of the field, and discuss recent advances in the development of robust and aggregation resistant antibody therapeutics.

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1. Introduction

The number of human monoclonal antibody candidates has increased rapidly in recent years, and now represent the largest single class of molecules entering clinical studies [1]. Despite the rapid growth of the antibody therapeutics market over the past twenty to thirty years, hurdles remain that limit their manufacture. Key factors relate to the variable and often limited stability of human antibodies, which negatively impact on many production processes including expression [2,3], purification [4] and formulation [5].

Antibodies are complex multidomain proteins and mechanisms governing their thermodynamic [6] and, in particular, colloidal [7] stability are not fully understood. Human antibodies of the commonly used immunoglobulin G (IgG) type consist of a total of twelve domains, which can be further divided into two chains (heavy and light) and variable (V_H, V_L) and constant (C_H1, C_H2, C_H3, C_L) domains (Fig. 1). Only limited diversity is observed among constant domains, with a total of four isotype (IgG1–4) and two light chain ([κ] kappa and [λ] lambda) classes expressed in humans. Consequently, isotype differences can explain only a limited proportion of the observed stability variation in the human antibody repertoire [8]. A higher proportion of sequence diversity is observed among antibody variable domains, which are assembled through genetic recombination of variable, diverse (V_H only) and joining segments (VDJ-recombination). Further diversity is then introduced into framework, and in particular complementarity determining regions (CDR), through somatic hypermutation, followed by clonal selection of the repertoire. As a result of these processes, the overwhelming majority of sequence variation within the human antibody repertoire is observed within the CDR regions of a limited range of human variable domain families. In this review, we summarize the influence of the observed antibody diversity on colloidal and thermodynamic stabilities and discuss recent advances to improve these properties through engineering approaches.

2. Protein aggregation

Aggregation is a complex process by which proteins can form alternative colloidal states, which are different from the native state, but otherwise energetically favorable [9,10]. It is generally believed that such aggregate species are predominantly formed via unfolded or partially unfolded states [11]. Protein aggregation is increasingly recognized as a problem affecting the manufacturability of human therapeutic antibodies, shelf life and efficacy [12]. Importantly, the presence of aggregates has also been linked to increased immunogenicity, with effects ranging from mild skin irritation to anaphylaxis [13].

Although it can be assumed that stabilization of the native state over alternative aggregate states has occurred during evolution [14], it is important to note that the production of monoclonal antibodies exposes these molecules to a wide range of non-physiological

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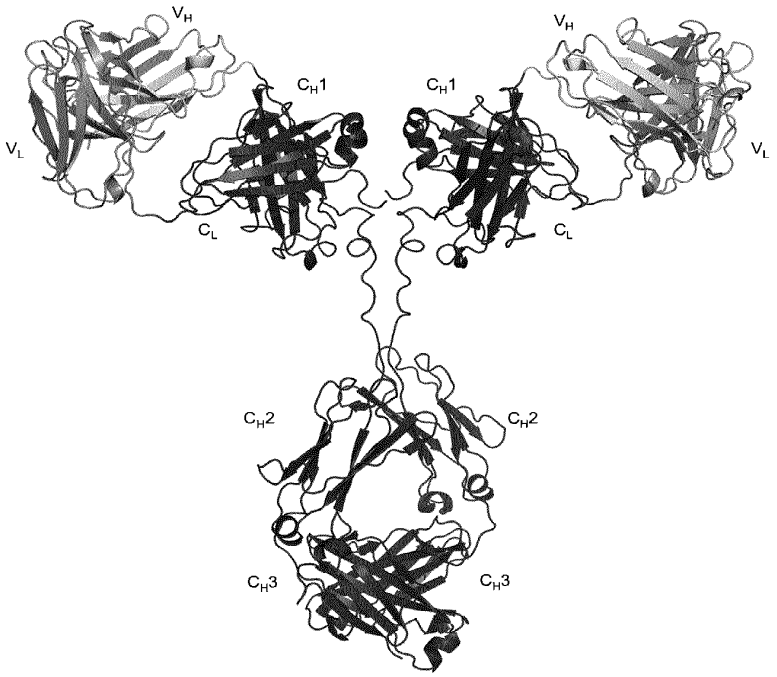


Fig. 1. Structure of the human IgG molecule. The molecule is formed by two heavy chains (consisting of V_H, C_H1, C_H2 and C_H3 domains) and two light chains (consisting of V_L and C_L). Figure was generated using PYMOL.

processes and conditions. These include recombinant expression, purification, concentration, viral inactivation, filtration, formulation, freeze/drying, transport and long-term storage. Throughout these steps the antibody molecule may encounter several stress factors that can dramatically increase its propensity to aggregate (including variations of temperature, pH, protein concentrations, ionic strength, exposure to air–water interfaces and mechanical stress). A further driver of protein aggregation has been a growing trend towards formulations that allow sub-cutaneous administration routes. This requires formulation at high protein concentrations (at around 100 mg/ml) in syringes for self-injection, which places increased demands on colloidal stability [15].

3. Stability of human antibody isotypes and constant domains

The four human IgG isotypes differ in their stabilities and biological functions, namely their potential to induce cellular killing through antibody dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). While the human IgG1 isotype induces powerful ADCC and CDC responses, this is not the case for human IgG2, which is particularly suitable for applications where cellular killing is not required (such as neutralization of soluble ligands) [16]. Unlike the other human isotypes, IgG4 also has a naturally occurring potential to form a bispecific molecule [17] (it has been recently shown that this dual specificity can be grafted onto other isotypes [18]). Human IgG3 is not commonly used due to its longer hinge region, which renders it susceptible to proteolysis [19]. The vast majority of human antibody therapeutics currently in clinical practice and development are of the IgG1 isotype [16].

Conflicting evidence exists in respect to the colloidal stability of the various human IgG isotypes. A recent study of eleven different IgG1 and IgG2 antibodies concluded that, after high temperature storage, the IgG2 isotype is more prone to aggregation [20]. Similar findings were also obtained when subjecting these isotypes to high salt conditons [21] and from isotype switching studies [22]. The latter study confirmed that the human IgG1 isotype is less prone to aggregation when compared to IgG2 or IgG4. However, the authors also found that this isotype is more prone to fragmentation, especially at low pH, due to a non-enzymatic site in the upper hinge region. The hinge regions have also been implicated in the observed differences in aggregation propensity as the IgG2 hinge contains two additional cysteines in each heavy chain compared to IgG1, and is prone to the display of free cysteine residues [23,24]. Differences in the thermal stability of human constant domains have also been reported, with the C_H3 domains of human IgG1 and IgG2 displaying similar melting temperatures, much higher than what was observed for IgG4 [8]. In general, C_H3 domains exhibit more favorable biophysical properties than C_H2 domains [25]. This is reflected by melting temperatures of 8–10 °C higher than those observed for C_H2 domains [8,26].

Although human IgG1 therefore appears less aggregation prone than other isotypes, this is not universally the case. For example, a recent study found that the IgG1 isotype variant of an anti-LINGO-1 antibody exhibited a much higher propensity to aggregate than an IgG2 variant containing identical variable regions [27]. Studies such as these demonstrate that predicting the aggregation propensities of IgG molecules based on isotype can be difficult at the best of times. Moreover, evidence suggests that the aggregation propensity of human IgG molecules is greatly influenced by their variable domains [8,27,28]. This has the effect that antibodies with identical constant domains, but different variable domains, can vary widely in their stability profiles [5].

Samsung Bioepis Exhibit No. 1002
Page 5406

4. Stability of human antibody variable domains

Human antibody variable domains can be grouped into several homologous families, which differ in their biophysical properties. A seminal study of consensus domains of human V_H and V_L families has revealed considerable differences of expression yields and thermodynamic stability [6]. In the case of human V_H domains, stabilities ranging from ΔG_{N-U} 14–53 kJ/mol were observed, with soluble expression yields ranging from 1–2.4 mg/l (Table 1). Intriguingly, Ewert et al. also reported that members of the (odd numbered) V_H 1, 3 and 5 families generally displayed superior biophysical properties in comparison with the (even numbered) 2, 4 and 6 families. These differences were observed for both thermodynamic stabilities and expression yield. In particular, the human V_H3 family was identified as uniquely stable and well expressed. This family is also among the most abundant in the human repertoire [29]. The overall stability trends were confirmed in a more recent study, which analyzed the effect of pairing of different human variable domain families on expression, thermal and colloidal stabilities of Fab fragments and IgG monoclonals [30].

More limited differences in biophysical properties were observed among human V_L families, with thermodynamic stabilities ranging from ΔG_{N-U} 15–24 kJ/mol for lambda and ΔG_{N-U} 29–35 kJ/mol for kappa [6]. Similarly, higher expression yields were observed for kappa variable light domains (5–17 mg/l) than for lambda domains (0.3–2 mg/l). The observed differences between antibody variable light domains were much less pronounced when pairing these with human V_H in an scFv format, while considerable differences between variable heavy domain families remained. Overall, the authors found the V_H/V_L combinations H3κ3, H1bκ3, H5κ3 and H3κ1 to be superior in respect to expression yield and thermodynamic stability [6]. However, despite the clear influence of variable domain family (and framework regions), the study also observed a strong influence of CDR sequence on biophysical properties. Indeed, initial attempts of the authors to express human V_H domain in isolation failed to yield protein (with the exception of V_H3). Only the grafting of a solubilizing CDR3 region allowed the soluble expression of human V_H1, and of V_H2, V_H4, V_H5 and V_H6 through refolding from inclusion bodies.

Although several of the human variable domain families are thermodynamically stable, they nevertheless often display poor colloidal stability and readily aggregate when heated above their melting temperatures. This behavior is exemplified by the V_H3 model domain DP47 [31] and by studies of synthetic antibody repertoires on phage, which have demonstrated that as few as one in a thousand VH domains resists heat induced aggregation [32].

Table 1
Biophysical properties of human antibody variable domains. Adapted from Ref. [6].

	Human family	Solubilizing CDR3 required	Expression yield	ΔG_{N-U}
V_H	1a	Y	+	+
	1b	Y	+	++
	2	Y	Refold	nd
	3	N	+	+++
	4	Y	Refold	nd
	5	Y	Refold	+
	6	Y	Refold	nd
V_L	κ1	N	+	++
	κ2	N	+++	++
	κ3	N	+++	+++
	κ4	N	++	-
	λ1	N	+	++
	λ2	N	+	+
	λ3	N	+	+

5. Increasing antibody stability through formulation

The most common method for controlling the biophysical properties of monoclonal antibodies and other biologics is through changes of buffer conditions and excipients. Formulation allows for the control of ionic strength, pH and the addition of excipient agents such as sugars or surfactants, which have been shown to stabilize monoclonal antibody preparations [33]. Although formulation can shield proteins from some chemical and physical challenges (including deamidation, oxidation, hydrolysis/fragmentation and isomerization), aggregation remains difficult to control [12]. Consequently, finding appropriate formulation conditions for an individual product is often an arduous process. This is exemplified by the therapeutic monoclonal antibody cetuximab (Erbitux). Its initial formulation conditions (2 mg/ml in phosphate buffer pH 6.0) generated visible aggregates under mechanical stress [34]. To address this issue, conditions had to be empirically optimized and were changed to 5 mg/ml in citrate buffer pH 5.5 with the addition of glycine and polysorbate 80 as stabilizers.

In recent years, more high-throughput techniques for formulation optimization have been developed [35], and stabilizers and excipients with more general applicability have been identified. Among these, arginine is commonly used to improve refolding yields [36,37], and more recently has been used in formulation to reduce heat-induced aggregation [38]. Another commonly used class of excipient molecules are polysorbates, which accumulate at the air–solvent interface and offer protection against shaking and other mechanical stresses [39]. 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 [5].

6. Stability engineering of human antibody constant domains

Many efforts have been made to increase the stability of the different human antibody isotypes. Early work in this field focused on sequences analyses to predict stabilizing mutations. This approach allowed the identification of a set of substitutions (371K, 376G and 392L), which considerably increased thermal stability (from 76 °C to 86 °C) of the C_H3 domain [40]. The study was based on frequency analyses within 19 mammalian species to generate a consensus sequence. Structural approaches have also been utilized to increase stability of the C_H1/C_L interface [41]. More recently, it was shown that the introduction of additional intra-domain disulfide bridges in the human C_H3 domain resulted in the thermal stabilization of an isolated IgG1 Fc fragment [42] (Fig. 2). In particular, the introduction of a link between the N-terminal A strand (position 343) and the F strand (position 431) caused a 8 °C increase in the melting temperature, while a linkage between positions 375 and 396 resulted in a 4 °C increase (combined 13 °C). These findings were successfully applied to a HER2 specific Fcab molecule (Fc fragment with engineered binding loops) resulting in an increased in the thermal stability of 19 °C. A similar finding was observed for a human IgG1 C_H3 single domain, which could be stabilized by introduction of an intra-domain disulfide bridge [43]. The authors used a two-step approach, by initially introducing mutations that rendered the C_H3 domain monomeric, resulting in a overall low thermal stability (41 °C compared to 82 °C for the native dimeric form). Using structure-based design, two additional cysteines (at positions 343 and 431) were then introduced to generate a disulfide bridge, which increased the thermal stability to 76 °C (a

Samsung Bioepis Exhibit No. 1002
Page 5407

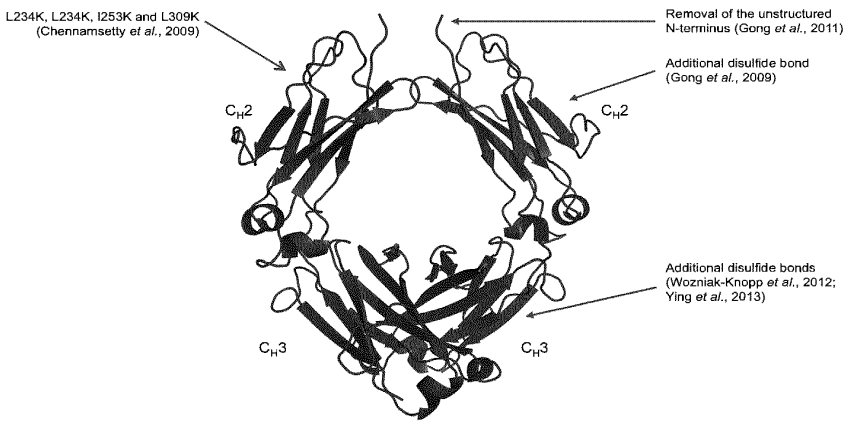


Fig. 2. Stability engineering of human constant domains.

35 °C increase) [43]. Similar strategies have been also reported for C_H2 domains [26]. Here the authors reported the engineering of an isolated human C_H2 domain, resulting in an increase in thermal stability of 20 °C (from 54 °C to 74 °C) [26]. More recently, the same group reported the stabilization of a C_H2 domain through removal of an unstructured region at the N-terminus. Combined with the intra-domain disulfide bond, this resulted in a further increase of melting temperature of 10 °C [44]. For all of the above studies, stability of constant domains was evaluated in the context of fragments or isolated domains.

Other studies have investigated the effects of such mutations on the stability of full-length human IgG. In the case of human IgG4, alteration of the disulfide bond network in the Fab region was shown to lead to an increase of thermal stability of between 3 and 7 °C [45]. This effect was achieved through removal of a cysteine at position 127 in the C_H1 domain, while an additional inter-domain disulfide bond between position 229 and C_L was introduced (similar to what is observed in human IgG1). Importantly, the authors reported that the introduced mutations did not affect antigen binding [45]. More recently, molecular dynamic simulations have been used to predict the spatial aggregation propensity (SAP) of human IgG molecules [46]. The authors later showed that by mutating identified surface-exposed hydrophobic patches within the hinge and Fc regions of an IgG, aggregation resistance and, to a more limited extent, thermodynamic stability could be improved [47].

7. Increased stability through charged fusion tags

In addition to the modification of constant domains, it was recently shown that the addition of charged fusion tags to the N-terminus of human variable domains could considerably improve aggregation propensity [48]. The authors noted that batches of the same IgG monoclonal expressed in yeast were less prone to aggregation than those expressed in mammalian cells. Mass spectrometry and sequencing analyses revealed the presence of a non-processed "EAEA" leader sequence within both heavy and light chains. In contrast to colloidal stability, the authors did not observe improved thermodynamic stability, and the effect seemed to be particularly pronounced for antibodies with a low global net charge. In addition to terminal fusion tags, a different study explored the internal use of a "DED" motif [49]. It was shown that the insertion of the tag into the CDR3 region of aggregation-prone V_H domain antibodies reduced their aggregation propensity, while reducing retention on gel filtration matrices.

8. Stability engineering of human antibody variable domains

8.1. Engineering of human V_H domains through mutation of the light chain interface

The poor biophysical properties of isolated human antibody variable domains are in marked contrast to those of the variable domains of camels and llamas [50]. Although these immunoglobulin domains (V_{HH}) display a relatively high level of sequence identity with human V_H domains, they are generally well expressed and soluble [51] and reversibly unfold after incubation at temperatures as high as 90 °C [52]. In addition to the observed differences in biophysical nature, camelid domains also display an important difference in chain structure, as they are naturally devoid of light chain partners [50]. Their single domain nature results in unique structural features, compared to human antibody V_H domains. These include CDR length and conformation, as well as framework residues. A particular feature of V_{HH} domains is their long flexible CDR3 loop capable of folding back on itself, thereby protecting an area forming the V_H/V_L interface in human antibodies. In addition, these naturally occurring single domains display a marked reduction in hydrophobicity of the former light chain interface, due to a limited number of changes in their respective germlines (Fig. 3). The most common and significant changes compared to humans, coined the "V_{HH} tetrad", are located at positions: G44Q, L45R/C, W47G/I and V37F/Y (human/camelid). The latter residue is nucleating a small core involving Y91, W103, R45 and other hydrophobic residues within the CDR3 loop [53]. Structural investigation of V_{HH} has also shown the CDR3 loop covering residue 37, thereby shielding hydrophobicity in an area corresponding to the light chain interface in human V_H [54]. In addition, camelid V_{HH} often contain an additional disulfide bridge between CDR1 and CDR3, which increases their thermal stability [55,56].

In order to improve the biophysical properties of human V_H domains, elements of the V_{HH} tetrad have been incorporated into human frameworks ("camelization") [57]. Although the introduced framework changes improved the solubility of human V_H, they also reduced expression levels, induced conformational changes, decreased thermodynamic stability and did not considerably improve the colloidal stability of the domains [58,59]. In contrast, combining the approach with the introduction of an additional disulfide bond between CDR1 and CDR3, yielded domains with high colloidal stability, although at the expense of antigen binding affinity [60].

As an alternative to camelid domains, shark single domain antibodies (V_{NAR}) could also potentially serve as a blueprint for improving

Samsung Bioepis Exhibit No. 1002
Page 5408

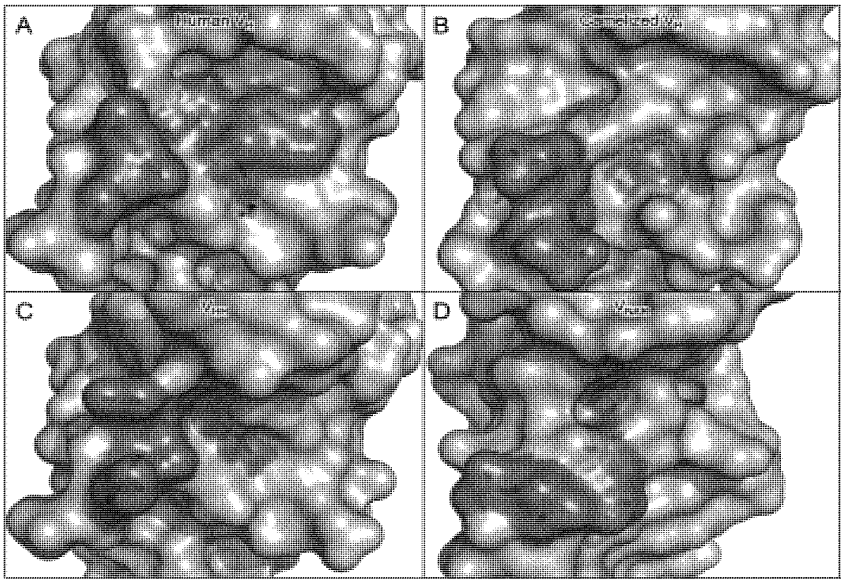


Fig. 3. Engineering of human VH domains through mutation of the light chain interface. Shown are surface representations of the light chain interface in human V_H (PDB 2VXS) and of the equivalent surfaces in camelized V_H (PDB 1VHP), camelid V_{HH} (PDB 1ZVH) and shark V_{NAR} domains (PDB 1T6V). Residues forming the camelid 'tetrad' at positions 37, 44, 45 and 47 are highlighted in green. Camelized V_H, V_{HH} and V_{NAR} domains display a considerable reduction in surface hydrophobicity in comparison to human V_H domains. Figure was generated using PYMOL.

the stability of human antibody V_H domains (although they are more distantly related to human domains [61]). As observed for camelid domains, shark V_{NAR} domains are characterized by a long protruding CDR3 [62]. The CDR loop is constrained by additional disulfide bonds, thereby folding over an area equivalent to the former V_H/V_L interface of human antibodies [61]. A recent study revealed that V_{NAR} domains are resistant to thermal denaturation, acidic denaturation, and lyophilisation denaturation and are capable of returning to their native state after thermal unfolding [63].

Rather than focusing on direct grafting of camelid residues, more recent approaches for the generation of autonomous single domains have focused on the identification of novel mutations. This has been possible due to the use of phage display based selection strategies and the use of protein A to select for folded domains (pA superantigen binds to folded human V_H3 domains, but not to unfolded or aggregated domains). Using this approach, an extensive mutagenesis study has identified hydrophilic substitutions within the V_H3/V_L interface [53]. The authors were able to demonstrate that their mutations (including H35G, Q39R, L45E, R50S) improved solubility, thermal refolding and melting temperature. Importantly, some of the mutations improved protein A binding (and presumably stability), independently of CDR3 sequence diversity. As CDR3 of V_H provides the majority of binding energy in antibody–antigen interactions, this indicated broad compatibility with antigen binding and suggested that the engineered frameworks could be used for the construction of stable repertoires of autonomous V_H domains.

8.2. Engineering of human V_H domains through CDR grafting and framework mutations

In addition to their use in the engineering of autonomous antibody single domains, homology-based approaches have also been used for the identification of mutations outside the interface regions, thereby retaining the ability of human V_H to interact with variable light domains. One of the earliest examples of such strategies relates to the use of CDR grafting for the improvement of biophysical properties. Developed by Jones and Winter in the mid-1980s, CDR grafting was initially used to reduce the immunogenicity of mouse monoclonals, by transplantation of murine CDR regions onto a human framework [64]. However, it was later shown that by grafting onto a stable and well-behaved framework (such as the Herceptin 4D5 [V_H3] framework) considerably improved expression levels and thermodynamic stability, while maintaining binding affinity of the parental antibody fragment [65]. A second approach is based on the use of consensus sequences to improve stability [66]. This approach has been applied to a range of protein scaffolds [67], and utilizes amino acid observed with high frequency within the repertoire [68]. This approach was also used in the construction of the HuCAL human antibody library, for which the authors have reported good expression yields [69]. More recent studies from the same group have identified additional framework mutations that transfer biophysical properties between human V_H3 and V_H6 families [70,71].

As an alternative to homology-based approaches, other studies have focused on the computational prediction of aggregation hotspots in antibody sequences. Early studies on peptides and proteins involved in neurodegenerative diseases have highlighted physicochemical properties that correlate with an increase in aggregate formation, namely hydrophobicity, β-sheet propensity and reduced net charge [72]. Algorithms have been developed to detect such aggregation-prone regions [73–76]. Two of these algorithms, TANGO and PAGE, have been used to identify potential aggregation-prone regions in commercial therapeutic antibodies, which are predominately located in variable domains, and particularly within CDR and adjacent framework residues [28].

8.3. Engineering of human variable domains through CDR mutations

The role of CDR residues in determining thermodynamic and in particular colloidal stability of human antibody variable domains is further highlighted by studies on the biophysical properties of the

Samsung Bioepis Exhibit No. 1002
Page 5409

HEL4 model domain [31]. HEL4 was originally isolated through phage display selection from a synthetic CDR-only repertoire based on the human V_H3-23 DP47 germline [77]. As phage is remarkably resistant to chemical, proteolytic and thermal denaturation [78–80], the authors were able to heat the phage displayed antibody fragments at high temperature (80 °C), followed by cooling and binding to antigen (hen egg white lysozyme). The method thereby selected for domains that unfolded reversibly, and could be captured by binding to antigen or superantigen. Unlike other human V_H3 domains, HEL4 displayed highly favorable biophysical properties, including heat-refoldability, high expression levels in bacteria, and the absence of 'stickiness' on gel-filtration [31]. Indeed, it was later shown that aggregation resistance on phage is a general indicator of the solution behavior of human V_H domains [81]. In addition to HEL4, several other V_H domains, that were isolated using this method, showed a high level of resistance to aggregation. Intriguingly, despite their high level of colloidal stability, all of the domains actually had lower thermodynamic stabilities than the DP47 domain from which they had been derived from (ΔG_{N-U} = 15–20 kJ/mol vs 35 kJ/mol). In contrast, DP47 readily aggregated upon heating, further highlighting differences between thermodynamic and colloidal stability. As an alternative to heat-based selection on phage, a more recent study explored the use of pH stress (pH 3.2) for the selection of aggregation-resistant V_H domains [82]. In contrast to domains isolated after thermal denaturation, the selected V_H domains displayed increased thermodynamic stabilities and increased colloidal stability at low pH, reflecting the different denaturation conditions experienced during the selection process.

Although the denaturation method on phage was capable of selecting human V_H domains with CDR sequences that promoted a high level of aggregation resistance, it was also evident that such domains were rare within the repertoire. To increase the frequency of such clones, a human V_H library was constructed through combinatorial assembly of CDR regions that had been pre-selected for aggregation resistance in phage. Binders were successfully selected from this repertoire, which exhibited good antigen-binding properties together with favorable biophysical properties [32].

Despite clear improvements over other human V_H domains, detailed determinants of the observed aggregation resistance initially had remained unclear. As HEL4 and other clones had been selected from synthetic CDR-only repertoires, it was apparent that the

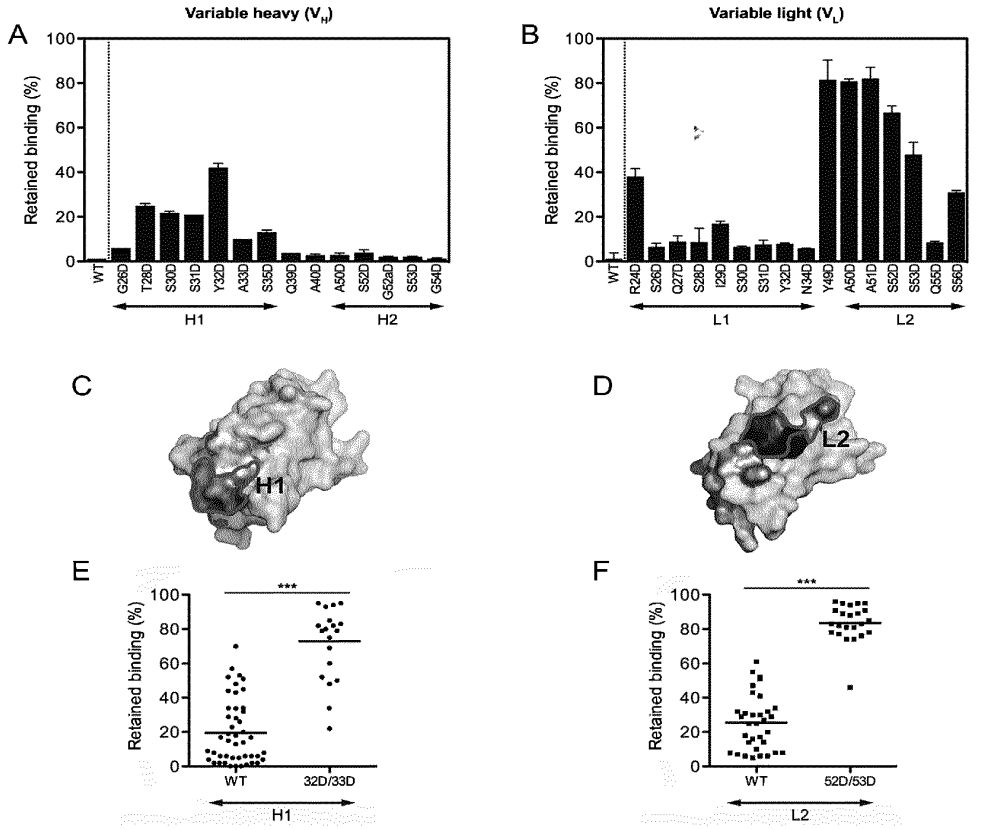


Fig. 4. Effect of mutations in human antibody V_H and V_L domains on aggregation resistance. Surface residues in variable heavy and light domains were targeted for substitution with aspartate. Aggregation resistance of the domains was determined by measuring binding to protein A and protein L superantigen after heating to 80 °C on phage. Complementarily determining regions are indicated (A) Mutations in human V_H. (B) Mutations in human V_L. Introduction of aspartate into CDR H1 and CDR L2 increases aggregation resistance of human variable domains up to 40-fold for V_H and up to 80-fold for V_L. (C) Mutations mapped onto the human V_H surface. (D) Mutations mapped onto the human V_L surface (blue: 100% retained superantigen binding, white: 0%). (E) Stability engineering of human V_H repertoire. (F) Stability engineering of human V_L repertoire. The mutant repertoires were generated through introduction of aspartate mutations in CDR H1 and CDR L2 (at positions 32/33 and 52/53, respectively). Introduction of the mutations significantly increases the aggregation resistance of human antibody repertoires, independent of sequence diversity at other CDR positions. Adapted from Ref. [7].

Samsung Bioepis Exhibit No. 1002
Page 5410

exhibited biophysical properties were a result of changes in the complementarity determining regions, without any further influence of framework residues [31]. Crystal structure of the HEL4 model domain had indicated a potential effect of a serine to glycine mutation at position 35 [77]. Indeed, introduction of this mutation into DP47, resulted in increased solubility and reduced "stickiness" on gel-filtration. However, the mutant domain continued to readily aggregate when heated above its melting temperature. The authors also reported that many of the selected domains had a low isoelectric point (pI). Similar findings were also obtained in a second study, which reported that a low pI, together with the non-canonical inter-CDR disulfide linkages, contribute to improved biophysical properties human V_H domains [83]. Using transient heating to pre-select for proteins reversible thermal unfolding properties, the authors found that many of the isolated domains had pI values of less than six, although they also observed domains with values higher than eight. The authors concluded that the improved biophysical characteristics of these V_H domains were caused by a combination of global pI effects, together with an increase of stability through inter-CDR disulfide linkage (other studies by the same group demonstrated that, although the additional disulfide bridge significantly increased thermal stability and protease resistance [84], it also induced structural changes in human V_H as indicated by a marked reduction of protein A superantigen binding [85]). A global increase in charge was also observed in a different study, analyzing larger sets of V_H domain sequences after heat selection on phage, together with an overall reduction of hydrophobicity and a decrease in beta-sheet propensity [86].

More recently, an extensive mutagenesis study on phage has provided detailed insights into mutations that increase the colloidal stability of human V_H and V_L antibody domains [7]. Using superantigen binding after heat denaturation on phage as a mean to select for reversible unfolding, the investigators analyzed the effect of charged mutations at all CDR positions (excluding CDR3 to limit effects on antigen binding). This revealed that the introduction of negative charge (and in particular aspartate) at CDR-H1 positions (28, 30–33, 35) considerably increased the aggregation resistance of human V_H domains (up to 40-fold) (Fig. 4A). This is in excellent agreement with the observations for the model HEL4 domain, which contains negatively charged amino acids at a subset of the identified positions (as highlighted by CDR grafting studies [87–89]).

Intriguingly, this was not the case for human V_L domains, for which the introduction of mutations at equivalent positions had little effect. In contrast to what was observed for human V_H, increased aggregation resistance (up to 80-fold) was instead endowed by the introduction of asparate at CDR-L2 positions (49, 50–53, 56) (Fig. 4B). As observed in other studies [81], the improved properties on phage correlated with increased aggregation resistance in solution, improved expression yields and reduced retention on gel-filtration. Importantly, the observed increases in aggregation resistance were not only observed in model domains, but also when introducing CDR-H1 and CDR-L2 mutations into diverse antibody libraries [7] (Fig. 4E/F).

In addition, and unlike what had been observed for camelized and other engineered variable domains [53,59,85], crystal structures of mutant V_H and V_L domains showed no detectable conformational changes in either CDR conformations or the V_H/V_L interface. Taken together, these results indicated broad compatibility with high affinity antigen binding, which was confirmed by the introduction of CDR-H1 and CDR-L2 mutations into the antibody therapeutic Herceptin [7]. Further evidence for the influence of the identified mutations on colloidal stability has recently been reported in a study of an aggregation-prone human IgG1 monoclonal [90]. The authors found that the aspartic acid at position 49 of the V_L domain considerably reduced aggregation rate of the antibody during long-term stability studies (from 35% to 0.2% per month).

9. Conclusion – towards stable human antibody therapeutics

The increasing number of human monoclonals in development and clinical practice, together with a trend towards high concentration formulations for subcutaneous delivery places increasing demands on the stability of human antibody therapeutics. While the thermodynamic stability of human antibodies is relatively well studied and can be further increased through homology, disulfide and computational approaches [71,83,47], determinants of their colloidal stability are just beginning to emerge due to a combination of rational and high-throughput approaches on phage [7,31,46,91]. In particular, the recent availability of human V_L domains with high levels of aggregation resistance [7] will greatly assist in the generation of paired antibody fragments and IgG. Further advances in the generation of human antibody therapeutics with high thermodynamic and colloidal stability will not only improve the production and formulation of human antibody therapeutics, but may also open up new administration routes and therapeutic applications.

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Samsung Bioepis Exhibit No. 1002
Page 5411

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Samsung Bioepis Exhibit No. 1002
Page 5412

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Samsung Bioepis Exhibit No. 1002
Page 5413

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Effective induction of naive and recall T-cell responses by targeting antigen to human dendritic cells via a humanized anti–DC-SIGN antibody

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Current dendritic cell (DC)–based vaccines are based on ex vivo–generated autologous DCs loaded with antigen prior to readministration into patients. A more direct and less laborious strategy is to target antigens to DCs in vivo via specific surface receptors. Therefore, we developed a humanized antibody, hD1V1G2/G4 (hD1), directed against the C-type lectin DC-specific intercellular adhesion molecule 3–grabbing nonintegrin (DC-SIGN) to explore its capacity to serve as a target receptor for vaccination purposes. hD1 was cross-linked to a model antigen, keyhole limpet hemocyanin (KLH). We observed that the chimeric antibody-protein complex (hD1-KLH) bound specifically to DC-SIGN and was rapidly internalized and translocated to the lysosomal compartment. To determine the targeting efficiency of hD1-KLH, monocyte-derived DCs and peripheral blood lymphocytes (PBLs) were obtained from patients who had previously been vaccinated with KLH-pulsed DCs. Autologous DCs pulsed with hD1-KLH induced proliferation of patient PBLs at a 100-fold lower concentration than KLH-pulsed DCs. In addition, hD1-KLH–targeted DCs induced proliferation of naive T cells recognizing KLH epitopes in the context of major histocompatibility complex (MHC) classes I and II. We conclude that antibody-mediated targeting of antigen to DCs via DC-SIGN effectively induces antigen-specific naive as well as recall T-cell responses. This identifies DC-SIGN as a promising target molecule for DC-based vaccination strategies. (Blood. 2005;106:1278-1285)

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Introduction

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that play a key role in regulating antigen-specific immunity. DCs capture antigens, process them into peptides, and present these to T cells.[1] The interaction between DC and T-cell controls the type and magnitude of the resulting immune response. Recently, preclinical and clinical studies have exploited DCs in an attempt to improve vaccine efficacy.[2] Most of these studies involve ex vivo antigen loading of autologous monocyte-derived DCs that are readministrated to the patient, a laborious and costly procedure. A more direct strategy involves targeting of antigens specifically to antigen uptake receptors on the DC in vivo. Potential candidate receptors highly expressed by DCs include Fc receptors[3-5] and members of the C-type lectin family.[6,7] Whereas Fc receptors are expressed by many different cell types, the expression of some members of the C-type lectin family are more DC restricted.[8]

C-type lectins bind sugar residues in a calcium-dependent manner via a highly conserved carbohydrate recognition domain. C-type lectin receptors expressed by DCs are implicated in immunoregulatory processes, such as antigen capture, DC trafficking, and DC–T-cell interactions.[8] Based on the location of the amino (N) terminus, 2 types of membrane-bound C-type lectins can be distinguished on DCs. Type I C-type lectins have their N terminus located outside, while type II C-type lectins have their N terminus located inside the cell. Several studies have been conducted on antigen targeting to C-type lectin receptors for vaccination purposes, mainly focusing on the type I C-type lectins mannose receptor (MR)[9] and DEC-205.[6,10,11] Vaccines based on natural MR ligands have been shown to effectively induce humoral and cellular responses.[9] However, these ligands lack specificity for the MR, and may target multiple lectins with overlapping binding specificities, including soluble lectins and lectin receptors expressed by cells that are not specialized in antigen presentation. More specific receptor targeting can be obtained by the use of antibodies directed against specific C-type lectins, a strategy that has been successfully applied in MR[7,12] and DEC-205[6,10,11] targeting studies. Antibody-mediated targeting of antigen to the MR on human DCs results in antigen presentation and activation of naive T cells in vitro.[7,12] Moreover, in vivo studies on antibody-mediated targeting of DEC-205 in mice demonstrate presentation of the antigen to naive $CD4^+$ and $CD8^+$ T cells.[6,10]

DC-specific intercellular adhesion molecule 3 (ICAM-3)–grabbing nonintegrin (DC-SIGN) represents a member of the

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Samsung Bioepis Exhibit No. 1002
Page 5414

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type II C-type lectin family. We have previously demonstrated that DC-SIGN is an endocytic receptor mediating antigen presentation.[13] A major advantage of targeting DC-SIGN over other C-type lectin receptors is its expression pattern. In humans, DC-SIGN expression is restricted to professional APCs and expression levels are high. Human DC-SIGN is abundantly expressed by DCs residing in lymphoid tissues and at mucosal surfaces, dermal DCs, and by specialized macrophages in placenta and lung.[14,15]

Targeting constructs that are to be used in humans should consist of antibodies that do not elicit immune responses directed against the antibody itself. Recent developments in antibody engineering provide the tools for the production of either humanized or human antibodies.[16] Undesired interactions between the targeting antibody and Fc receptors can be avoided by use of single-chain Fv constructs or composite immunoglobulin G (IgG) molecules,[17] thus enhancing targeting specificity.

Here we evaluate the effectiveness of targeting antigen to human DCs via DC-SIGN. For targeting purposes, keyhole limpet hemocyanin (KLH), a large globular protein containing a large array of immunogenic epitopes, was chosen as a model antigen. KLH is widely used in clinical DC-based vaccination trials for immunomonitoring purposes, and is thought to stimulate cytotoxic T-cell responses by recruiting bystander T-cell help.[2] KLH was chemically cross-linked to a humanized anti–DC-SIGN IgG2/IgG4 composite antibody (hD1), resulting in the chimeric hD1-KLH protein. The results demonstrate that hD1-KLH was capable of inducing T-cell responses at a 100-fold lower concentration than KLH alone.

Patients, materials, and methods

Antibodies and reagents

The following antibodies were used: AZN-D1 (IgG1, mouse antihuman DC-SIGN),[14] AZN-L19 (IgG1, mouse antihuman CD18),[18] W6/32 (IgG2a, mouse anti–HLA-A, –HLA-B, –HLA-C; ATCC, Manassas, VA), IVA-12 (IgG1, mouse anti–HLA-DR, –HLA-DP, –HLA-DQ; ATCC), mouse IgG1 isotype (R&D Systems, Abingdon, United Kingdom), total mouse IgG (Jackson ImmunoResearch; Brunschwig Chemie B.V., Amsterdam, the Netherlands), Alexa Fluor 647–labeled goat anti–human IgG (Molecular Probes, Leiden, the Netherlands), and Alexa Fluor 647–labeled goat anti–mouse IgG1 (Molecular Probes). Endotoxin-free KLH was purchased from Calbiochem (La Jolla, CA).

Recombinant antibodies

The humanized antihuman DC-SIGN antibody hD1V1G2/G4 (hD1) was generated by complementarity determining region (CDR) grafting of AZN-D1 hypervariable domains into human framework regions. The humanized variable heavy and variable light regions were then genetically fused with a human hybrid IgG2/IgG4 constant domain[17] and a human kappa chain constant domain, respectively. This construct was cloned into a mammalian expression vector and the final construct transfected into NSO cells. Stable transfectants were obtained using glutamate synthetase (GS) selection (Lonza Biologics, Portsmouth, NH). Supernatants containing hD1 were purified over a protein A column. An isotype control antibody, h5G1.1-mAb (5G1.1, eculizamab; Alexion Pharmaceuticals) containing the same IgG2/IgG4 constant region, is specific for the human terminal complement protein C5.[19]

Generation of hD1-KLH

The chemical cross-linker sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sSMCC; Pierce, Rockford, IL) was conjugated to KLH according to the manufacturer's protocol. Protected sulfhydryl groups were introduced to the hD1 antibody with N-succinimidyl-S-acetylthiopropionate (SATP; Pierce) and were reduced with hydroxylamine hydrochloride (Pierce) using the manufacturer's protocol. Subsequently, hD1 was added to sSMCC-treated KLH in phosphate-buffered saline (PBS, pH 7.4) and allowed to react for 16 hours at 4°C. Unbound sites were alkylated by adding iodoacetamide (Sigma-Aldrich, St Louis, MO) to a final concentration of 25 mM, followed by 30-minute incubation at room temperature. The protein mixture was loaded onto a Superose 6 column (24-mL bed volume; Amersham Pharmacia Biotech, Uppsala, Sweden), and fractions were collected and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing hD1-KLH were pooled and fractions containing free hD1 were discarded. The efficiency of the cross-linking reaction was estimated by comparing the amount of hD1 relative to KLH before the reaction to the hD1–to–hD1-KLH ratio after cross-linking. We calculated that, on average, each KLH molecule had reacted with 10 hD1 molecules (data not shown). Endotoxin levels of the pooled hD1-KLH fractions were below detection levels (< 0.04 pg/μg protein) in the QCL-1000 *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD).

Monocyte-derived DCs and PBLs

Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats of healthy individuals and were purified using Ficoll density centrifugation. Peripheral blood lymphocytes (PBLs) and immature DCs (iDCs) were obtained from PBMCs as reported elsewhere.[14] In brief, PBMCs were allowed to adhere for one hour at 37°C. Nonadherent cells (PBLs) were gently removed, washed, and cryopreserved. The adherent monocytes were cultured in the presence of interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF, 500 and 800 U/mL, respectively; Schering-Plough International, Kenilworth, NJ) for 6 days to obtain immature DCs. Mature DCs (mDCs) were obtained by culturing iDCs in the presence of 2 μg/mL lipopolysaccharide (LPS) for 24 hours. DCs were cryopreserved until use. Unless indicated otherwise, cells were cultured in X-VIVO 15 medium (Cambrex, Verviers, Belgium) supplemented with 2% human serum.

Tritiated thymidine incorporation assays

Tritiated thymidine (1 μCi [0.037 MBq]/well; MP Biomedicals, Amsterdam, the Netherlands) was added to the cell cultures. Tritiated thymidine incorporation was measured after 16 hours in a β-scintillation counter. Proliferation indices higher than 2 were considered positive.

Clinical vaccination protocol

PBLs and monocyte-derived DCs were isolated from melanoma patients participating in a clinical vaccination trial, as described by de Vries et al.[20] Patients were determined to have stage IV disease according to the American Joint Committee on Cancer (AJCC) criteria.[21] The study was approved by the institutional review board (Radboud University Nijmegen Medical Centre, Commissie Mensgebonden Onderzoek). Informed consent was provided according to the Declaration of Helsinki. The vaccination protocol consisted of 2 parts. In the first part, antigen-pulsed mDCs were administered intravenously and intradermally, 3 times at biweekly intervals. In the second part, patients received 3 monthly intradermal vaccinations with peptides alone (100 μg) and KLH (2 μg). Patients who remained free of disease progression after the first vaccination cycle were eligible for maintenance cycles at 6-month intervals, each consisting of 3 biweekly intranodal vaccinations in a clinically tumor-free lymph node region under ultrasound guidance with mDCs alternately pulsed with wild-type or modified glycoprotein 100 (gp100) peptides,[20] tyrosinase peptides, and KLH. Clinical grade DCs for vaccination purposes were generated from PBMCs as described previously.[22]

Humoral responses to KLH

Humoral responses to KLH were determined by enzyme-linked immunosorbent assays (ELISA) as described by Holtl et al.[23] Briefly, 96-well plates were coated overnight at 4°C with the protein KLH (25 μg/mL) in PBS (0.1

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Page 5415

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mL/well). Subsequently, plates were incubated with serial dilutions of patient serum, obtained before and during the third maintenance cycle, for one hour at room temperature. After extensive washing, human IgG-specific antibody labeled with horseradish peroxidase was allowed to bind for one hour at room temperature. Peroxidase activity was revealed using 3,3′ 5,5′ tetramethyl-benzidine as substrate and measured in a microtiter plate reader at 450 nm. A signal detected at 1:400 or higher dilution of serum was considered positive.

Cellular responses to KLH

Cryopreserved PBMCs, isolated from peripheral blood samples taken from the patients, were thawed, washed, and plated at 1×10^5 PBMCs per well of a 96-well tissue culture microplate either in the presence or absence of 10 μg/mL KLH. After 4 days of culture, a tritiated thymidine incorporation assay was performed.

Binding and internalization assays

Binding of hD1 and hD1-KLH to iDCs was assessed by immunofluorescence and flow cytometry. iDCs were incubated with or without 10 μg/mL hD1 or hD1-KLH. In some experiments, DC-SIGN was blocked by pretreating iDCs with 100 μg/mL AZN-D1. After a one-hour incubation at 4°C, cells were washed and incubated with Alexa Fluor 647–labeled anti–human IgG antibody. Cells were analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences, San Jose, CA).

Internalization of hD1 by iDCs was determined by flow cytometry as described previously.[24] Briefly, iDCs were incubated with 10 μg/mL hD1, AZN-D1, AZN-L19, or mouse IgG1 and 5G1.1 isotype control antibodies at 4°C for 30 minutes, washed, and incubated for 0, 15, 30, or 45 minutes at 37°C. Subsequently, some of the cells were fixed, while others were fixed and permeabilized in PBS/0.1% (vol/wt) saponin (Sigma-Aldrich) before addition of the Alexa Fluor 647–labeled anti–human IgG secondary antibody. The amount of internalized antibody was calculated by subtracting the mean fluorescence in fixed cells (surface bound) from that recorded with fixed and permeabilized cells (internalized and surface bound) at the various time points.

Internalization of hD1 and hD1-KLH was confirmed by confocal laser scanning microscopy (CLSM). iDCs were incubated with 10 μg/mL hD1, hD1-KLH, AZN-D1, or isotype control 5G1.1 and mouse IgG1 antibodies for one hour at 37°C. Cells were fixed on poly-L-lysine–coated glass slides, followed by intracellular staining with Alexa Fluor 647–labeled secondary antibodies. Cells were imaged with a Bio-Rad MRC 1024 confocal system operating on a Nikon Optiphot microscope and a Nikon × 60 planApo 1.4 oil immersion lens (Bio-Rad, Hercules, CA). Pictures were analyzed with Bio-Rad Lasersharp 2000 and Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA) software.

KLH binding and internalization by iDCs was assessed by direct labeling of KLH using the Alexa Fluor 488 labeling kit (Molecular Probes). iDCs were incubated with 10 μg/mL labeled KLH for one hour at either 4°C or 37°C. Subsequently, cells were washed and analyzed by flow cytometry.

Live imaging of hD1-KLH and KLH uptake by iDCs

iDCs were labeled with LysoTracker Red (Molecular Probes) in PBS for 10 minutes at room temperature. Subsequently, cells were transferred to RPMI 1640 without phenol red (Gibco, Life Technologies, Breda, the Netherlands) supplemented with 1% human serum. Labeled cells were analyzed at 37°C with a Zeiss LSM 510 microscope equipped with a type S heated stage CO_2 controller and PlanApochromatic 63 × 1.4 oil immersion DIC lens (Carl Zeiss, Jena, Germany). KLH and hD1-KLH, directly labeled with the Alexa Fluor 488 labeling kit (Molecular Probes), were added to the medium at 10 μg/mL. Cells were imaged using Zeiss LSM Image Browser version 3.2 (Carl Zeiss) and processed with Image J version 1.32j software (National Institutes of Health, http://rsb.info.nih.gov/ij).

Targeting experiments

iDCs were incubated with hD1, hD1-KLH, or KLH for one hour at 4°C or 37°C. Where indicated, iDCs were matured with LPS. Subsequently, DCs were washed and cocultured with KLH-responsive PBLs (ratio 1:10) at 37°C. In some experiments, iDCs were matured with LPS before addition of PBLs. After 16 hours of coculture, IL-2, IL-4, IL-5, IL-10, tumor necrosis factor α (TNF-α), and interferon γ (IFN-γ) were measured by cytometric bead array (CBA) (T helper 1 [Th1]/Th2 Cytokine CBA 1; BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. After 4 days of coculture, a tritiated thymidine incorporation assay was performed.

Presentation of KLH epitopes over time

iDCs were incubated with 5 μg/mL KLH or 5 μg/mL hD1-KLH for one hour at 37°C. Following washing, KLH-responsive PBLs were added to the iDCs either on the same day, or 2 or 4 days later. Four days after addition of the PBLs, proliferative responses were determined in a tritiated thymidine incorporation assay.

Activation and expansion of naive T cells

Experiments were performed essentially as described previously.[7] Briefly, PBLs from a healthy donor were used as a source of T cells. Autologous DCs were incubated with 10 μg/mL hD1-KLH for one hour at 37°C, washed, and matured. Targeted mDCs were incubated with PBLs (ratio, 1:10) in the presence of IL-7 (10 ng/mL, day 0), followed by addition of IL-10 (10 ng/mL) on day 1 and IL-2 (20 U/mL) on day 2. IL-2 was added to the culture every 3 to 4 days. PBLs were restimulated each week. Restimulations were performed with iDCs treated with 5 μg/mL hD1-KLH after 1 week, and with iDCs treated with 2.5 μg/mL hD1-KLH after 2, 3, and 4 weeks. Finally, PBLs were harvested and cocultured with autologous mDCs that had been pulsed with KLH, as described in "Targeting experiments." Proliferative responses were determined by tritiated thymidine incorporation assays.

Results

Humanized anti–DC-SIGN antibody hD1 is internalized by iDCs

The CDRs of the mouse antihuman DC-SIGN antibody AZN-D1 were grafted onto a human IgG2/IgG4 composite antibody to generate a humanized antibody for DC-SIGN targeting, hD1. We have previously shown that the human hybrid IgG2/IgG4 constant domain prevents antibodies from binding to Fc receptors.[17] The binding affinity of hD1 for DC-SIGN was similar to that of AZN-D1 as determined by surface plasmon resonance (3.7 ± 0.7 nM and 3.8 ± 1.1 nM, respectively, data not shown).

Flow cytometric analysis revealed specific binding of hD1 to DC-SIGN on iDCs, as preincubation of iDCs with AZN-D1 efficiently reduced binding (Figure 1A). Furthermore, hD1 bound to DC-SIGN–expressing K562 cells after transfection with DC-SIGN cDNA, whereas it did not bind to untransfected K562 cells (data not shown). A time-course internalization experiment revealed that both the hD1 and the AZN-D1 antibodies were rapidly internalized by iDCs, although the AZN-D1 antibody was internalized slightly more efficiently than hD1. As expected, the control AZN-L19 antibody, directed against CD18, was not internalized (Figure 1B). Analysis by confocal microscopy confirmed internalization of the hD1 and AZN-D1 antibodies by iDCs, whereas the control antibodies 5G1.1, directed against human terminal complement protein C5, and mouse IgG1 isotype were not internalized (Figure 1C).

hD1-KLH is rapidly internalized and translocated to the lysosmal compartment

To further explore the potential of DC-SIGN as a targeting receptor for DC vaccination, we studied the uptake of KLH complexed to

Samsung Bioepis Exhibit No. 1002
Page 5416

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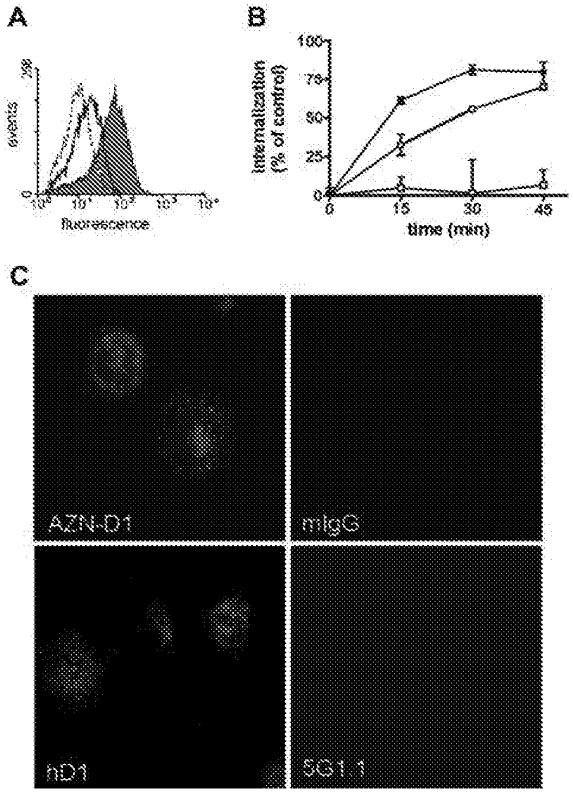


Figure 1. Binding of hD1 to DC-SIGN and internalization by DCs. (A) iDCs were treated with 10 μg/mL hD1 (gray shaded curve), 100 μg/mL AZN-D1 (open dotted curve), or pretreated with 100 μg/mL AZN-D1 followed by 10 μg/mL hD1 incubation (open solid curve), followed by incubation with an Alexa Fluor 647–labeled goat anti–human IgG antibody. Binding of hD1 was analyzed by flow cytometry. (B) iDCs were incubated with AZN-D1 (●), hD1 (○), or AZN-L19 (□) at 4°C for one hour, and transferred to 37°C. Cells were fixed at various time points, and stained with Alexa Fluor–labeled secondary antibodies with or without prior permeabilization. The mean fluorescence was determined by flow cytometric analysis, and the amount of internalized antibody was plotted as a percentage of the amount of total cell-associated antibody. Data represent experiments performed in triplicate ± SD. (C) Internalization of hD1 was confirmed by CSLM. iDCs were incubated with hD1, AZN-D1, or their isotype controls 5G1.1 and mouse IgG1 (mIgG) for one hour at 37°C. Cells were stained with Alexa Fluor 647–labeled secondary antibodies (blue), followed by microscopic analysis. The image represents the middle focal plane of the DCs, with iris set at 2 nm. Original magnification, ×600.

the humanized anti–DC-SIGN antibody hD1. As was observed with the hD1 antibody, hD1-KLH bound to iDCs (Figure 2A). hD1-KLH binding to DCs is mediated by DC-SIGN, as binding could be blocked by pretreating iDCs with AZN-D1 (Figure 2A). Analysis by CLSM revealed that hD1-KLH was rapidly internalized by iDCs (Figure 2B). After one hour of incubation at 37°C, hD1-KLH could be detected in 74% of the iDCs (data not shown). In contrast to hD1-KLH, KLH itself did not bind to iDCs, but was internalized by iDCs following one hour of incubation at 37°C (Figure 2C). These findings demonstrate that internalization of KLH by DCs is not receptor mediated, and likely depends on macropinocytosis.

Foreign antigens are taken up by DCs and shuttled, via endosomes, to the lysosomal compartment where they can be processed. In order to establish whether hD1-KLH reaches the lysosomal compartment, and to study uptake-kinetics and routing of KLH and D1-KLH, these proteins were directly labeled with Alexa Fluor 488 dye. Live imaging by CLSM revealed rapid uptake of hD1-KLH and KLH by iDCs. Both hD1-KLH and KLH were detected in the lysosomal compartment within one hour after their addition to the culture medium (Figure 2D; Supplemental Videos S1 and S2; see the Supplemental Videos link at the top of the online article, at the *Blood* website).

Induction of KLH responses by DC vaccination

In order to compare the antigen-presentation capacity of DCs pulsed with KLH to that of DCs targeted by hD1-KLH, we required KLH-specific T cells. To this end, PBLs were isolated from melanoma patients participating in a vaccination study. This study involved vaccination of patients with autologous DCs pulsed ex vivo with KLH and melanoma-associated antigens. Monocyte-derived DCs and PBLs from patients showing humoral as well as cellular responses against KLH were used for the targeting studies.

All 31 vaccinated patients participating in the clinical vaccination trial showed responses against KLH (data not shown). Humoral responses were readily detected in the patients' serum. KLH-specific IgGs were present after the first vaccination cycle, and their serum levels increased upon the second vaccination (Figure 3A). Cellular responses were analyzed using PBMCs obtained from patients one week before the start of the third vaccination cycle. Typically, PBMCs pulsed with KLH showed an increased proliferative response compared with unpulsed PBMCs, revealing the presence of KLH-reactive T cells in peripheral blood

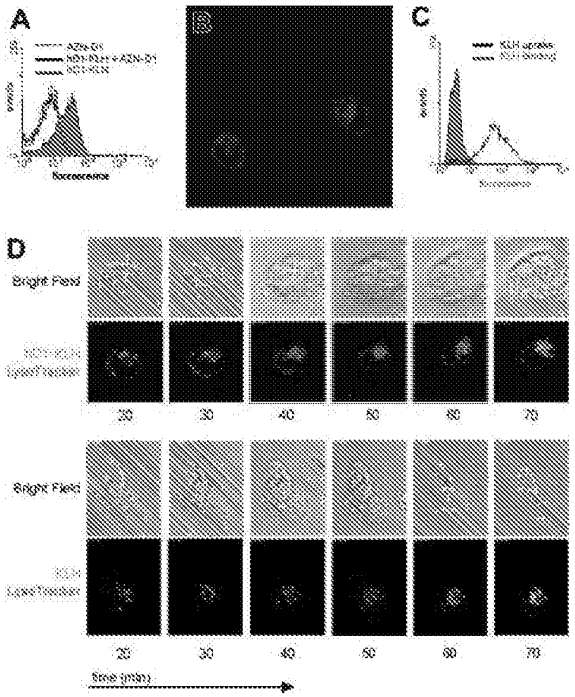


Figure 2. Binding and uptake of hD1-KLH by DCs. (A) iDCs were treated with 10 μg/mL hD1-KLH, 100 μg/mL AZN-D1, or pretreated with 100 μg/mL AZN-D1 followed by 10 μg/mL hD1 incubation, followed by incubation with an Alexa Fluor 647–labeled goat anti–human IgG antibody. Binding of hD1-KLH was analyzed by flow cytometry. (B) Internalization of hD1-KLH was confirmed by CSLM. iDCs were incubated with Alexa Fluor 647–labeled hD1-KLH (blue) for one hour at 37°C, followed by microscopic analysis. The image represents the middle focal plane of the DCs, with iris set at 2 nm. Original magnification, ×600. (C) iDCs were incubated with Alexa Fluor 488–labeled KLH for one hour at 4°C or 37°C. Subsequently, cells were fixed and analyzed by flow cytometric analysis. (D) iDCs were labeled with LysoTracker Red, followed by addition of Alexa Fluor 488–labeled (green) hD1-KLH or KLH. Cells were imaged by CSLM. Data represent bright field and corresponding fluorescent images of cells at various time points after addition of hD1-KLH or KLH to the culture medium. Original magnification, ×630.

Samsung Bioepis Exhibit No. 1002
Page 5417

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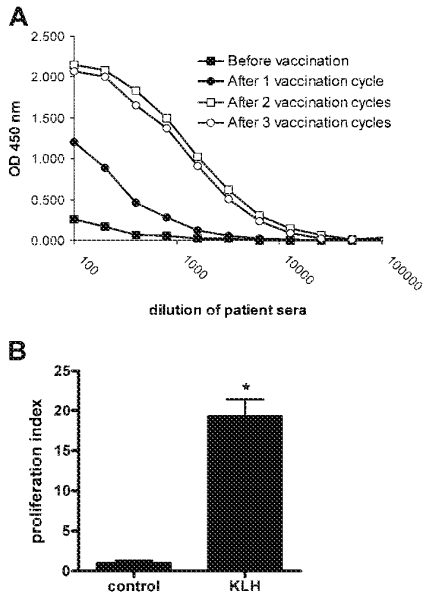


Figure 3. Humoral and cellular responses against KLH in patients vaccinated with KLH-pulsed DCs. (A) Patient serum was obtained both before the first, and after the first, second, and third vaccination cycles. Total IgG antibodies specific for KLH were detected by ELISA. Data represent optical density (OD) 450 values of serially diluted serum samples for a representative patient. (B) Patient PBMCs were isolated one week prior to the third vaccination cycle and cultured in the absence (control) or presence of 10 μg/mL KLH (KLH). Cellular responses were assessed in a tritiated thymidine incorporation assay. Data represent mean ± SD of experiments performed in 6-fold for a representative patient. Significant difference from control according to Student *t* test: $^*P < .001$.

(Figure 3B). These findings show that KLH-pulsed mDCs effectively induced both humoral and cellular responses in vivo.

Targeting of KLH to DC-SIGN results in presentation of KLH antigen epitopes

To demonstrate that antibody-mediated targeting of antigen to DC-SIGN leads to antigen presentation, autologous iDCs were incubated with 5 μg/mL KLH (± 0.63 nM) or hD1-KLH (± 0.53 nM) at 4°C for one hour, washed to remove unbound protein, and added to PBLs. Presentation of KLH epitopes to specific T cells was determined by measuring proliferative responses (Figure 4A-B) and the levels of cytokines secreted in the medium (Figure 4C). As endocytotic processes are inhibited at 4°C, and KLH does not bind to iDCs (Figure 2C), hD1-KLH can be internalized only after binding to DC-SIGN. iDCs incubated with hD1-KLH induced cellular responses by the PBLs, whereas iDCs incubated with KLH did not (Figure 4A-C), showing that targeting of KLH to DC-SIGN results in antigen presentation. We have previously demonstrated that the clinical vaccination protocol used in our studies results in a Th1-type immune response.[20] Indeed, stimulation of patient PBLs with hD1-KLH–treated iDCs resulted in enhanced secretion of IL-2, IFN-γ, and TNF-α (Figure 4C), while IL-4, IL-5, and IL-10 levels were not detectable (data not shown). As expected, iDCs incubated with KLH at 37°C induced proliferative responses, as did iDCs incubated with hD1-KLH (Figure 4A).

Presentation of KLH epitopes by iDCs incubated with hD1-KLH at 4°C was abolished by preincubation of iDCs with the hD1 antibody, but was unaffected by preincubation with the isotype control 5G1.1 (Figure 4B-C). This confirms that hD1-KLH targeted specifically to DC-SIGN, resulting in presentation of KLH epitopes. Cross-linking of hD1 to KLH was required for induction of DC-SIGN–mediated presentation of KLH epitopes, since iDCs incubated with both KLH and hD1 antibody did not induce cellular responses (Figure 4B-C).

Targeting KLH to DC-SIGN enhances its immunogenicity

To determine whether DC-SIGN targeting increases the efficiency of antigen presentation, immature monocyte-derived DCs from previously immunized patients were incubated at 37°C with various concentrations of KLH or hD1-KLH. Subsequently, iDCs were washed, and a portion of the iDCs was matured with LPS. hD1-KLH by itself did not induce maturation of iDCs (data not shown). Both hD1-KLH–treated iDCs (Figure 5A) and mDCs (Figure 5B) induced cellular responses at 100-fold lower concentrations than KLH-pulsed DCs. Thus, antigen targeting via DC-SIGN significantly enhances immunogenicity.

KLH epitopes are presented up to 4 days after targeting DC-SIGN

After antigen uptake in the periphery, DCs migrate to the draining lymph nodes to present the processed antigen to T cells. It is

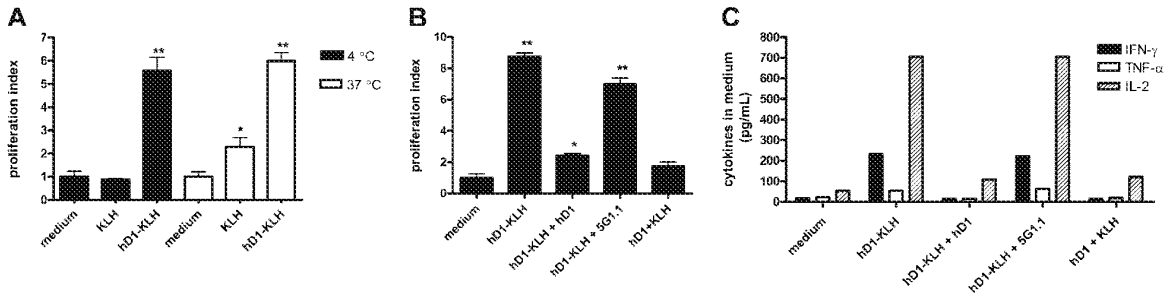


Figure 4. Antibody-mediated targeting of antigen to DC-SIGN results in antigen presentation. (A) iDCs were incubated with medium (control), 5 μg/mL KLH, or 5 μg/mL hD1-KLH for one hour at 4°C or 37°C. Subsequently, iDCs were washed and cocultured with autologous KLH-responsive PBLs, derived from the patients enrolled in the vaccination trial. After 4 days, cellular responses were assessed in a proliferation assay. Data are mean proliferation indices relative to medium control for experiments performed in triplicate ± SD. Significant difference from medium control according to analysis of variance (ANOVA) and Bonferroni test: $^*P < .05$; $^{**}P < .001$. (B) iDCs were incubated with medium (control), 5 μg/mL hD1-KLH, 5 μg/mL hD1-KLH and 100 μg/mL hD1 (hD1-KLH + hD1), 5 μg/mL hD1-KLH and 100 μg/mL 5G1.1 (hD1-KLH + 5G1.1), or 5 μg/mL hD1 and 5 μg/mL KLH (hD1 + KLH) for one hour at 4°C. Subsequently, iDCs were washed and cocultured with autologous KLH-responsive PBLs, derived from patients enrolled in the vaccination trial. After 4 days, cellular responses were assessed in a proliferation assay. Data are mean proliferation indices relative to medium control for experiments performed in triplicate ± SD. Significant difference from medium control according to ANOVA and Dunnett test: $^*P < .05$; $^{**}P < .01$. (C) Production of IFN-γ, TNF-α, and IL-2 in the coculture experiment described in panel B. After 16 hours of coculturing iDCs and PBLs, supernatants were taken and cytokine levels were determined. Data represent cytokine levels in pooled samples of experiments performed in triplicate.

Samsung Bioepis Exhibit No. 1002
Page 5418

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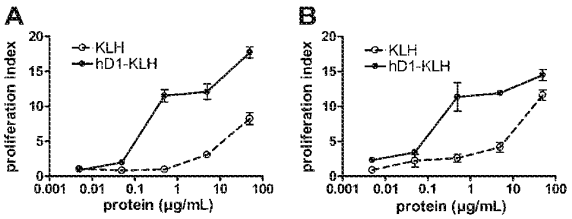


Figure 5. Targeting of antigen to DC-SIGN enhances its immunogenicity. (A) iDCs were incubated with medium, 5 μg/mL KLH, or 5 μg/mL hD1-KLH for one hour at 37°C. Subsequently, iDCs were washed and cocultured with autologous KLH-responsive PBLs, derived from patients enrolled in the vaccination trial. After 4 days, cellular responses were assessed in a proliferation assay. Data are mean proliferation indices relative to medium control for experiments performed in triplicate ± SD. (B) Experiment performed as described in panel A, except that antigen-treated iDCs were matured with LPS before addition of PBLs.

imperative that DCs present the antigens over a prolonged period of time to initiate a significant immune response. We evaluated the efficiency of antigen presentation over time following targeting by incubating patient PBLs with autologous iDCs that had been pulsed with KLH or hD1-KLH 0, 2, or 4 days earlier. The results demonstrate that DCs targeted with hD1-KLH presented KLH epitopes up to 4 days after targeting. Moreover, at all time points evaluated, DCs targeted with hD1-KLH induced stronger proliferative responses than DCs pulsed with KLH (Figure 6).

hD1-KLH targeted to DCs activates both major histocompatibility complex (MHC) class I– and II–restricted naive T cells recognizing KLH epitopes

To evaluate whether hD1-KLH, besides triggering recall responses, is able to activate naive T cells, hD1-KLH was tested in an autologous in vitro culture system. PBLs from a healthy donor were stimulated by repetitive coculturing with hD1-KLH–pulsed autologous DCs. IL-10 was added to the cultures to stimulate expansion of $CD8^+$ T cells.[25] After 5 rounds of stimulation, PBLs were cocultured with DCs pulsed with KLH or hD1 to evaluate KLH-specific proliferative responses. The results demonstrate that hD1-KLH–targeted DCs activated KLH-specific naive T cells (Figure 7). Presentation of KLH epitopes was mediated by MHC class I and class II, since both class I and class II blocking antibodies significantly reduced the proliferative response, while an antibody control did not. DCs pulsed with hD1 antibody did not induce proliferative responses by the PBLs (Figure 7), demonstrating that the T-cell response is specific for KLH epitopes rather than epitopes contained within the hD1 antibody.

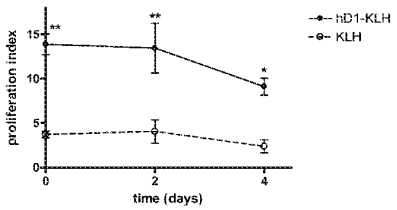


Figure 6. Antigen targeted to DC-SIGN is presented for at least 4 days. iDCs were incubated with medium, 5 μg/mL KLH, or 5 μg/mL hD1-KLH for one hour at 37°C. Subsequently, iDCs were washed and cultured for 0, 2, or 4 days before addition of autologous KLH-responsive PBLs, derived from patients enrolled in the vaccination trial. Four days after addition of PBLs, cellular responses were assessed in a proliferation assay. Data are mean proliferation indices relative to medium control for experiments performed in triplicate ± SD. Significant difference from KLH according to ANOVA and Bonferroni test: $^*P < .05$; $^{**}P < .01$.

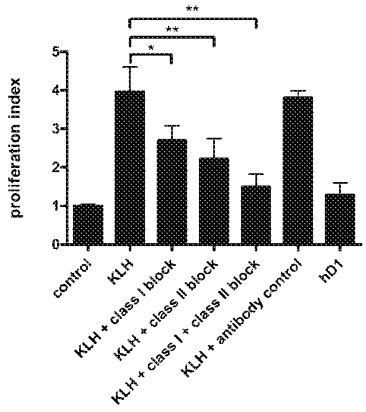


Figure 7. hD1-KLH–treated DCs activate naive T cells recognizing KLH epitopes. iDCs derived from a healthy donor were incubated with medium (control), 10 μg/mL KLH, or 10 μg/mL hD1 for one hour at 37°C. Subsequently, cells were washed and matured with LPS. mDCs were cocultured with autologous PBLs that had been repeatedly stimulated with DCs treated with hD1-KLH as described in "Patients, materials, and methods." Some cocultures of KLH-pulsed mDCs and PBLs were supplemented with w6/32 antibody (KLH + class I block), IVA-12 (KLH + class II block), both w6/32 and IVA-12 (KLH + class I + class II block), or total mouse IgG (KLH + antibody control). Data are mean proliferation indices relative to medium control for experiments performed in triplicate ± SD. Significant difference according to ANOVA, followed by the Student-Newman-Keuls test: $^*P < .01$; $^{**}P < .001$.

Discussion

In the present study, we explored the capacity of the type II C-type lectin DC-SIGN to function as a target receptor for vaccination purposes. The results demonstrate that antibody-mediated targeting of antigen to human DC-SIGN was 100-fold more efficient than pulsing DCs with antigen, and that antigen epitopes were presented for at least 4 days after targeting.

A critical step in designing a suitable targeting construct is the choice of targeting antibody. Various antibodies directed against the same receptor might bind distinct receptor epitopes, thus influencing biologic outcome. For example, not all antibodies directed against DC-SIGN are internalized following binding,[13] and the anti-MR antibody PAM-1 induces maturation of iDCs, in contrast to a different isotype-matched anti-MR antibody.[26] The mouse antihuman DC-SIGN antibody AZN-D1 has been previously described to be internalized by DCs,[13] a prerequisite for successful targeting. However, the use of murine antibodies in humans presents numerous problems, including a short half-life and high immunogenicity.[27] We were able to graft the CDR of AZN-D1 hypervariable domains onto a human composite IgG2/IgG4 antibody without loss of binding characteristics. The resulting hD1 antibody exhibited a similar binding affinity for DC-SIGN as AZN-D1, and did not induce maturation of iDCs (data not shown). hD1 binding induced rapid internalization of DC-SIGN, while internalization did not result in a lasting down-regulation of DC-SIGN expression (data not shown), thus minimizing the effect a therapeutic intervention might have on the biologic function of DC-SIGN. hD1 rapidly targeted antigen to the DC lysosomal compartment resulting in antigen presentation, and did not induce hD1-specific proliferative responses in the naive T-cell stimulation experiments. Thus, humanization of AZN-D1 resulted in an effective targeting antibody, and will facilitate introduction into clinical trials.

Vaccination strategies aimed at inducing cytotoxic T-cell help, such as antitumor therapies, require antigen presentation in the

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Page 5419

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context of MHC class I. In addition, the induction of CTL responses requires bystander $CD4^+$ T-cell help.[28] Targeting DCs with hD1-KLH resulted in the activation of naive T cells in the context of both MHC class I and II. Although the major route for presentation of exogenous antigens is via class II, presentation via class I can occur via the process of cross-presentation. This process provides internalized proteins access to cytosolic proteasomes and their derived peptides access to the endoplasmatic reticulum (ER)–based class I processing machinery.[29] Particulate antigens taken up by phagocytosis have access to this machinery since phagosomes fuse with the ER soon after or during their formation.[30] Particulate antigens are more efficiently cross-presented than soluble antigens.[31,32] However, a recent study by Ackerman et al reveals that internalized soluble proteins can escape proteolysis and also gain access to the lumen of the ER.[33] This might explain how antibody-mediated targeting of the type I C-type lectins MR[7] and DEC-205[10] results in class I responses. Although we cannot exclude the possibility that, besides receptor-mediated endocytosis, a portion of our chimeric protein was taken up via macropinocytosis, our current findings strongly suggest that antibody-mediated targeting of DC-SIGN results in cross-presentation.

The technique commonly used in current clinical trials to load DCs with antigens involves ex vivo incubation with MHC class I and II binding peptides. Other techniques involve loading with tumor lysates or apoptotic tumor cells, and the introduction of genetic material to drive expression of specific antigens by the DC itself.[2] Ideally, vaccines should deliver antigens to the DCs in vivo. Antibody-mediated targeting of antigens to DC surface receptors to stimulate antigen presentation in vivo has been shown to be far more potent than immunization with antigen in complete Freund adjuvant or splenic DCs pulsed with antigen ex vivo.[6] However, mere targeting of antigens to DCs is not sufficient for induction of immunity. Antibody-mediated targeting of antigen to DEC-205 in mice leads to tolerance, and coadministration of a DC maturation stimulus is required to induce immunity.[6,10,11] These findings are consistent with DC-based vaccination studies in humans, showing that DC maturation is a prerequisite for induction of immunity.[20] Vaccination strategies for transplantation, allergy, autoimmunity, and chronic inflammatory diseases could exploit the finding that targeted iDCs induce tolerance. However, strategies aimed at inducing immunity will require a combination of antigens and DC activation factors. Agents that have been shown to activate DCs in vivo include anti-CD40 antibody,[11] α-galactosylceramide,[34] and the Toll-like receptor ligands LPS[35] and cytosine-phosphate-guanosine (CpG) oligonucleotides.[36] A better understanding of differences in DC subsets, their activation pathways, and antigen uptake receptors will provide the information necessary for development of effective vaccines.

In the present study, we used a neoantigen to study targeting efficiency. A major challenge in cancer therapy is to break immunologic tolerance to tumor-associated self-antigens. Tolerance can be the result of clonal deletion, active suppression of antigen-specific T cells by regulatory T cells, or inadequate activation stimuli provided by tumor cells upon antigen presentation, resulting in T-cell anergy.[37] One way of breaking tolerance is by disruption of negative regulatory mechanisms directly at the T-cell level. Antibody-mediated blockade of the cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) breaks tolerance against moderately immunogenic tumors[38] and improves the efficiency of tumor cell–based vaccines against poorly immunogenic tumors.[39] Depletion of $CD25^+$ regulatory T cells prior to blocking of CTLA-4 improves vaccine efficiency even further.[40] A second way of breaking tolerance is to recruit DCs as potent APCs. DCs can overcome the heightened threshold of anergic T cells[41] and restore responsiveness of tolerogenic tumor-specific T cells[42] in vitro. Moreover, studies in mice demonstrate that DC-based vaccines can break tolerance against (tumor-associated) self-antigens,[43-47] resulting in regression of established tumors.[47] Thus, a combination of antibody-mediated strategies targeting tumor-associated antigens to DCs and strategies modulating regulatory mechanisms at the T-cell level might provide effective cancer therapies.

In conclusion, antibody-mediated targeting of antigens to DC surface receptors represents an exciting way to induce immune responses. Our results demonstrate efficient delivery of antigen to DCs via DC-SIGN, resulting in naive as well as recall responses by T cells. These data expose DC-SIGN as a promising target molecule for antibody-mediated antigen delivery to DCs.

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Samsung Bioepis Exhibit No. 1002
Page 5420

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Samsung Bioepis Exhibit No. 1002
Page 5421

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Primer

The complement system

Philip Taylor, Marina Botto and Mark Walport

The system of plasma proteins called complement was so-named because it complements the activity of antibody in the lysis of bacteria. It has a central role in host defence against many micro-organisms and in the modulation of inflammatory reactions, an activity that has been illuminated by the study of humans with naturally occurring deficiencies of complement.

Complement is activated by three pathways: the classical, alternative and mannan-binding lectin (MBL) pathways (Figure 1 and Table 1). The classical pathway is primarily activated by antibody bound to antigens (immune complexes). The alternative pathway is activated by the direct binding of complement component C3 to pathogens in the absence of antibody. The recently discovered MBL pathway is initiated by the binding of MBL to mannose groups, present on many bacterial cell walls. All three pathways lead to the cleavage of C3, the central component of complement, which functions as an opsonin, tagging pathogens and immune complexes for recognition and uptake, mediated by specific complement receptors on phagocytic cells. Subsequent activation of the terminal complement pathway, known as the membrane attack complex (MAC), leads to cell lysis.

An important current focus of complement research is the physiological activities of complement *in vivo*, on which we will concentrate in this primer.

Complement and inflammation

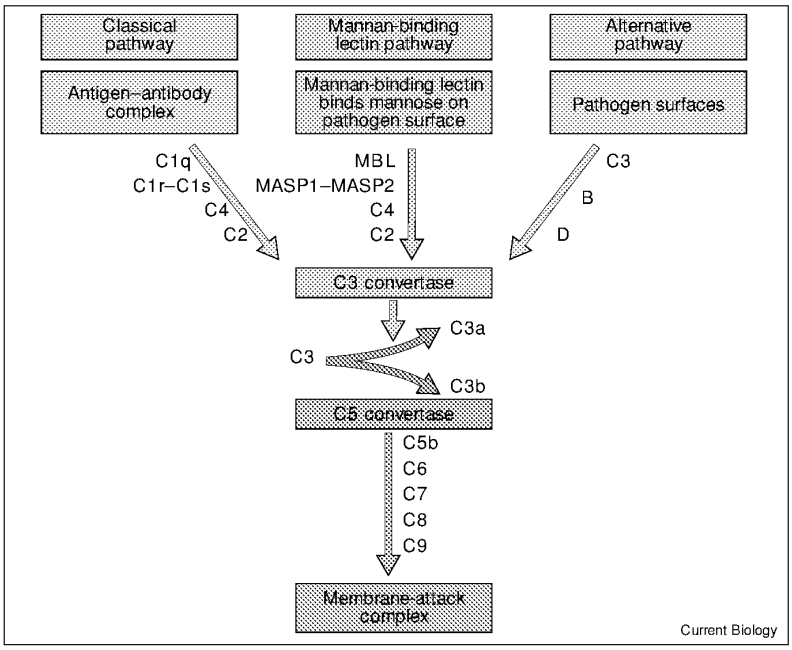
Inflammation is a complex process involving vasodilation, increased vascular permeability and extravasation of plasma. This is accompanied by the adhesion of leukocytes to vascular endothelium and their emigration into surrounding tissue. Complement plays an important part in inflammation, in helping to recruit effector cells and in promoting the killing and clearance of pathogens.

But complement can be both friend and foe. In some circumstances the activation of complement in response to immune complexes may cause harm: acutely in the context of massive complement activation occurring in patients with overwhelming Gram-negative bacterial sepsis, and chronically, when an antibody response develops in the context of antigens that cannot be cleared. Examples of this are infections at anatomically-protected sites — such as heart valves in bacterial endocarditis — or infections by certain viruses, such as hepatitis C, in patients who are unable to clear the infection. The second category in which there is persistent formation of immune complexes is autoimmune disease, in which, by definition, the antigen is a permanent feature of the host.

An understanding of how immune complexes cause tissue injury developed from study of such diseases in humans and animals, and from the induction of experimental immune complex disease. In these situations, immune complexes were shown to cause tissue injury through activation of complement, as opsonin (binding to complement receptors on leukocytes) and as the source of anaphylatoxins (stimulating leukocyte chemotaxis and degranulation). But the development of gene-targeted mice with deficiencies of complement proteins and of receptors for the Fc part of antibodies has led to re-appraisal of the role of complement and antibodies in inflammatory injury.

Some studies of the formation of immune complexes in the skin of these animals (the Arthus reaction)

Figure 1



The main pathways and components of the complement activation system.

Samsung Bioepis Exhibit No. 1002
Page 5424

Table 1

Effector activities of the complement system

Activity	Complement protein	Complement receptor mediating effect
Opsonin for phagocytosis	C3b, iC3b	CR1, CR3
	C4b	CR1
	C1q, MBL	C1q receptor
Peptide mediators of inflammation	C5a	C5a receptor
	C3a	C3a receptor
Lytic membrane attack complex	C5b, C6, C7, C8, C9	
Processing and clearance of antigen–antibody immune complexes	C1q	C1q receptor
	C3b, iC3b	CR1, CR3
	C4b	CR1
Localization of antigen in the form of immune complexes on antigen-presenting cells	C3b, iC3b, C3dg	CR1, CR2, CR3
Activation of B lymphocytes	C3dg	CR2
Clearance of apoptotic cells	C1q?	C1q receptor?

The activites of the complement system are shown in the left-hand column. In the centre are shown the complement proteins that mediate the effects. (C3b, iC3b and C4b are large activation products of these complement proteins that bind covalently to the targets of complement activation.) The column on the right shows the receptors that are bound by the relevant complement ligands in the centre column.

showed that ligation of Fc receptors — which bind to antibodies complexed with antigens — on mast cells is critical for the induction of inflammatory injury by immune complexes, and that complement plays little or no part. This view has been supported by the study of the effects of Fc receptor deficiency in a spontaneous mouse model of an autoimmune disease, systemic lupus erythematosus, in which immune complexes are prominent. Fc receptors were shown to be required for the development of severe renal disease.

It is probably too early, however, to dismiss completely the role of complement in inflammation in immune complex-mediated disease. Studies of immune complex formation in mice deficient in complement component C5, or in the receptor for the anaphylatoxin C5a, demonstrated a more substantial role for complement in the mediation of inflammatory reactions in the lungs and peritoneum. Similar results were obtained in studies of peritoneal infection in C3- and C4-deficient mice. Complement activation was shown to mediate the initial release of the pro-inflammatory cytokine TNFα by mast cells, and the subsequent neutrophil influx.

Complement and adaptive immunity

It has been known since the seminal experiments of Pepys in 1974 that complement is involved in the induction and maintenance of antibody responses. Recently, there has been an increasing appreciation of the importance of the role of complement at the interface between innate and adaptive immunity. Here, complement plays a part in the localization of antigens to antigen-presenting cells — cells specialized to present antigens to lymphocytes and to activate them — and to B cells, both of which bear receptors for major cleavage products of C3.

This activity of complement was beautifully demonstrated by experiments in which antibody responses were measured in animals immunized with antigens covalently linked by genetic engineering to the relevant cleavage product of C3. The addition of increasing numbers of C3 molecules to each antigen molecule dramatically reduced the threshold for activation of B lymphocytes.

Evasion of complement by pathogens

One of the most fascinating aspects of the study of complement is the exploration of the mechanisms that pathogens have evolved to evade and, in some cases, exploit the complement system. Pathogens that cause disease are pathogenic by virtue of their ability to escape, at least in part, the primary mechanisms of defence of the innate immune system. To evade complement pathogens have stolen and mimicked regulatory molecules of the complement system (Figure 2).

Complement and autoimmunity

Homozygous deficiency of classical pathway proteins is very strongly associated with the development of the autoimmune disease systemic lupus erythematosus (SLE). There is a hierarchy of susceptibility and severity of autoimmune disease according to the position of the deficient protein in the classical pathway, with C1q deficiency showing the highest prevalence and causing the most severe disease. These clinical observations imply that there is a, so far unknown, physiological activity of the early proteins of the classical pathway that protects against the development of SLE.

Some recent data point to the discovery of a possible new physiological activity of the complement system that could link complement deficiency and SLE. There is evidence that apoptotic cells might be the source of the autoantigens that stimulate autoimmunity in SLE and that C1q might be involved in the processing of apoptotic cells. If these findings are confirmed, then failure of clearance of apoptotic cells could perhaps explain the link between complement deficiency and SLE. The development of C1q-deficient mice will help to resolve this.

Samsung Bioepis Exhibit No. 1002
Page 5425

Therapeutic inhibition of complement

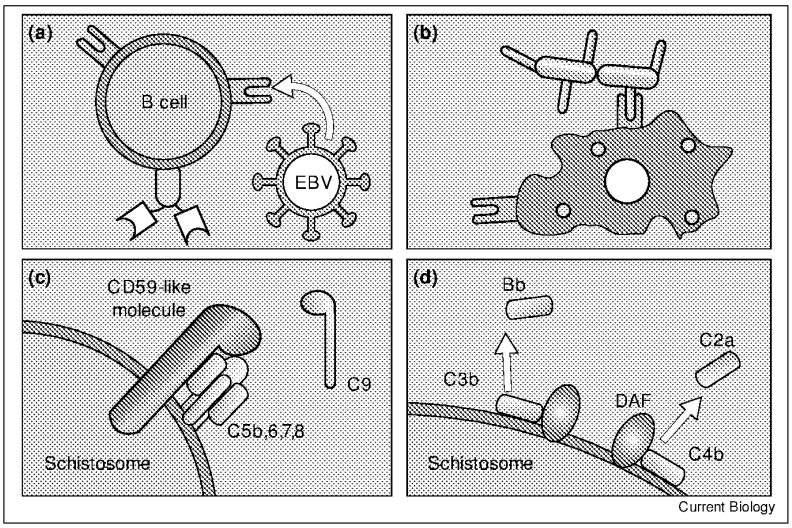
Transplantation of organs between species that are distantly related in phylogenetic terms — such as humans and pigs — is followed by rapid organ rejection, which is mediated by massive complement activation, caused by antibody binding to abundant carbohydrate epitopes on the pig tissues. Because xenotransplantation is an attractive option (if ethical and viral issues permit) for overcoming the severe shortage of human organs for transplantation, there has been much interest in inhibiting complement-mediated injury to xenotransplants. Pigs have been developed that are transgenic for the expression of human complement regulatory proteins. Organs from such animals show significant protection from hyperacute rejection mediated by complement when transplanted into non-human primates.

A clinically-important example of complement-mediated tissue injury is that following infarction. For example, the MAC might be found in the heart following myocardial infarction. Soluble fragments of the complement receptor and regulatory protein CR1 (CD35) reduce the infarct size in experimental models of myocardial infarction. This has yet to be translated into clinical practice, but complement activation in infarction and ischaemic-reperfusion injury remains a very attractive target, especially if a small molecule could be discovered with efficient inhibitory activity for complement.

Conclusion

When one of the authors of this article, MW, entered complement research he was told, possibly tongue in cheek, by a distinguished Professor of Medicine that complement research was dead. We hope this primer will persuade that professor that the complement system is alive and well and that the study of complement continues to make major and surprising contributions to our understanding of immunobiology.

Figure 2



Four strategies by which pathogens use the complement system as part of their pathogenesis. **(a)** The Epstein–Barr virus (EBV) uses a B lymphocyte receptor for complement, CR2 (pink), to gain entry to the cell. (Cell-surface immunoglobulin is shown in yellow.) **(b)** A mycobacterium (green) fixes a complement enzyme, C2a, to its surface. The C2a cleaves C3, resulting in the binding of C3b (red) to the mycobacterial surface. The mycobacterium then enters macrophages (blue) via C3 receptors (pink) on the cell surface. **(c)** Schistosomes synthesize an endogenous protein (purple) that mimics a host complement regulatory protein, CD59, and inhibits the formation of the MAC (red) on the parasite. **(d)** Schistosomes absorb a complement regulatory protein, decay accelerating factor (DAF; green), from the host plasma that protects against the activation of complement by inhibiting the formation of the C3 convertase enzyme.

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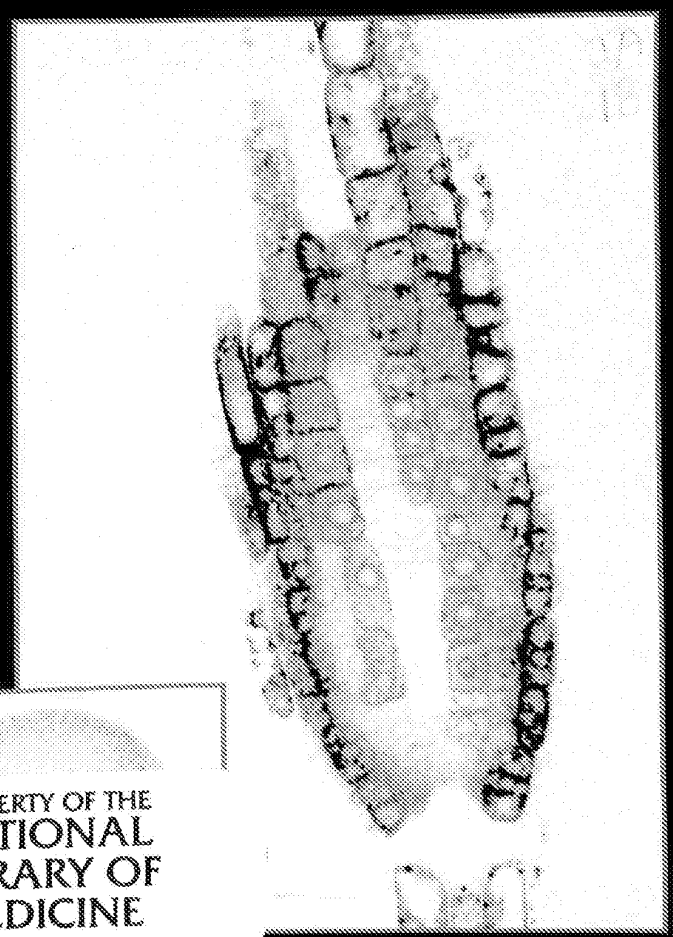
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Antibody-based therapy

Humanized antibodies

Greg Winter and William J. Harris

Hybridoma technology enabled rodent monoclonal antibodies to be created against human pathogens and cells, but these had limited clinical utility. Protein engineering, reviewed here by Greg Winter and William Harris, *is now generating antibodies for treatment of infectious disease, autoimmune disease and cancer by 'humanizing' rodent antibodies. Humanized antibodies have improved pharmacokinetics, reduced immunogenicity and have been used to clinical advantage.*

The antibody is an adaptor molecule containing binding sites for antigen at one end and for effector molecules at the other, and has evolved to bind to a vast range of antigens. Binding alone may be sufficient to neutralize some toxins and viruses, however, more commonly, the antibody triggers the complement system and cell-mediated killing. Although antibodies are natural therapeutic agents, it has proved difficult to make human monoclonal antibodies (mAbs) by hybridoma technology.

Rodent mAbs unfortunately have serious disadvantages: a short half-life in serum; only some of the different classes can trigger human effector functions; and the mAbs can also elicit an unwanted immune response in patients (human anti-mouse antibodies or HAMA). HAMA can result in enhanced clearance of the antibody from the serum, blocking of its therapeutic effect and hypersensitivity reactions. These problems have prompted the use of protein engineering technologies to 'humanize' rodent mAbs by transplanting antigen-binding sites from rodent to human antibodies. In principle, humanizing allows access to a large pool of well-characterized rodent mAbs for therapy, including those with specificities against human antigens that are difficult to elicit from a human immune response[1].

The engineering of antibodies is facilitated by the modular arrangement of protein domains: the heavy- and light-chain variable (V) domains are responsible for binding to antigen, and the constant domains to effector functions. As both complement and cell-mediated killing require fully glycosylated antibody, the engineered mAbs are expressed in mammalian hosts. Each antibody domain is encoded by a different genetic exon and, to build recombinant antibodies, the exons are pasted together. The exons encoding the variable domains (*V* genes) can be cloned from the genomic DNA of a B-cell hybridoma; more conveniently, the *V* genes of hybridomas are isolated

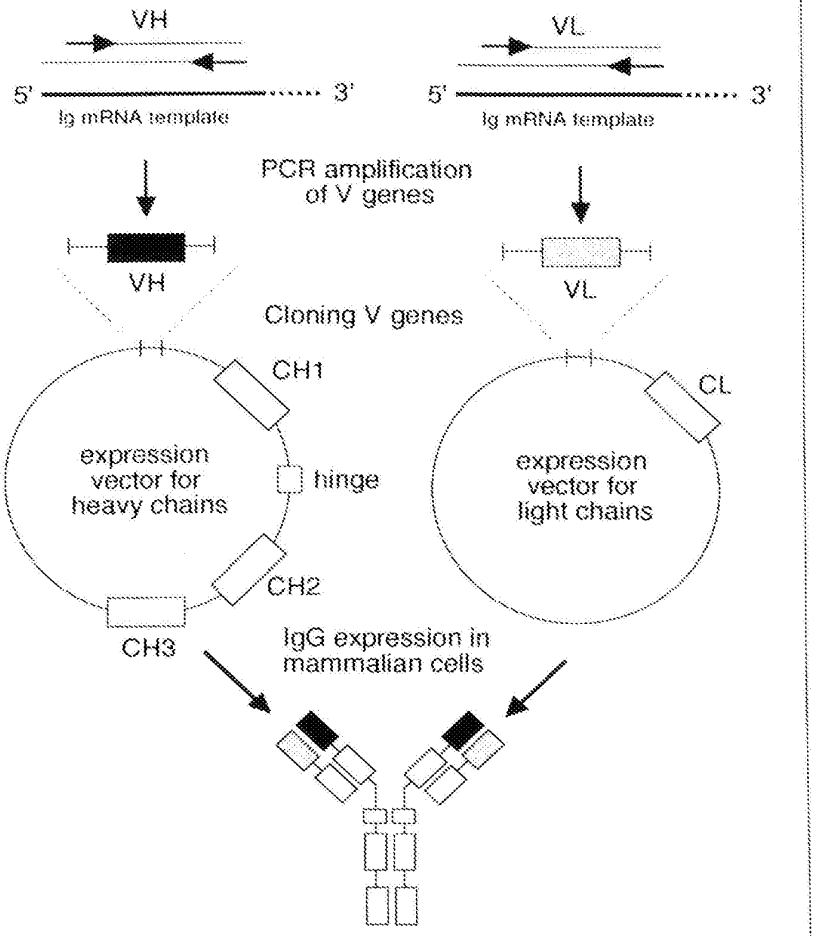


Fig. 1. Cloning of heavy- **(VH)** *and light-* **(VL)** *chain V genes from the mRNA of a mouse B-cell hybridoma into vectors comprising (human) genes encoding constant domains, for expression of mouse human chimaeric antibodies in mammalian cells.*

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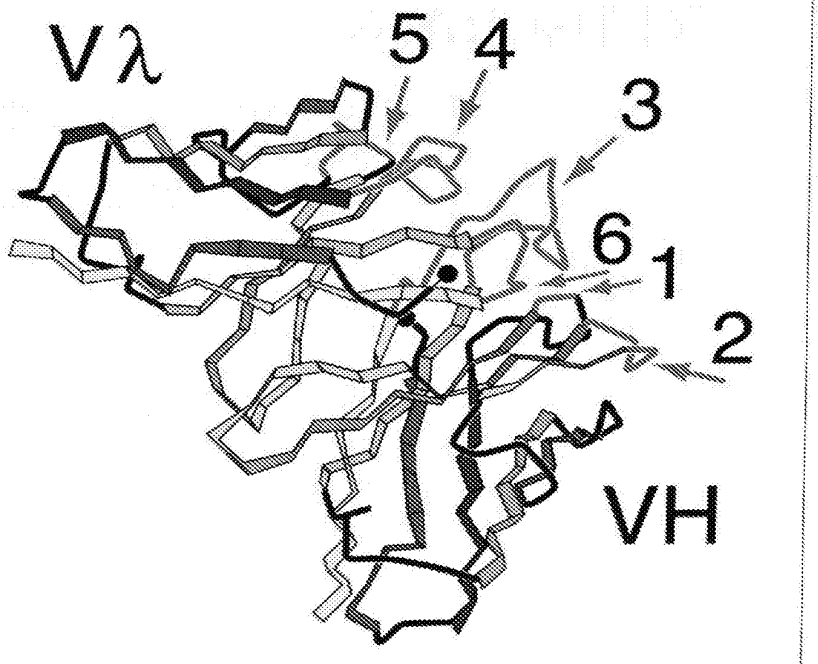


Fig. 2. The β-sheet framework structure of heavy- **(VH)** *and light-* **(Vλ)** *chain variable domains with hypervariable loops 1–6.* Reproduced, with permission, from Winter, G. and Milstein, C. (1991) *Nature* 349, 293–299.

from the mRNA by use of the polymerase chain reaction (PCR). The *V* genes are readily linked to those exons encoding constant domains for expression of mAbs[3] (Fig. 1). Expression vectors have been built with both antibody and viral promoters and enhancers, with *V* and *C* genes as different exons ('genomic') or linked together ('cDNA'). Different markers for selection of transformed cells are available and in both myeloma and CHO hosts[3,4], mAb expression is greatly enhanced by amplifying the number of integrated copies, resulting in yields of up to 0.7 gl^{-1} in fermenters[5].

Building humanized antibodies

The first generation of humanized antibodies were simple chimaeric mAbs, in which the variable domains of a rodent mAb are transplanted to the constant domains of human antibodies (Fig. 1). This reduces the immunogenicity of the rodent mAb (see below) and allows the effector functions to be selected for the therapeutic application. Thus, the human γ1 isotype appears to be the most effective for complement and cell-mediated killing, while the human γg4 isotype appears more suitable for imaging and blocking[6].

The second generation of humanized antibodies were the so-called CDR-grafted antibodies, in which the antigen-binding loops of the rodent mAb were built into a human antibody. The architecture of each antibody V-domain consists of a β-sheet 'sandwich' surmounted by antigen-binding loops (complementarity determining regions or CDRs): in different antibodies, these CDR loops are hypervariable in sequence (Fig. 2). It is this hypervariability that allows the antibody repertoire to bind a potentially vast array of antigens. By transplanting (or grafting) the CDRs from rodent mAb to human antibody, the antigen-binding site can also be transferred[7]; indeed the same human framework can be used for mounting different antigen-binding sites[7–9]. However, to recreate the antigen-binding site it is also necessary to consider other possible interactions between the β-sheet framework and the loops. With the help of molecular modelling it is possible to design framework substitutions that maintain key contacts with the CDR loops.

For example, with the rat antibody CAMPATH-1 directed against the CDw52 antigen of human lymphocytes, the simple grafting of the CDRs failed to transplant the binding activity to a human antibody. When the three-dimensional folding of the VH-CDR1 loop of the rat antibody and its contacts with the rat framework were modelled by computer graphics, the framework amino acid residue Phe27 was predicted to pack against the loop. However, in the human framework of the CDR-grafted antibody, Phe27 was replaced by Ser27; indeed when Ser27 was mutated to Phe in the CDR-grafted antibody, the binding activity was restored[8]. In other examples, enhancement of antigen affinity was achieved stepwise by combining several framework substitutions[10,11]. Indeed analysis of antibody structures is leading towards the identification of sets of framework residues that may exert an influence on CDR structure[11] and also on the packing of the strands of the β-sheet[12].

The first CDR-grafted antibodies were based on the known crystallographic structures of the human myeloma proteins[7–9]. CDR-grafted antibodies have also been built with consensus human frameworks based on several human heavy chains[13]. The use of a single or a limited number of human frameworks offers the prospect of a range of therapeutic antibodies that are almost identical, apart from the CDR sequences. Conversely a range of framework structures should be capable of supporting the CDR loops, and 'hyperchimaeric' CDR-grafted antibodies have used mouse–human frameworks. For example, to humanize a mouse antibody directed against the human IL-2 receptor (anti-Tac), a human framework sequence was selected by homology. A molecular model was then used to identify those framework residues of the rodent antibody that might interact with the antigen-binding loops, and these were built into the selected human framework[14] (Fig. 3). Chimaeric frameworks have also been proposed in which the

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internal residues that pack between the domains and with the antigen-binding loops are derived from the rodent sequence and the solvent-accessible residues are taken from a human sequence[15].

Most generally, all of the rodent CDRs are transplanted from mouse to human antibody. However, some CDRs are more important than others for binding of antigen, as evident from the crystallographic structures of antibody–antigen complexes. The interaction of antibody loops with antigen involves both main-chain and sidechain contacts: as the CDR loops of mouse and human antibodies fold in a limited number of ways[16], it is possible to maintain some main-chain contacts while varying some of the sidechains (and sequence) of the CDRs[17].

There is some loss in binding affinity on CDR grafting but, in combination with some framework alterations, it is usually possible to obtain a reshaped antibody with an affinity within three-fold of the parent monoclonal antibody. High binding affinities may be critical for neutralization of a cytokine or toxin in the serum; they appear to be less important where multiple interactions can occur with high avidities, as with multimeric (cell surface) antibody binding to repeated epitopes on a viral coat[9]. However, small improvements in affinity have been seen for some CDR-grafted antibodies[18], and binding affinities can also be improved *in vitro*, by chain shuffling[19], or random mutation[20].

Using humanized antibodies

Both chimaeric and CDR-grafted antibodies appear to have better pharmacokinetics than rodent mAbs, with extended serum half-life (>75 hours) in humans and cynomolgus monkeys. Likewise the immunogenicity is reduced. Much of the HAMA response to mouse antibodies in patients is directed against the constant region; chimaeric antibodies and CDR-grafted antibodies appear to elicit much less response with the immunogenic epitopes being located in the variable regions. Indeed much of this response is directed against the antigen-binding site (for review see Ref. 4).

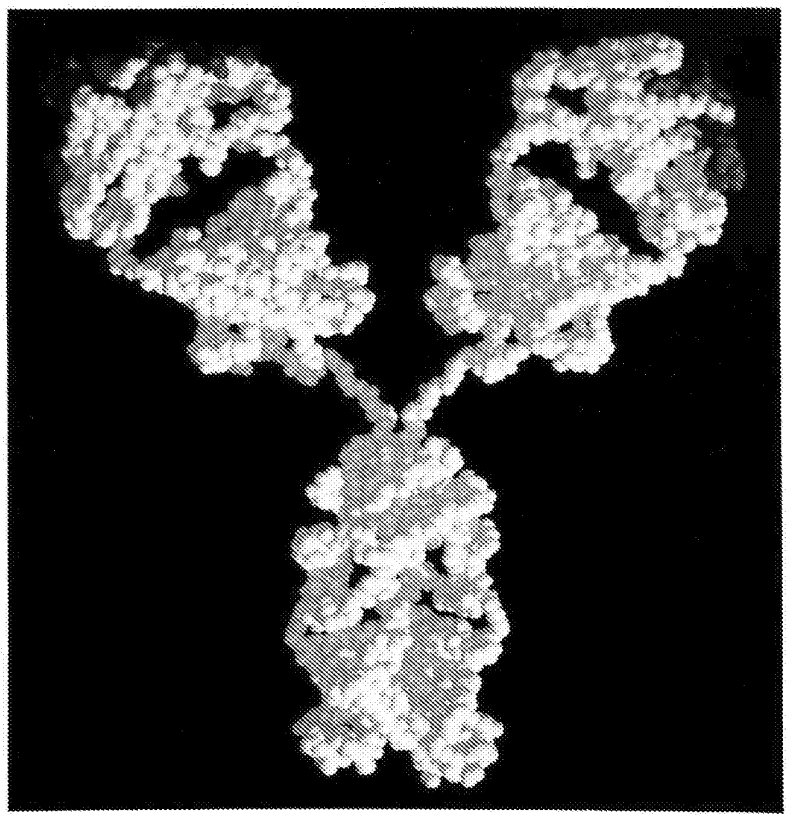


Fig. 3. Computer model of the main-chain backbone of the humanized anti-Tac antibody. **Red**, *complementarity-determining regions;* **blue**, *altered framework residues.* Reproduced, by kind permission of L. Korn, from the prospectus of Protein Design Laboratories.

No antibody response was detected against the CDR-grafted anti-CDw52 antibody during the treatment of two patients with non-Hodgkin's lymphoma for up to 43 days with escalating doses of antibody ranging from 1 to 20 mg per day[21]. Also, no response was reported when the antibody was used in a single course of therapy for rheumatoid arthritis patients[22] or in conjunction with an anti-CD4 antibody in treatment of a patient with intractable systemic vasculitis[23]. However, an antibody response was detected on further treatment of the rheumatoid patients[22]. Antibody responses were not detected against other CDR-grafted antibodies used for radio-imaging in tumour patients[24] or treating acute graft-versus-host disease[25].

Chimaeric and CDR-grafted antibodies have been constructed against a wide range of viral and bacterial pathogens, and against human cell-surface markers including tumour cell antigens. Some of the targets are summarized in Table I. CDR-grafted antibodies against lymphocyte markers have already been used to clinical advantage. The anti-CDw52 antibody was used to deplete a large tumour mass in two lymphoma patients, and to achieve clinical remission[21]. Likewise, this antibody resulted in significant clinical benefit for a patient with systemic vasculitis[23], and for rheumatoid arthritis patients[22]. The anti-Tac antibody was used for immunosuppression following allogeneic marrow transplantation, and resulted in improvement in several cases of acute graft-versus-host disease[25].

Future prospects

As shown above, humanized antibodies can be engineered from rodent mAbs. Their use has been demonstrated in the clinic, and they have a longer serum half-life and reduced immunogenicity

3
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TABLE I. CDR-grafted antibodies for therapy (see also Ref. 3)

Target	Clinical potential
CDw52	lymphomas, systemic vasculitis, rheumatoid arthritis
CD3	organ transplantation
CD4	organ transplants, rheumatoid arthritis, Crohn's disease
IL-2 receptor	leukaemias and lymphomas, organ transplants, graft-versus-host disease
Tumour necrosis factor β	septic shock
Human immunodeficiency virus	AIDS
Rous sarcoma virus	respiratory syncytial virus infection
Herpes simplex virus	neonatal, ocular and genital herpes infection
Lewis-Y	cancer
$p185^{HER-2}$	cancer
Placental alkaline phosphatase	cancer
Carcinoembryonic antigen	cancer

compared to rodent mAbs. They therefore appear to be promising as therapeutics, especially for a single course of treatment. It is not yet clear whether humanized (or even human) antibodies will elicit a blocking immune response over longer or several courses of treatment.

The immunogenicity of humanized antibodies is likely to depend on several factors, including the immune state of the patients, and the dose and regimen of antibody administration. The target is also likely to be important; antibodies directed against cell-bound antigens might be expected to be more immunogenic than those binding to soluble antigen. Furthermore, since foreign framework regions can elicit an immune response[26], we might also expect that the differing strategies to select and mutate human frameworks (as described above) could lead to reshaped antibodies with differing immunogenicity. For example, human antibody frameworks that are mutated (*in vivo* or *in vitro*) with respect to the germ-line segments could prove immunogenic: even buried residues could form the critical element of a T-cell epitope if presented as a denatured peptide by a class II MHC molecule[27]. Indeed it may be desirable to design CDR-grafted antibodies by using framework regions based on human germ-line *V*-gene segments.

The design of antibodies for therapy would certainly be revolutionized if *in vitro* assays were available to test the immunogenicity of different constructs. However the key practical issues are not whether the immune response can be avoided entirely, but how it can be bypassed, for example by changing the idiotype of the engineered antibody, and whether the antibodies can be used for long enough to achieve clinical benefit.

So far we have focussed almost entirely on the construction and use of glycosylated antibodies expressed in mammalian cells. However, the use of antibody fragments may be advantageous in some applications, since they penetrate tissues more readily, and are cleared more rapidly from the serum. This may help in neutralizing and clearing drugs from the serum, or in imaging tumours with radioactive entities coupled to the fragments[1]. Although antibody fragments, lacking the glycosylated Fc portion, cannot trigger effector functions, they could in principle be equipped to do so, for example by chemically linking Fab fragments together[28] as bispecific antibody fragments (with one arm binding a tumour cell antigen and the other binding and triggering effector cells such as cytotoxic T cells or monocytes).

Furthermore, antibody fragments can be expressed by secretion from bacteria[29,30], and can be readily derived from the *V* genes of hybridomas, or from *V* gene repertoires. The repertoires are cloned for display on the surface of filamentous bacteriophage by fusion of the encoded antibody fragment to a coat protein of the phage, and phage with the desired activities selected by binding to antigen. Indeed this technology mimics the strategy of immune selection, and human antibody fragments with specificities against many different foreign and human self-antigens have been isolated from the same 'single pot' of phages (see Refs 31, 32 for review).

Over the past century we have seen three generations of antibody therapeutics: polyclonal animal antibodies, rodent monoclonal antibodies and now humanized antibodies. We anticipate that the use of 'repertoire selection' technologies to make human antibodies and fragments will provide the next generation. However, in the meantime it seems likely that humanized antibodies will prove clinically useful for treating several diseases, and the experience should prove valuable for designing and formulating the next generation of antibody therapeutics.

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The use of monoclonal antibodies to achieve immunological tolerance

Herman Waldmann and Stephen Cobbold

Monoclonal antibodies are potentially useful immunosuppressive agents. Short courses of CD4/CD8 monoclonal antibody can be used to guide the immune system of experimental animals to accept organ grafts and to arrest autoimmunity. This reprogramming, reviewed by Herman Waldmann and Stephen Cobbold, *is accompanied by potent T-cell dependent, 'infectious' regulatory mechanisms. A goal for therapeutic immunosuppression should be to understand and harness these innate immunoregulatory mechanisms.*

The ideal form of therapeutic immunosuppression would be one that could be given over a short-term period to achieve long-term unresponsiveness to the desired antigen, without impairing the response to infectious agents. Current immunosuppressive regimens are relatively non-antigen-specific, require long-term administration and incur a sustained risk of infection and undesirable side-effects.

If we are to achieve tolerance as a therapeutic goal in autoimmunity or in transplantation, then it is essential that we understand how the body normally establishes 'self tolerance'. From this basis we can determine which of these natural mechanisms might be exploited for pharmaceutical control. As thymus-derived cells (T cells) are required for driving most forms of immune response it is appropriate that we focus our discussion on how to control their functions.

T-cell recognition of nonself

T cells recognize foreign or 'nonself' antigen as peptide fragments displayed to them in the clefts of major histocompatibility complex (MHC) class I and II molecules expressed on the surfaces of many body cells. In addition, T cells have unique clonally distributed receptors that respond to these antigens displayed to them on specialized cells in the lymphoid tissues (antigen-presenting cells or APC). They then proliferate and differentiate to effector mode (Fig. 1). It is now clear that the cells with the greatest ability to present antigen and activate T cells are the dendritic cells[1,2]. Two features of dendritic cells that endow them with this property are their possession of a particular array of cell surface ligands complementary to an array of adhesion molecules on T cells, and their abundant MHC class II expression. A further requirement for T-cell activation is one of collaboration or help from other T cells responsive to the same antigen[3–9] (Fig. 2).

H. Waldmann is Kay Kendall Professor of Therapeutic Immunology and S. Cobbold is Senior Research Associate at the Immunology Division, Department of Pathology, Tennis Court Road, Cambridge, UK CB2 1QP.

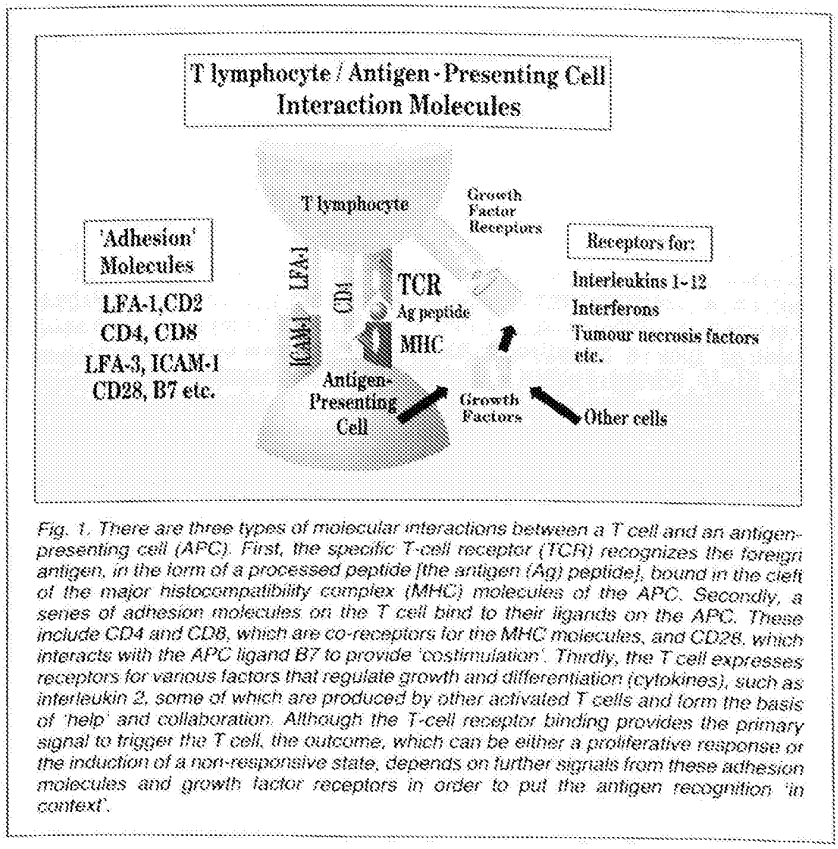


Fig. 1. There are three types of molecular interactions between a T cell and an antigen-presenting cell (APC). First, the specific T-cell receptor (TCR) recognizes the foreign antigen, in the form of a processed peptide [the antigen (Ag) peptide], bound in the cleft of the major histocompatibility complex (MHC) molecules of the APC. Secondly, a series of adhesion molecules on the T cell bind to their ligands on the APC. These include CD4 and CD8, which are co-receptors for the MHC molecules, and CD28, which interacts with the APC ligand B7 to provide 'costimulation'. Thirdly, the T cell expresses receptors for various factors that regulate growth and differentiation (cytokines), such as interleukin 2, some of which are produced by other activated T cells and form the basis of 'help' and collaboration. Although the T-cell receptor binding provides the primary signal to trigger the T cell, the outcome, which can be either a proliferative response or the induction of a non-responsive state, depends on further signals from these adhesion molecules and growth factor receptors in order to put the antigen recognition 'in context'.

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Trends in Pharmacological Sciences
including TOXICOLOGICAL SCIENCES

Introduction
Why immunopharmacology?, *Richard B. Gallagher, Robert Brines and Debbie Girdlestone* 137
Introduction to immunopharmacology, *John W. Hadden and Tadamitsu Kishimoto* 138

Antibody-based therapy
Humanized antibodies, *Greg Winter and William J. Harris* 139
The use of monoclonal antibodies to achieve immunological tolerance, *Herman Waldmann and Stephen Cobbold* 143
Immunotoxins: magic bullets or misguided missiles?, *Ellen S. Vitetta, Philip E. Thorpe and Jonathan W. Uhr* 148

Cytokine therapy
Modalities for reducing interleukin 1 activity in disease, *Charles A. Dinarello* 155
The IL-2/IL-2 receptor system: a target for rational immune intervention, *Thomas A. Waldmann* 159
Cytokine regulation of T-cell function: potential for therapeutic intervention, *Fiona Powrie and Robert L. Coffman* 164

Immunomodulation
Immunostimulants, *John W. Hadden* 169
Adjuvants: current status, clinical perspectives and future prospects, *Françoise M. Audibert and Luc D. Lise* 174
Selective immunosuppression, *Luciano Adorini, Jean-Charles Guéry, Gemma Rodriguez-Tarduchy and Sylvie Trembleau* 178
FK506 and ciclosporin: molecular probes for studying intracellular signal transduction, *Jun Liu* 182
Structure-based drug design: applications in immunopharmacology and immunosuppression, *Manuel A. Navia and Debra A. Peattie* 189

Clinical perspectives
Challenges in the therapy of HIV infection, *Robert Yarchoan, Hiroaki Mitsuya and Samuel Broder* 196
Cancer vaccines, *Drew M. Pardoll* 202
Immunopharmacology of asthma, *John Morley* 208
Immunosuppressive therapy of autoimmune diseases, *Jean-François Bach* 213
Donor cell chimerism permitted by immunosuppressive drugs: a new view of organ transplantation, *Thomas E. Starzl, Anthony J. Demetris, Noriko Murase, Angus W. Thomson, Massimo Trucco and Camillo Ricordi* 217

Job Trends XVIII

Diary XX

Index to advertisers/fax back form XXVI

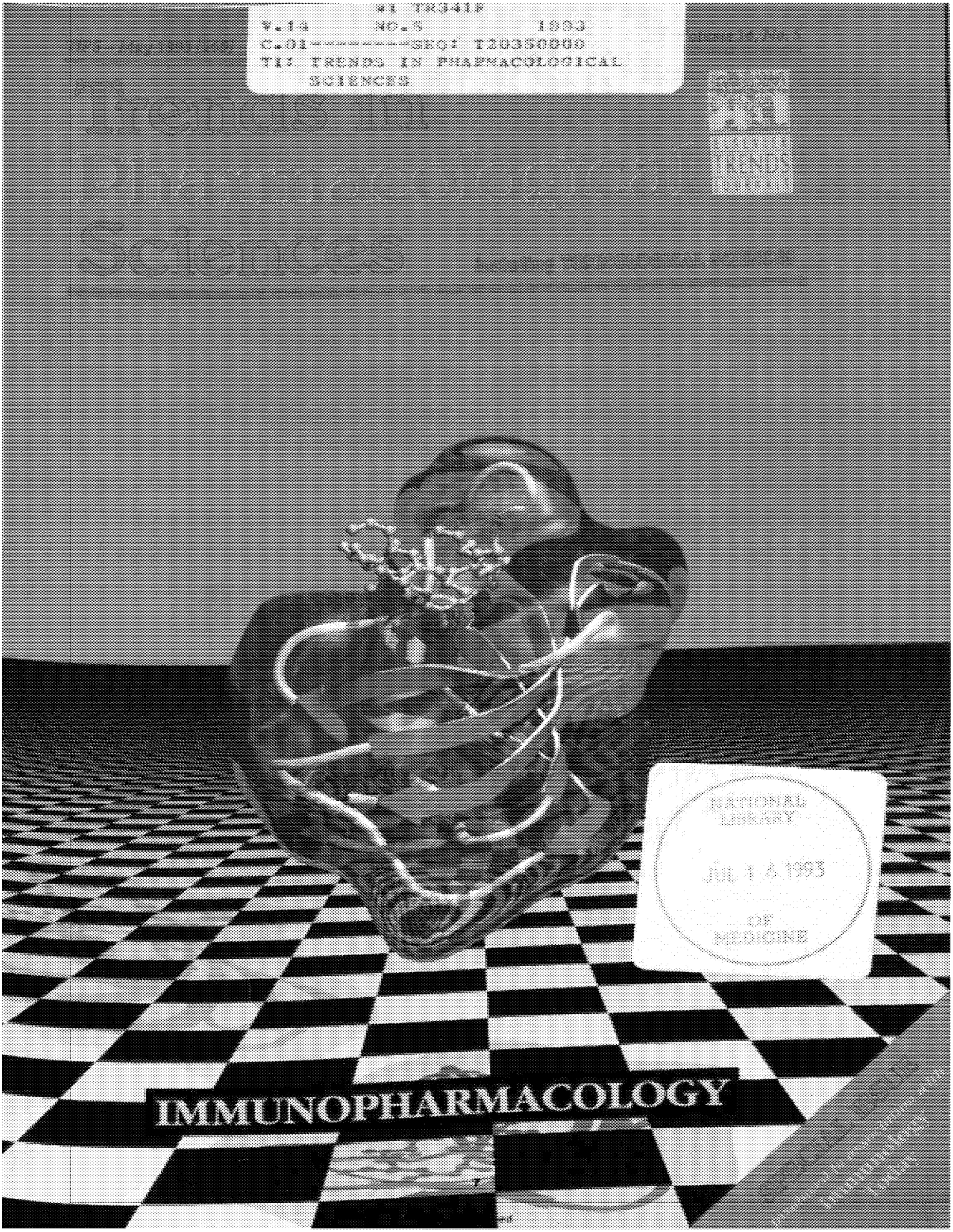
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Results. In 2004, 439 (4.2%) of a total of 10,525 units of O-RBC were transfused due to the policy. In 2005, 689 (6.1%) of a total of 11,293 units of O-RBC were transfused due to the policy. Increase in the use of O-RBC from 2004 to 2005 was by 218 units (6.7%), while the increase in the use of group O-RBC for the policy increased disproportionately by 210 units (43.8%). Over the 2 year period, a monthly median excess of 43 units (range 24-85) of O-RBC were issued in accordance with the policy. Daily excess use of O-RBC for the policy was a median of 1 unit (range 0-19). There were 291 days when no policy-related O-RBC were used, and only 3 days when more than 10 units were used. The interval between a 1st and 2nd (confirmatory) typing, when achieved, occurred within 24 hours for 15.4% of patients, but was a median of 23 days, and ranged from minutes to 21.5 years. Confirmatory re-typing activity within less than 72h of the 1st occurred in a minority of 28%, possibly reflecting nonessential repetition and/or deliberate action to release patients from policy-related O-RBC restrictions. Patients who were protracted users of Group O policy blood (no confirmatory type and/or repeatedly indeterminate typing) were neonates (310 units [26.5%]) and recipients of ABO-incompatible hemato-poietic stem cell transplants (76 units [6.5%]). The blood bank identified misdrawn blood through typing discrepancies in 6 patients over 2 years. Transfusion occurred inconsistently in 3 of these 6, as the misdraw was made apparent by comparison with prior typings on file in 2, and patient knowledge of correct type in 1. The misdrawn specimen was the 1st one in 2 of 6. Of the 3 who were not transfused, the misdraw suggested a type that would have led in all to the transfusion of clinically incompatible blood, had the knowledge of prior type, and the policy, both been absent. ABO-incompatible red cell transfusion errors did not occur, although this was a greater function of luck than the policy in the interval.

Conclusions. A policy instituted to prevent ABO-incompatible transfusion errors, by providing O-RBC to those with unconfirmed blood types, appeared to be both feasible, and potentially misransfusion-preventative, at a high-volume tertiary-care teaching hospital.

Disclosure: No relevant conflicts of interest to declare.

Bone Marrow Failure

Abstract# 971 **Poster Board #-Session: 99-I**

Safety and Efficacy of the Terminal Complement Inhibitor Eculizumab in Patients with Paroxysmal Nocturnal Hemoglobinuria: Interim Shepherd Phase III Clinical Study.

Neal S. Young,[1] Elisabetta Antonioli*,[2] Bruno Rotoli,[3] Hubert Schrezenmeier*,[4] Jörg Schubert*,[5] Alvaro Urbano-Ispizua*,[6] Luke Coyle,[7] Carlos de Castro,[8] Chieh-Lin Fu,[9] Jaroslaw P. Maciejewski,[10] Christopher F. Mojcik*,[11] Russell P. Rother*,[12] Peter Hillmen[13] *[1]NHLBI, NIH, Bethesda, MD, USA; [2]Azienda Ospedaliera Univ. Careggi, Firenze, Italy; [3]Universita degli Studi di Napoli, Naples, Italy; [4]Institut für Klinische Transfusionmedizin und Immungenetik, Helmholtzstr, Germany; [5]Saarland Univ. Med. School, Homburg/Saar, Germany; [6]Hospital Clinic i Provincial, Barcelona, Spain; [7]Royal North Shore Hospital, St Leonards, Australia; [8]Duke Univ. Medical Center, Durham, NC, USA; [9]Cleveland Clinic Florida, Weston, FL, USA; [10]The Cleveland Clinic Foundation, Cleveland, OH, USA; [11]Alexion Pharmaceuticals, Inc, Cheshire, CT, USA; [12]Haematology, Leeds General Infirmary, Leeds, United Kingdom.*

In paroxysmal nocturnal hemoglobinuria (PNH), lack of the GPI-anchored terminal complement inhibitor CD59 from blood cells renders erythrocytes susceptible to chronic hemolysis resulting in anemia, fatigue, thrombosis, poor quality of life (QoL), and a dependency on transfusions. Eculizumab, a terminal complement inhibitor, reduced intravascular hemolysis and transfusion requirements in transfusion dependent patients with normal or near-normal platelet counts in a randomized placebo-controlled trial (TRIUMPH). SHEPHERD, an open-label, non-placebo controlled 52-week phase III clinical study, is underway to evaluate the safety and efficacy of eculizumab in a broader PNH population including patients with significant thrombocytopenia and/or lower transfusion requirements. Eculizumab was dosed as follows: 600 mg IV every 7 days x 4; 900 mg 7 days later; and then 900 mg every 14±2 days. Eculizumab was administered to 97 patients at 33 international sites. In a pre-specified 6-month interim analysis, the most frequent adverse events were headache (50%), nasopharyngitis (23%), and nausea (16%); most were mild to moderate in severity. No infections or serious adverse events were reported as "probably" or "definitely" related to drug. Intravascular hemolysis, the central clinical manifestation in PNH and the primary surrogate efficacy endpoint of the trial, was significantly reduced in eculizumab patients as assessed by change in lactate dehydrogenase (LDH) area under the curve ($p<0.001$). LDH levels decreased from a median of 2,051 U/L at baseline to 270 U/L at 26 weeks ($p<0.001$; normal range 103-223 U/L). Control of intravascular hemolysis resulted in an improvement in anemia as transfusion requirements decreased from a median of 4.0 PRBC units/patient pre-treatment to 0.0 during treatment ($p<0.001$); approximately 50% of the patients were rendered transfusion independent ($p<0.001$), and hemoglobin levels increased ($p<0.001$). Fatigue, as measured by both the FACIT-Fatigue and EORTC QLQ-C30 instruments, was significantly improved with eculizumab treatment as compared to baseline ($p<0.001$ for each). Other EORTC QLQ-C30 patient reported outcomes demonstrating improvement included global health status ($p<0.001$), all 5 patient functioning subscales ($p<0.001$) and 7 of 9 symptom/single item subscales ($p<0.05$). These results demonstrate that the beneficial effects of eculizumab in PNH are applicable to a much broader patient population than previously studied and further underscore that eculizumab treatment markedly reduces intravascular hemolysis, thereby providing clinical benefit to treated patients. The trial will complete in September 2006 and the final results from this 52-week study will be presented.

Disclosure: There is no label for eculizumab as it is an investigational drug.; Drs Mojcik and Rother are employees of Alexion Pharmaceuticals, Inc.; Drs Hillmen, Schubert and Maciejewski have served as consultants for Alexion Pharmaceuticals, Inc.; Drs Mojcik and Rother have equity ownership in Alexion Pharmaceuticals, Inc.; Dr Hillmen has received grant support from Alexion Pharmaceuticals, Inc. Dr Young reports that Alexion made an unrestricted donation to the NHLBI in support of this research.; Drs Hillmen and Schubert have received lecture fees from Alexion Pharmaceuticals, Inc.; Drs Rotoli, Schubert, Urbano, Fu and Maciejewski have served on Advisory Committees for Alexion Pharmaceuticals, Inc.

Abstract# 972 **Poster Board #-Session: 100-I**

Blockade of Intravascular Hemolysis in PNH with the Terminal Complement Inhibitor Eculizumab Unmasks Low-Level Hemolysis Potentially Occurring through C3 Opsonization.

Anita Hill*,[1] Russell P. Rother*,[2] Antonino M. Risitano,[3] Duncan S. Cole*,[1] Matthew J. Cullen*,[1] Stephen J. Richards,[1] Carmine Selleri,[3] Patrizia Ricci*,[3] Bruno Rotoli,[3] Lucio Luzzatto,[3] Peter Hillmen.[1] *[1]Department of Haematology, Leeds General Infirmary, Leeds, United Kingdom; [2]Dept of Research, Alexion Pharmaceuticals, Inc, Cheshire, CT, USA; [3]Division of Hematology, Federico II University of Naples, Naples, Italy; [4]Dept Medical Biochemistry and Immunology, University Hospital of Wales, Cardiff, United Kingdom*

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hemolytic anemia characterized by intravascular hemolysis, often resulting in the need for red blood cell (RBC) transfusions. PNH RBCs lack two complement regulatory molecules: CD59, a terminal complement inhibitor, and CD55, a C3 convertase inhibitor. Eculizumab, a humanized monoclonal antibody that inhibits terminal complement by binding to C5, effectively controls intravascular hemolysis as determined by a dramatic reduction in lactate dehydrogenase (LDH) to levels in or just above the normal range. Control of intravascular hemolysis in these patients led to a reduction in, or cessation of, RBC transfusions. During eculizumab treatment, a majority of patients demonstrate evidence of residual, low-level hemolysis; LDH levels remain slightly elevated, haptoglobin levels are low or undetectable, and bilirubin levels are above normal. We hypothesized that this low-level residual hemolysis may be due to clearance of PNH RBCs through a C3b-mediated mechanism. Therefore we investigated C3 deposition on RBC in PNH patients before and on eculizumab. A direct antiglobulin test (DAT) using monoclonal anti-C3d was positive in 29 out of 39 PNH patients on eculizumab. Of these 29 DAT-positive patients, who were all receiving transfusions, 25 had DAT testing prior to eculizumab therapy and only one of these was positive. DAT was negative in all of 8 normal volunteers. By two-color flow cytometric analysis with anti-CD59 and anti-C3, the majority of patients on eculizumab demonstrated three distinct RBC populations: (i) CD59+/C3- (normal RBCs); (ii) CD59-/C3- (PNH RBCs without C3 coating); and (iii) CD59-/C3+ (PNH RBCs coated by C3). No CD59+/C3+ RBCs were observed. Of 25 DAT positive eculizumab treated patients tested, the median proportion of total RBCs that were C3dc positive was 17.6%. 18 of 29 [62%] eculizumab patients with a positive DAT received at least one transfusion during eculizumab therapy compared with 1 of 10 [10%] for DAT negative patients ($p<0.01$), although even patients who did not become transfusion independent during eculizumab treatment showed a marked reduction in transfusion requirement. The median hemoglobin value for the 29 DAT positive eculizumab patients was 9.8 g/dl, compared with 11.3 g/dl, in the 10 DAT negative eculizumab patients ($p=0.08$). No apparent relationship between LDH and DAT positivity was observed. It is proposed that resolution of intravascular hemolysis in PNH patients on eculizumab results in deposition of C3b on the surface of PNH RBC's which may explain, at least in part, the residual low level hemolysis occurring in some patients. This appears to be a previously undescribed mechanism of RBC clearance in PNH, most likely obscured by the rapidity of intravascular hemolysis in the absence of eculizumab therapy. Despite the low-level residual hemolysis, patients continue to receive significant benefit from eculizumab treatment.

Disclosure: RPR is an employee of Alexion Pharmaceuticals, Inc., PH and SJR serve as consultants to Alexion Pharmaceuticals, Inc.; RPR has equity ownership in Alexion Pharmaceuticals, Inc.; PH receives grant support from Alexion Pharmaceuticals, Inc.; AH, SJR, LL and PH receive lecture fees from Alexion Pharmaceuticals, Inc.; AH, RR and LL have served on an Advisory Committee for Alexion Pharmaceuticals, Inc.

Abstract# 973 **Poster Board #-Session: 101-I**

Paroxysmal Nocturnal Hemoglobinuria (PNH): Long-Term Epidemiological Study.

Regis Peffault de Latour*,[1] Jean Y. Mary*,[2] Celia Salanoubat*,[3] Louis Terriou*,[4] Gabriel Etienne*,[5] Mohamad Mohty*,[6] Sophie Roth*,[7] Sophie de Guibert*,[8] Jean Y. Cahn*,[9] Gerard Socie*,[1] (Intr. by Gerard Socie) *[1]Hematologie-Greffe, Hopital Saint Louis, Paris, France; [2]Unite INSERM U717, Hopital Saint Louis, Paris, France; [3]Service d Hematologie Clinique, Hopital Henri Dieu, Paris, France; [4]Service des Maladies du Sang, Hospital Claude Huriez, Lille, France; [5]Service de Medecine Interne, Groupe Hospitalier Sud, Bordeaux, France; [6]Unite de Transplantation et de Therapie Cellulaire, Institut Paoli Calmettes, Marseille, France; [7]Service de Medecine Interne, Hopital Archet, Nice, France; [8]Service d Hematologie Clinique, Centre Hospitalier Pontchaillou, Rennes, France; [9]Service d Onco-Hematologie, Hopital Michallon, Grenoble, France.*

PNH is a rare acquired disorder. We aimed to analyze a large cohort of patients on the long term to better determine prognostic factors. We already reported the analysis on 220 patients in 1998 (Société *et al*, Lancet). Data were updated and we collected additional data on 247 patients (total of 467 patients, 252 females), diagnosed from 1950 to 2005 from 58 centers. The date of diagnosis was based on flow cytometry analysis if there was no prior positive Ham's test. The age at diagnosis was 34 years (±13.3). The median follow-up time was 6.8 years (±0.5). Kaplan-Meier 10-year survival estimate was 76.3% (±2.6). Sixty seven (14.6%) patients developed pancytopenia during follow-up [10-year cumulative incidence

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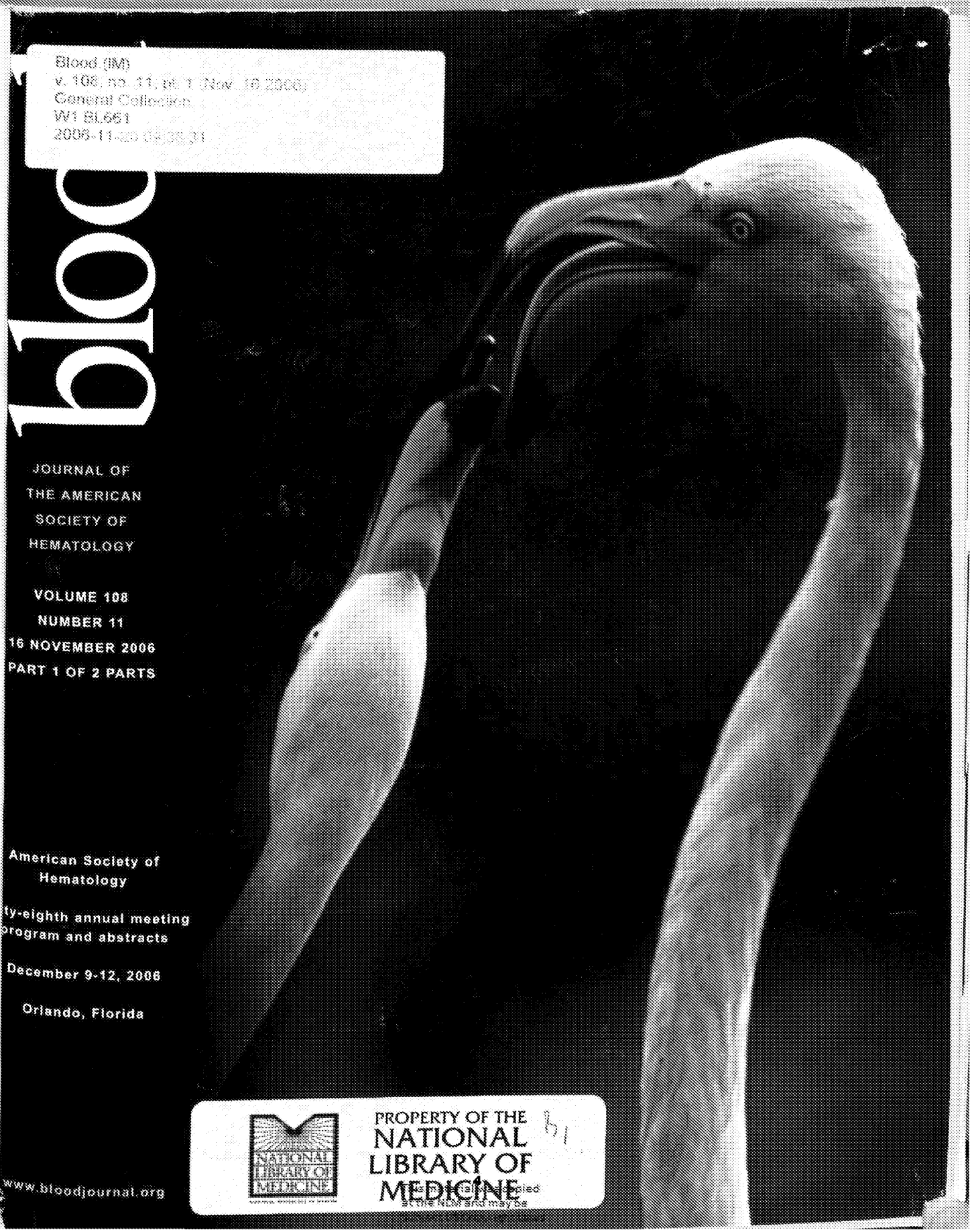
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WARNING
CARDIOMYOPATHY:
HERCEPTIN administration can result in the development of ventricular dysfunction and congestive heart failure. Left ventricular function should be evaluated in all patients prior to and during treatment with HERCEPTIN. Discontinuation of HERCEPTIN treatment should be strongly considered in patients who develop a clinically significant decrease in left ventricular function. The incidence and severity of cardiac dysfunction was particularly high in patients who received HERCEPTIN in combination with anthracyclines and cyclophosphamide. (See WARNINGS.)

DESCRIPTION
HERCEPTIN (Trastuzumab) is a recombinant DNA-derived humanized monoclonal antibody that selectively binds with high affinity in a cell-based assay (Kd = 5 nM) to the extracellular domain of the human epidermal growth factor receptor2 protein, HER2.[1,2] The antibody is an IgG_1 kappa that contains human framework regions with the complementarity-determining regions of a murine antibody (4D5) that binds to HER2.

The humanized antibody against HER2 is produced by a mammalian cell (Chinese Hamster Ovary) [CHO] suspension culture in a nutrient medium containing the antibiotic gentamicin. Gentamicin is not detectable in the final product.

HERCEPTIN is a sterile, white to pale yellow, preservative-free lyophilized powder for intravenous (IV) administration. Each vial of HERCEPTIN contains 440mg Trastuzumab, 9.9mg L-histidineHCl, 6.4mg L-histidine, 400 mg α,α-trehalose dihydrate, and 1.8 mg polysorbate 20, USP. Reconstitution with 20 mL of the supplied Bacteriostatic Water for Injection, (BWFI) USP, containing 1.1%benzyl alcohol as a preservative, yields 21mL of a multi-dose solution containing 21mg/mL Trastuzumab, at a pH of approximately 6.

CLINICAL PHARMACOLOGY
General
The HER2 (or c-erbB2) proto-oncogene encodes a transmembrane receptor protein of 185kDa, which is structurally related to the epidermal growth factor receptor.[1] HER2 protein overexpression is observed in 25%–30% of primary breast cancers. HER2 protein overexpression can be determined using an immunohistochemistry-based assessment of fixed tumor blocks.[3]

Trastuzumab has been shown, in both *in vitro* assays and in animals, to inhibit the proliferation of human tumor cells that overexpress HER2.[4-6]

Trastuzumab is a mediator of antibody-dependent cellular cytotoxicity (ADCC).[7,8] *In vitro*, HERCEPTIN-mediated ADCC has been shown to be preferentially exerted on HER2 overexpressing cancer cells compared with cancer cells that do not overexpress HER2.

Pharmacokinetics
The pharmacokinetics of Trastuzumab were studied in breast cancer patients with metastatic disease. Short duration intravenous infusions of 10 to 500 mg once weekly demonstrated dose-dependent pharmacokinetics. Mean half-life increased and clearance decreased with increasing dose level. The half-life averaged 1.7 and 12 days at the 10 and 500 mg dose levels, respectively. Trastuzumab's volume of distribution was approximately that of serum volume (44 mL/kg). At the highest weekly dose studied (500 mg), mean peak serum concentrations were 377 microgram/mL.

In studies using a loading dose of 4 mg/kg followed by a weekly maintenance dose of 2 mg/kg, a mean half-life of 5.8 days (range = 1 to 32 days) was observed. Between weeks 16 and 32, Trastuzumab serum concentrations reached a steady-state with a mean trough and peak concentrations of approximately 79microgram/mL and 123 microgram/mL, respectively.

Detectable concentrations of the circulating extracellular domain of the HER2 receptor (shed antigen) are found in the serum of some patients with HER2 overexpressing tumors. Determination of shed antigen in baseline serum samples revealed that 64% (286/447) of patients had detectable shed antigen, which ranged as high as 1880 ng/mL (median = 11 ng/mL). Patients with higher baseline shed antigen levels were more likely to have lower serum trough concentrations. However, with weekly dosing, most patients with elevated shed antigen levels achieved target serum concentrations of Trastuzumab by week 6.

Data suggest that the disposition of Trastuzumab is not altered based on age or serum creatinine (up to 2.0 mg/dL). No formal interaction studies have been performed.

Mean serum trough concentrations of Trastuzumab, when administered in combination with paclitaxel, were consistently elevated approximately 1.5-fold as compared with serum concentrations of Trastuzumab used in combination with anthracycline plus cyclophosphamide. In primate studies, administration of Trastuzumab with paclitaxel resulted in a reduction in Trastuzumab clearance. Serum levels of Trastuzumab in combination with cisplatin, doxorubicin or epirubicin plus cyclophosphamide did not suggest any interactions; no formal drug interaction studies were performed.

CLINICAL STUDIES
The safety and efficacy of HERCEPTIN were studied in a randonized, controlled clinical trial in combination with chemotherapy (469patients) and an open-label single agent clinical trial (222 patients). Both trials studied patients with metastatic breast cancer whose tumors overexpressthe HER2 protein. Patients were eligible if they had 2+ or 3+ levels of overexpression (based on a 0-3+ scale) by immunohistochemical assessment of tumor tissue performed by a central testing lab.

A multicenter, randomized, controlled clinical trial was conducted in 469patients with metastatic breast cancer who had not been previously treated with chemotherapy for metastatic disease. Patients were randomized to receive chemotherapy alone or in combination with HERCEPTIN given intravenously as a 4 mg/kg loading dose followed by weekly doses of HERCEPTIN at 2mg/kg. For those who had received prior anthracycline therapy in the adjuvant setting, chemotherapy consisted of paclitaxel (175 mg/m^2 over 3hours every 21 days for at least six cycles); for all other patients, chemotherapy consisted of anthracycline plus cyclophosphamide (AC: doxorubicin 60 mg/m^2 or epirubicin 75 mg/m^2 plus 600 mg/m^2 cyclophosphamide every 21 days for six cycles). Compared with patients in the AC subgroups (n = 281), patients in the paclitaxel subgroups (n = 188) were more likely to have had the following poor prognostic factors (premenopausal status, estrogen or progesterone receptor negative tumors, positive lymph nodes), prior therapy (adjuvant chemotherapy, myeloablative chemotherapy, radiotherapy), and a shorter disease-free interval.

Compared with patients randomized to chemotherapy alone, the patients randomized to HERCEPTIN and chemotherapy experienced a significantly longer time to disease progression, a higher overall response rate (ORR), a longer median duration of response, and a higher one-year survival rate. (See Table1.) These treatment effects were observed both in patients who received HERCEPTIN plus paclitaxel and in those who received HERCEPTIN plus AC, however the magnitude of the effects was greater in the paclitaxel subgroup. The degree of HER2 overexpression was a predictor of treatment effect. (See CLINICAL STUDIES: *HER2 protein overexpression*.)

Table 1
Phase III Clinical Efficacy in First-Line Treatment

	Combined Results		Paclitaxel subgroup		AC subgroup	
	HERCEPTIN + All Chemotherapy (n = 235)	All Chemotherapy (n = 234)	HERCEPTIN + Paclitaxel (n = 92)	Paclitaxel (n = 96)	HERCEPTIN + AC[a] (n = 143)	AC (n = 138)
Primary Endpoint						
Time to Progression[b,c]						
Median (months)	7.2	4.5	6.7	2.5	7.6	5.7
95% confidence interval	6.9, 8.2	4.3, 4.9	5.2, 9.9	2.0, 4.3	7.2, 9.1	4.6, 7.1
p-value (log rank)	<0.0001		<0.0001		0.002	
Secondary Endpoints						
Overall Response Rate[b]						
Rate (percent)	45	29	38	15	50	38
95% confidence interval	39, 51	23, 35	28, 48	8, 22	42, 58	30, 46
p-value (χ2-test)	<0.001		<0.001		0.10	
Duration of Response[b,c]						
Median (months)	8.3	5.8	8.3	4.3	8.0	6.4
25%, 75% quantile	5.5, 14.8	3.9, 8.5	5.1, 11.0	3.7, 7.4	5.8, 14.8	4.5, 8.5
1 Year Survival[c]						
Percent alive	79	68	73	61	83	73
95% confidence interval	74, 84	62, 74	66, 80	51, 71	77, 89	66, 82
p-value (Z-test)	<0.01		0.08		0.04	

[a] AC = anthracycline (doxorubicin or epirubicin) and cyclophosphamide.
[b] Assessed by an independent Response Evaluation Committee.
[c] Kaplan-Meier Estimate

HERCEPTIN was studied as a single agent in a multicenter, open-label, single-arm clinical trial inpatients with HER2 overexpressing metastatic breast cancer who had relapsed following one or two prior chemotherapy regimens for metastatic disease. Of 222 patients enrolled, 66% had received prior adjuvant chemotherapy, 68% had received two prior chemotherapy regimens for metastatic disease, and 25% had received prior myeloablative treatment with hematopoietic rescue. Patients were treated with a loading dose of 4 mg/kg IV followed by weekly doses of HERCEPTIN® (Trastuzumab) at 2mg/kg IV. The ORR (complete response+ partial response), as determined by an independent Response Evaluation Committee, was 14%, with a 2% complete response rate and a 12% partial response rate. Complete responses were observed only in patients with disease limited to skin and lymph nodes. The degree of HER2 overexpression was a predictor of treatment effect. (See CLINICAL STUDIES: *HER2 protein overexpression*.)

HER2 protein overexpression
Relationship to Response. In the clinical studies described, patient eligibility was determined by testing tumor specimens for overexpression of HER2 protein. Specimens were tested with a research-use-only immunohistochemical assay (referred to as the Clinical Trial Assay, CTA) and scored as 0, 1+, 2+, or 3+ with 3+ indicating the strongest positivity. Only patients with 2+ or 3+ positive tumors were eligible (about 33% of those screened).

Data from both efficacy trials suggest that the beneficial treatment effects were largely limited to patients with the highest level of HER2 protein overexpression (3+). (See Table 2.)

Table 2
Treatment Effect versus Level of HER2 Expression

	Single-Arm Trial	Treatment Subgroups in Randomized Trial			
	HERCEPTIN	HERCEPTIN + Paclitaxel	Paclitaxel	HERCEPTIN + AC	AC
Overall Response Rate					
2+	4%	21%	16%	40%	43%
overexpression	(2/50)	(5/24)	(3/19)	(14/35)	(18/42)
3+	17%	44%	14%	53%	36%
overexpression	(29/172)	(30/68)	(11/77)	(57/108)	(35/96)
Median time to progression (months) (95% CI)					
2+	N/A[a]	4.4	3.2	7.8	7.1
overexpression		(2.2, 6.6)	(2.0, 5.6)	(6.4, 10.1)	(4.8, 9.8)
3+	N/A[a]	7.1	2.2	7.3	4.9
overexpression		(6.2, 12.0)	(1.8, 4.3)	(7.1, 9.2)	(4.5, 6.9)

[a] N/A = Not Assessed

Immunohistochemical Detection: In clinical trials, the Clinical Trial Assay (CTA) was used for immunohistochemical detection of HER2 protein overexpression. The DAKO HercepTest™, another immunohistochemical test for HER2 protein overexpression, has not been directly studied for its ability to predict HERCEPTIN treatment effect, but has been compared to the CTA on over 500 breast cancer histology specimens obtained from the National Cancer Institute Cooperative Breast Cancer Tissue Resource. Based upon these results and an expected incidence of 33% of 2+ or 3+ HER2 overexpression in tumors from women with metastatic breast cancer, one can estimate the correlation of the HercepTest™ results with CTA results. Of specimens testing 3+ (strongly positive) on the HercepTest™, 94% would be expected to test at least 2+ on the CTA (i.e., meeting the study entry criterion) including 82% which would be expected to test 3+ on the CTA (i.e., the reading most associated with clinical benefit). Of specimens testing 2+ (weakly positive) on the HercepTest™, only 34% would be expected to test at least 2+ on the CTA, including 14% which would be expected to test 3+ on the CTA.

INDICATIONS AND USAGE
HERCEPTIN as a single agent is indicated for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein and who have received one or more chemotherapy regimens for their metastatic disease. HERCEPTIN in combination with paclitaxel is indicated for treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein and who have not received chemotherapy for their metastatic disease. HERCEPTIN should only be used in patients whose tumors have HER2 protein overexpression. (See CLINICAL STUDIES: *HER2 protein overexpression* for information regarding HER2 protein testing and the relationship between the degree of overexpression and the treatment effect.)

CONTRAINDICATIONS
None known.

WARNINGS
Cardiotoxicity:
Signs and symptoms of cardiac dysfunction, such as dyspnea, increased cough, paroxysmal nocturnal dyspnea, peripheral edema, S_3 gallop, or reduced ejection fraction, have been observed in patients treated with HERCEPTIN. Congestive heart failure associated with HERCEPTIN therapy may be severe and has been associated with disabling cardiac failure, death, and mural thrombosis leading to stroke. The clinical status of patients in the trials who developed congestive heart failure were classified for severity using the New York Heart Association classification system (I--IV, where IV is the most severe level of cardiac failure). (SeeTable 3.)

Table 3
Incidence and Severity of Cardiac Dysfunction

	HERCEPTIN[a] alone n = 213	HERCEPTIN+ Paclitaxel[b] n = 91	Paclitaxel[b] n = 95	HERCEPTIN+ Anthracycline+ cyclophosphamide[b] n = 143	Anthracycline+ cyclophosphamide[b] n = 135
Any Cardiac Dysfunction	7%	11%	1%	28%	7%
Class III-IV	5%	4%	1%	19%	3%

[a] Open-label, single-agent Phase 2 study (94% received prior anthracyclines).
[b] Randomized Phase III study comparing chemotherapy plus HERCEPTIN to chemotherapy alone, where chemotherapy is either anthracycline/cyclophosphamide or paclitaxel.

Candidates for treatment with HERCEPTIN should undergo thorough baseline cardiac assessment including history and physical exam and one or more of the following: EKG, echocardiogram, and MUGA scan. There are no data regarding the most appropriate method of evaluation for the identification of patients at risk for developing cardiotoxicity. Monitoring may not identify all patients who will develop cardiac dysfunction.

Extreme cautionshould be exercised in treating patients with pre-existing cardiac dysfunction.

Patients receiving HERCEPTIN should undergo frequent monitoring for deteriorating cardiac function.

The probability of cardiac dysfunction was highest in patients who received HERCEPTIN concurrently with anthracyclines. The data suggest that advanced age may increase the probability of cardiac dysfunction.

Pre-existing cardiac disease or prior cardiotoxic therapy (e.g., anthracycline or radiation therapy to the chest) may decrease the ability to tolerate HERCEPTIN therapy; however, the data are not adequate to evaluate the correlation between HERCEPTIN-induced cardiotoxicity and these factors.

Discontinuation of HERCEPTIN therapy should be strongly considered in patients who develop clinically significant congestive heart failure. In the clinical trials, most patients with cardiac dysfunction responded to appropriate medical therapy often including discontinuation of HERCEPTIN. The safety of continuation or resumption of HERCEPTIN in patients who have previously experienced cardiac toxicity has not been studied. There are insufficient data regarding discontinuation of HERCEPTIN therapy in patients with asymptomatic decreases in ejection fraction; such patients should be closely monitored for evidence of clinical deterioration.

PRECAUTIONS
General: HERCEPTIN therapy should be used with caution in patients with known hypersensitivity to Trastuzumab, Chinese Hamster Ovary cell proteins, or any component of this product.

Drug Interactions: There have been no formal drug interaction studies performed with HERCEPTIN in humans. Administration of paclitaxel in combination with HERCEPTIN resulted in a two-fold decrease in HERCEPTIN clearance in a non-human primate study and in a 1.5-fold increase in HERCEPTIN serum levels in clinical studies (see Pharmacokinetics).

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Page 5440

Benzyl Alcohol: For patients with a known hypersensitivity to benzyl alcohol (the preservative in Bacteriostatic Water for Injection) reconstitute HERCEPTIN® (Trastuzumab) with Sterile Water for Injection (SWFI), USP. DISCARD THE SWFI-RECONSTITUTED HERCEPTIN VIAL FOLLOWING A SINGLE USE.

Immunogenicity: Of 903 patients who have been evaluated, human anti-human antibody (HAHA) to Trastuzumab was detected in onepatient, who had no allergic manifestations.

Carcinogenesis, Mutagenesis, Impairment of Fertility:
Carcinogenesis: HERCEPTIN has not been tested for its carcinogenic potential.

Mutagenesis: No evidence of mutagenic activity was observed in Ames tests using six different test strains of bacteria, with and without metabolic activation, at concentrations of up to 5000 µg/mL Trastuzumab. Human peripheral blood lymphocytes treated *invitro* at concentrations of up to 5000 µg/plate Trastuzumab, with and without metabolic activation, revealed no evidence of mutagenic potential. In an *invivo* mutagenic assay (the micronucleus assay), no evidence of chromosomal damage to mouse bone marrow cells was observed following bolus intravenous doses of up to 118 mg/kg Trastuzumab.

Impairment of Fertility: A fertility study has been conducted in female cynomolgus monkeys at doses up to 25times the weekly human maintenance dose of 2mg/kg HERCEPTIN and has revealed no evidence of impaired fertility.

Pregnancy Category B: Reproduction studies have been conducted in cynomolgus monkeys at doses up to 23 times the weekly human maintenance dose of 2 mg/kg HERCEPTIN and have revealed no evidence of impaired fertility or harm to the fetus. However, HER2 protein expression is high in many embryonic tissues including cardiac and neural tissues; in mutant mice lacking HER2, embryos died in early gestation.9 Placental transfer of HERCEPTIN during the early (Days20–50 of gestation) and late (Days120–150 of gestation) fetal development period was observed in monkeys. There are, however, no adequate and well-controlled studies in pregnant women. Because animal reproduction studies are not always predictive of human response, this drug should be used during pregnancy only if clearly needed.

Nursing Mothers: A study conducted in lactating cynomolgus monkeys at doses 25times the weekly human maintenance dose of 2mg/kg HERCEPTIN demonstrated that Trastuzumab is secreted in the milk. The presence of Trastuzumab in the serum of infant monkeys was not associated with any adverse effects on their growth or development from birth to 3 months of age. It is not known whether HERCEPTIN is excreted in human milk. Because human IgG is excreted in human milk, and the potential for absorption and harm to the infant is unknown, women should be advised to discontinue nursing during HERCEPTIN therapy and for 6 months after the last dose of HERCEPTIN.

Pediatric Use: The safety and effectiveness of HERCEPTIN in pediatric patients have not been established.

Geriatric Use: HERCEPTIN has been administered to 133 patients who were 65 years of age or over. The risk of cardiac dysfunction may be increased in geriatric patients. The reported clinical experience is not adequate to determine whether older patients respond differently from younger patients.

ADVERSE REACTIONS

A total of 958 patients have received HERCEPTIN alone or in combination with chemotherapy. Data in the table below are based on the experience with the recommended dosing regimen for HERCEPTIN in the randomized controlled clinical trial in 234 patients who received HERCEPTIN in combination with chemotherapy and four open-label studies of HERCEPTIN as a single agent in 352 patients at doses of 10—500 mg administered weekly.

Cardiac Failure/Dysfunction: For a description of cardiac toxicities, see WARNINGS.

Anemia and Leukopenia: An increased incidence of anemia and leukopenia was observed in the treatment group receiving HERCEPTIN and chemotherapy, especially in the HERCEPTIN and ACsubgroup, compared with the treatment group receiving chemotherapy alone. The majority of these cytopenic events were mild or moderate in intensity, reversible, and none resulted in discontinuation of therapy with HERCEPTIN.

Hematologic toxicity is infrequent following the administration of HERCEPTIN as a single agent, with an incidence of GradeIII toxicities for WBC, platelets, hemoglobin all<1%. No GradeIV toxicities were observed.

Diarrhea: Of patients treated with HERCEPTIN as a single agent, 25% experienced diarrhea. An increased incidence of diarrhea, primarily mild to moderate in severity, was observed in patients receiving HERCEPTIN in combination with chemotherapy.

Infection: An increased incidence of infections, primarily mild upper respiratory infections of minor clinical significance or catheter infections, was observed in patients receiving HERCEPTIN in combination with chemotherapy.

Infusion-Associated Symptoms: During the first infusion with HERCEPTIN, a symptom complex most commonly consisting of chills and/or fever was observed in about 40% of patients. The symptoms were usually mild to moderate in severity and were treated with acetaminophen, diphenhydramine, and meperidine (with or without reduction in the rate of HERCEPTIN infusion). HERCEPTIN discontinuation was infrequent. Other signs and/or symptoms may include nausea, vomiting, pain (in some cases at tumor sites), rigors, headache, dizziness, dyspnea, hypotension, rash, and asthenia. The symptoms occurred infrequently with subsequent HERCEPTIN infusions.

Table 4
Adverse Events Occurring in ≥ 5% of Patients or at
Increased Incidence in the HERCEPTIN Arm of the Randomized Study
(Percent of Patients)

	Single Agent n = 352	HERCEPTIN + Paclitaxel n = 92	Paclitaxel Alone n = 95	HERCEPTIN + AC n = 143	AC Alone n = 135
Body as a Whole					
Pain	47	61	62	57	42
Asthenia	42	62	57	54	55
Fever	36	49	23	56	34
Chills	32	41	4	35	11
Headache	26	36	28	44	31
Abdominal pain	22	34	22	23	18
Back pain	22	34	30	27	15
Infection	20	47	27	47	31
Flu syndrome	10	12	5	12	6
Accidental injury	6	13	3	9	4
Allergic reaction	3	8	2	4	2
Cardiovascular					
Tachycardia	5	12	4	10	5
Congestive heart failure	7	11	1	28	7
Digestive					
Nausea	33	51	9	76	77
Diarrhea	25	45	29	45	26
Vomiting	23	37	28	53	49
Nausea and vomiting	8	14	11	18	9
Anorexia	14	24	16	31	26
Heme & Lymphatic					
Anemia	4	14	9	36	26
Leukopenia	3	24	17	52	34
Metabolic					
Peripheral edema	10	22	20	20	17
Edema	8	10	8	11	5
Musculoskeletal					
Bone pain	7	24	18	7	7
Arthralgia	6	37	21	8	9
Nervous					
Insomnia	14	25	13	29	15
Dizziness	13	22	24	24	18
Paresthesia	9	48	39	17	11
Depression	6	12	13	20	12
Peripheral neuritis	2	23	16	2	2
Neuropathy	1	13	5	4	4
Respiratory					
Cough increased	26	41	22	43	29
Dyspnea	22	27	26	42	25
Rhinitis	14	22	5	22	16
Pharyngitis	12	22	14	30	18
Sinusitis	9	21	7	13	6
Skin					
Rash	18	38	18	27	17
Herpes simplex	2	12	3	7	9
Acne	2	11	3	3	<1
Urogenital					
Urinary tract infection	5	18	14	13	7

Other serious adverse events
The following other serious adverse events occurred in at least one of the 958 patients treated with HERCEPTIN® (Trastuzumab):

Body as a Whole: cellulitis, anaphylactoid reaction, ascites, hydrocephalus, radiation injury, deafness, amblyopia

Cardiovascular: vascular thrombosis, pericardial effusion, heart arrest, hypotension, syncope, hemorrhage, shock arrhythmia

Digestive: hepatic failure, gastroenteritis, hematemesis, ileus, intestinal obstruction, colitis, esophageal ulcer, stomatitis, pancreatitis, hepatitis

Endocrine: hypothyroidism

Hematological: pancytopenia, acute leukemia, coagulation disorder, lymphangiois

Metabolic: hypercalcemia, hypomagnesemia, hyponatremia, hypoglycemia, growth retardation, weight loss

Musculoskeletal: pathological fractures, bone necrosis, myopathy

Nervous: convulsion, ataxia, confusion, manic reaction

Respiratory: apnea, pneumothorax, asthma, hypoxia, laryngitis

Skin: herpes zoster, skin ulceration

Urogenital: hydronephrosis, kidney failure, cervical cancer, hematuria, hemorrhagic cystitis, pyelonephritis

OVERDOSAGE

There is no experience with overdosage in human clinical trials. Single doses higher than 800 mg have not been tested.

DOSAGE AND ADMINISTRATION

Usual Dose
The recommended initial loading dose is 4 mg/kg Trastuzumab administered as a 90-minute infusion. The recommended weekly maintenance dose is 2 mg/kg Trastuzumab and can be administered as a 30-minute infusion if the initial loading dose was well tolerated. HERCEPTIN may be administered in an outpatient setting. **DO NOT ADMINISTER AS AN IV PUSH OR BOLUS** (see ADMINISTRATION).

Preparation for Administration
Use appropriate aseptic technique. Each vial of HERCEPTIN should be reconstituted with 20mL of BWFI, USP, 1.1% benzyl alcohol preserved, as supplied, to yield a multi-dose solution containing 21 mg/mL Trastuzumab. Immediately upon reconstitution with BWFI, the vial of HERCEPTIN must be labeled in the area marked "Do not use after:" with the future date that is 28 days from the date of reconstitution.

If the patient has known hypersensitivity to benzyl alcohol, HERCEPTIN must be reconstituted with Sterile Water for Injection (see PRECAUTIONS). HERCEPTIN WHICH HAS BEEN RECONSTITUTED WITH SWFI MUST BE USED IMMEDIATELY AND ANY UNUSED PORTION DISCARDED. USE OF OTHER RECONSTITUTION DILUENTS SHOULD BE AVOIDED.

Determine the number of mg of Trastuzumab needed, based on a loading dose of 4 mg Trastuzumab/kg body weight or a maintenance dose of 2 mg Trastuzumab/kg body weight. Calculate the volume of 21 mg/mL Trastuzumab solution and withdraw this amount from the vial and add it to an infusion bag containing 250 mL of 0.9% sodium chloride, USP. **DEXTROSE (5%) SOLUTION SHOULD NOT BE USED.** Gently invert the bag to mix the solution. The reconstituted preparation results in a colorless to pale yellow transparent solution. Parenteral drug products should be inspected visually for particulates and discoloration prior to administration.

No incompatibilities between HERCEPTIN and polyvinylchloride or polyethylene bags have been observed.

Administration
Treatment may be administered in an outpatient setting by administration of a 4 mg/kg Trastuzumab loading dose by intravenous (IV) infusion over 90 minutes. **DO NOT ADMINISTER AS AN IV PUSH OR BOLUS.** Patients should be observed for fever and chills or other infusion-associated symptoms (see ADVERSE REACTIONS). If prior infusions are well tolerated, subsequent weekly doses of 2 mg/kg Trastuzumab may be administered over 30minutes.

HERCEPTIN should not be mixed or diluted with other drugs. HERCEPTIN infusions should not be administered or mixed with Dextrose solutions.

Stability and Storage
Vials of HERCEPTIN are stable at 2—8°C (36—46°F) prior to reconstitution. Do not use beyond the expiration date stamped on the vial. A vial of HERCEPTIN reconstituted with BWFI, as supplied, is stable for 28days after reconstitution when stored refrigerated at 2—8°C (36—46°F), and the solution is preserved for multiple use. Discard any remaining multi-dose reconstituted solution after 28days. If unpreserved SWFI (not supplied) is used, the reconstituted HERCEPTIN solution should be used immediately and any unused portion must be discarded. DO NOT FREEZE HERCEPTIN THAT HAS BEEN RECONSTITUTED.

The solution of HERCEPTIN for infusion diluted in polyvinylchloride or polyethylene bags containing 0.9% Sodium Chloride for Injection, USP, may be stored at 2—8°C (36—46°F) for up to 24 hours prior to use. Diluted HERCEPTIN has been shown to be stable for up to 24 hours at room temperature (2—25°C). However, since diluted HERCEPTIN contains no effective preservative, the reconstituted and diluted solution should be stored refrigerated (2—8°C).

HOW SUPPLIED

HERCEPTIN is supplied as a lyophilized, sterile powder containing 440mg Trastuzumab per vial under vacuum.

Each carton contains one vial of 440mg HERCEPTIN®(Trastuzumab) and one 30mL vial of Bacteriostatic Water for Injection, USP, 1.1% benzyl alcohol. NDC 50242-134-60.

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HERCEPTIN® (Trastuzumab)
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Page 5441

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First Named Inventor/Applicant Name:	Leonard BELL
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Non Patent Literature	AXJ-114BUSCN5_-_EX1027_P1pt1.pdf	23159831 7e5e1504342d9ce3b43de85807ad1bab159d8845	no	167

Warnings:

The page size in the PDF is too large. The pages should be 8.5 x 11 or A4. If this PDF is submitted, the pages will be resized upon entry into the Image File Wrapper and may affect subsequent processing					
Information:					
2	Non Patent Literature	AXJ-114BUSCN5_-_EX1027_P1pt2.pdf	15096388 a72153ccd49d088cb870a56331cfae7f94604ecc	no	167
Warnings:					
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3	Non Patent Literature	AXJ-114BUSCN5_-_EX1027_P1pt3.pdf	10709490 146d5995c8aae5798cdc0ffdc45545aae1528bb6	no	167
Warnings:					
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4	Non Patent Literature	AXJ-114BUSCN5_-_EX1027_P1pt4.pdf	12061051 596c9c1c76f1448d28a112971d37f033941e3afa	no	167
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5	Non Patent Literature	AXJ-114BUSCN5_-_EX1027_P1pt5.pdf	22903767 3c17bcfef1d0d08ac82179ca5d11c99d11da324c	no	167
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The page size in the PDF is too large. The pages should be 8.5 x 11 or A4. If this PDF is submitted, the pages will be resized upon entry into the Image File Wrapper and may affect subsequent processing					
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6	Non Patent Literature	AXJ-114BUSCN5_-_EX1027_P1pt6.pdf	18519669 e411ab332724b45fd82077fa68fed3c71484c780	no	167
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7	Non Patent Literature	AXJ-114BUSCN5_-_EX1027_P1pt7.pdf	10788338 7c6c14396b419dacbe9e13fc3ea74fbd7d2291c7	no	167
Warnings:					
The page size in the PDF is too large. The pages should be 8.5 x 11 or A4. If this PDF is submitted, the pages will be resized upon entry into the Image File Wrapper and may affect subsequent processing					
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8	Non Patent Literature	AXJ-114BUSCN5_-_EX1027_P1pt8.pdf	8045528 a7f876087c17957d03043f08ee02cc52c6afe817	no	167
Warnings:					
The page size in the PDF is too large. The pages should be 8.5 x 11 or A4. If this PDF is submitted, the pages will be resized upon entry into the Image File Wrapper and may affect subsequent processing					
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9	Non Patent Literature	AXJ-114BUSCN5_-_EX1027_P1pt9.pdf	9958663 17de8f7129b46856cc56ffbef8f0b5683f1e9261	no	167
Warnings:					
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10	Non Patent Literature	AXJ-114BUSCN5_-_EX1027_P1pt10.pdf	9352378 426a51fe630e90453d0d652fa9ad7c0affa72b3d	no	167
Warnings:					
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Information:					
11	Non Patent Literature	AXJ-114BUSCN5_-_EX1027_P1pt11.pdf	16001026 fd8965b4ba6963521762b8fcca4b7df49e14d0b9	no	167
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12	Non Patent Literature	AXJ-114BUSCN5_-_EX1027_P1pt12.pdf	13843417 91f9928e30ea5dca304b570e62622200d4a964b7	no	167
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13	Non Patent Literature	AXJ-114BUSCN5_-_EX1027_P1pt13.pdf	12960414 1b03da8623b06ec954166ce9128f8e226c0339a7	no	167
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14	Non Patent Literature	AXJ-114BUSCN5_-_EX1027_P1pt14.pdf	15262491 8a1dabc7a3528817 4e3d258088597e782a457683	no	167
Warnings:					
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Information:					
15	Non Patent Literature	AXJ-114BUSCN5_-_EX1027_P1pt15.pdf	16115448 25a2d56c78c0e5f1923a43c2e1a6d00c9f5c0059	no	167
Warnings:					
Information:					
16	Non Patent Literature	AXJ-114BUSCN5_-_EX1027_P1pt16.pdf	10456871 e700ba94afb17650fd01bd5fc1f90e6783b78e6a	no	180
Warnings:					
The page size in the PDF is too large. The pages should be 8.5 x 11 or A4. If this PDF is submitted, the pages will be resized upon entry into the Image File Wrapper and may affect subsequent processing					
Information:					
17	Non Patent Literature	AXJ-114BUSCN5_-_Herceptin.pdf	90390 1fbbe91d07b0c534431357d7726b012cd8e649e2	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	225325160	

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New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

Europäisches Patentamt
European Patent Office
Office européen des brevets

Notice of opposition to a European patent

I. Patent opposed

Patent No.	EP2359834
Application No.	EP11001632.6
Date of mention of the grant in the European Patent Bulletin (Art. 97(3), Art. 99(1) EPC)	09 November 2016
Title of the invention	Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement

II. Proprietor of the patent

first named in the patent specification	ALEXION PHARMACEUTICALS, INC.
Opponent's or representative's reference	AMG17203OP

III. Opponent

Name	Amgen Inc.
Address:	One Amgen Center Drive Thousand Oaks CA California 91320-1799 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

	Dörries, Mr H. Ulrich	
Company:	df-mp	
Address of place of business	Fünf Höfe Theatinerstraße 16 80333 Munich Germany	
Telephone/Fax	+49 89 2102960	+49 89 21029633
E-mail:	info@df-mp.com	

Multiple representatives (see additional sheet) ☐

Authorisation(s)

is/are enclosed ☐

has/have been registered under No. ☐

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:

IX. Evidence presented

A1	Patent document	EP2359834 (A1) original file name: A1_EP 2 359 834 A1.pdf attached as: Published-Evidence-3.pdf
D1	Patent document	US2005/0191298 (A1) particular relevance: Bell, Leonard et al.; September 1, 2005 original file name: D1.pdf attached as: Published-Evidence-18.pdf
D10	Non-patent literature - internet	U.S. Pharmacopeia, "Customized plastic packaging - applied to every need" , [cited 17.05.2017] Available from: [http://www.pharmacopeia.cn/v29240/usp29nf24s0_c1136.html] original file name: D10.pdf attached as: Published-Evidence-4.pdf
D11	Other evidence	Adis Data information, Drugs R. D. 2007, 8(1), 61-68 original file name: D11.pdf attached as: Other-evidence-1.pdf
D12	Non-patent literature - article	Hill, Anita et al., "Sustained response and long-term safety of eculizumab in paroxysmal nocturnal hemoglobinuria" Bloodjournal, Vol. 106, No. 7, 2007 particular relevance: 2559-2565 original file name: D12.pdf attached as: Published-Evidence-5.pdf
D13	Non-patent literature - article	Thomas, Thomas C. et al., "INHIBITION OF COMPLEMENT ACTIVITY VY HUMANIZED ANTI-C5 ANTIBODY AND SINGLE-CHAIN Fv." Moleculary Immunology, Vol. 33, No. 17/18, 1996 particular relevance: 1389-1401 original file name: D13.pdf attached as: Published-Evidence-6.pdf
D14	Non-patent literature - article	Mueller, John 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, Vol. 34, No. 6, 1997 particular relevance: 441-452 original file name: D14.pdf attached as: Published-Evidence-7.pdf
D15	Patent document	WO97/11971 (A1) particular relevance: Mueller, John et. al.; April 3, 1997 original file name: D15.pdf attached as: Published-Evidence-8.pdf
D16	Patent document	WO2005/007809 (A2) particular relevance: Rother, Russell et al., January 27, 2005 original file name: D16.pdf attached as: Published-Evidence-9.pdf
D17	Patent document	US6,355,245 (B1) particular relevance: Evans, Mark J. et al., March 12, 2002 original file name: D17.pdf attached as: Published-Evidence-10.pdf

D18	Non-patent literature - article	Hillmen, M.B., Ch.N., PhD., Peter et al., "The Complement Inhibitor Eculizumab in Paroxysmal Nocturnal Hemoglobinuria" THE NEW ENGLAND JOURNAL OF MEDICINE, 2006 original file name: D18.pdf attached as: Published-Evidence-11.pdf
D19	Other evidence	EMEA, CPMP, 20 February 2003, Note for guidance on excipients original file name: D19.pdf attached as: Other-evidence-2.pdf
D2	Non-patent literature - article	Hillmen, P. et al., "Oral Communication; Presidents Free Communications 257-280" Br. J. Hematol., Vol. 121, 2003 particular relevance: (Suppl. 1), 87-95 original file name: D2.pdf attached as: Published-Evidence-19.pdf
D20	Non-patent literature - article	Wei, Wang et al., "Anitbody Structure, Instability, and Formulation" Journal of Pharmaceutical Sciences, Vol. 96, No. 1, January 2007 original file name: D20.pdf attached as: Published-Evidence-12.pdf
D21	Non-patent literature - article	Daugherty, Ann L. et al., "Formulation and delivery issues for monoclonal antibody therapeutics" ScienceDirect - Advanced Drug Delivery Reviews, Vol. 58, May 2006 particular relevance: 686-706 original file name: D21.pdf attached as: Published-Evidence-13.pdf
D22	Patent document	US2005/0271660 (A1) particular relevance: Wang, Yi; December 8, 2005 original file name: D22.pdf attached as: Published-Evidence-14.pdf
D23	Other evidence	Expert declaration by Dr. Michael Treuheit; CV; Appendix A-C original file name: D23.pdf attached as: Other-evidence-3.pdf
D24	Other evidence	Soliris® (eculizumab) Highlights of prescribing information original file name: D24.pdf attached as: Other-evidence-4.pdf
D25	Patent document	US2012/0230982 (A1) particular relevance: Zhou, Xiao-Hong et al., September 13, 2012 original file name: D25.pdf attached as: Published-Evidence-15.pdf
D26	Patent document	WO2007/002571 (A2) particular relevance: Rother, January 4, 2007 original file name: D26.pdf attached as: Published-Evidence-16.pdf
D27	Non-patent literature - article	Hill, Anita et al., "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, Vol. 104, 2004 particular relevance: 2823-2824 original file name: D27.pdf

		attached as: Published-Evidence-17.pdf
D3	Non-patent literature - article	Hillmen, M.B., Ph.D., Peter et al., "Effect of Eculizumab on Hemolysis and Transfusion Requirements in Patients with Paroxysmal Nocturnal Hemoglobinuria" THE NEW ENGLAND JOURNAL OF MEDICINE, Vol. 350, No. 6, 2004 particular relevance: 552-559 original file name: D3.pdf attached as: Published-Evidence-20.pdf
D4	Non-patent literature - article	Harding et al., "Eculizumab - Huminazed Anti-C5 Monoclonal Antibody" Drugs of the Future, Vol. 29, No. 7, 2004 particular relevance: 673-676 original file name: D4.pdf attached as: Published-Evidence-21.pdf
D5	Patent document	US2005/0221382 (A1) particular relevance: Rother, P. Russell; October 6, 2005 original file name: D5.pdf attached as: Published-Evidence-22.pdf
D7	Non-patent literature - article	Morris et al., "Recurrence of neisserial meningococcemia due to deficiency of terminal complement component" Southern medical journal, Vol. 25, No. 19, 1992 ISSN: 1541-8243 particular relevance: 1030-1031 original file name: D7.pdf attached as: Published-Evidence-23.pdf
D8	Non-patent literature - article	Corvini MD, Michael et al., "Complement C7 deficiency presenting as recurrent aseptic meningitis" ANNALS OF ALLERGY, ASTHMA & IMMUNOLOGY, Vol. 93, No. 2, 2004 particular relevance: 200-205 original file name: D8.pdf attached as: Published-Evidence-24.pdf
D9	Non-patent literature - article	GVG, Sierra et al., "Vaccine Against Group B Neisseria Meningitidis: Protection Trial And Mass Caccination Results In Cuba" Niph Annals, Vol. 14, No. 2, 1991 ISSN: 0332-5652 original file name: D9.pdf attached as: Published-Evidence-25.pdf
P	Patent document	US2006/0783070 P (P) particular relevance: Bell, Leonard et. al.; March 15, 2006 original file name: P.pdf attached as: Published-Evidence-1.pdf
WO	Patent document	WO2007/106585 (A1) original file name: WO.pdf attached as: Published-Evidence-2.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
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Fees	Factor applied	Fee schedule	Amount to be paid
010 Opposition fee	1	785.00	785.00
Total:		EUR	785.00

	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	AMG17203OP_Notice of Opposition.pdf	OPPO.pdf

	C Attached evidence files	Original file name:	System file name:
C-1	1. Patent document	P.pdf	Published-Evidence-1.pdf
C-2	2. Patent document	WO.pdf	Published-Evidence-2.pdf
C-3	3. Patent document	A1_EP 2 359 834 A1.pdf	Published-Evidence-3.pdf
C-4	4. Patent document	D15.pdf	Published-Evidence-8.pdf
C-5	5. Patent document	D16.pdf	Published-Evidence-9.pdf
C-6	6. Patent document	D17.pdf	Published-Evidence-10.pdf
C-7	7. Patent document	D22.pdf	Published-Evidence-14.pdf

C-8	8. Patent document	D25.pdf	Published-Evidence-15.pdf
C-9	9. Patent document	D26.pdf	Published-Evidence-16.pdf
C-10	10. Patent document	D1.pdf	Published-Evidence-18.pdf
C-11	11. Patent document	D5.pdf	Published-Evidence-22.pdf
C-12	1. Non-patent literature - article	D12.pdf	Published-Evidence-5.pdf
C-13	2. Non-patent literature - article	D13.pdf	Published-Evidence-6.pdf
C-14	3. Non-patent literature - article	D14.pdf	Published-Evidence-7.pdf
C-15	4. Non-patent literature - article	D18.pdf	Published-Evidence-11.pdf
C-16	5. Non-patent literature - article	D20.pdf	Published-Evidence-12.pdf
C-17	6. Non-patent literature - article	D21.pdf	Published-Evidence-13.pdf
C-18	7. Non-patent literature - article	D27.pdf	Published-Evidence-17.pdf
C-19	8. Non-patent literature - article	D2.pdf	Published-Evidence-19.pdf
C-20	9. Non-patent literature - article	D3.pdf	Published-Evidence-20.pdf
C-21	10. Non-patent literature - article	D4.pdf	Published-Evidence-21.pdf
C-22	11. Non-patent literature - article	D7.pdf	Published-Evidence-23.pdf
C-23	12. Non-patent literature - article	D8.pdf	Published-Evidence-24.pdf
C-24	13. Non-patent literature - article	D9.pdf	Published-Evidence-25.pdf
C-25	14. Non-patent literature - internet	D10.pdf	Published-Evidence-4.pdf
C-26	1. Other evidence	D11.pdf	Other-evidence-1.pdf
C-27	2. Other evidence	D19.pdf	Other-evidence-2.pdf
C-28	3. Other evidence	D24.pdf	Other-evidence-4.pdf
C-29	4. Other evidence	D23.pdf	Other-evidence-3.pdf

Signature of opponent or representative

Place	**Munich, Germany**
Date:	**04 August 2017**
Signed by:	**Hans Doerries 13689**
Representative name:	**H. Ulrich Dörries**
Capacity:	**(Representative)**

dfmp
Dörries Frank-Molnia & Pohlman
Patentanwälte Rechtsanwälte PartG mbB

Patents – Trademarks – Designs

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European Patent Office
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80469 Munich

August 4, 2017

Opposition against European patent 2 359 834 (Application No. 11 001 632.6)

Title:	Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement
Proprietor:	Alexion Pharmaceuticals, Inc.
Opponent:	Amgen Inc.
Our Ref.:	AMG17203OP

On behalf of Amgen Inc.
One Amgen Center Drive
Thousand Oaks, CA 91320-1799
USA

we herewith file a **Notice of Opposition** pursuant to Article 99(1) EPC against

European Patent No. 2 359 834 B1
(European Appl. No. 11 001 632.6)

Publication and mention of the grant: 09.11.2016

Title of the patent: Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement

Proprietor: Alexion Pharmaceuticals Inc.
100 College Street
New Haven, CT 06510 / US

We request that the opposition fee in the amount of EUR 785.00 be debited from our deposit account No. 28001000 (see also the corresponding debit order on the form accompanying the online submission of the present Notice of Opposition).

H. Ulrich Dörries [1234]
Dr. sc. nat., Dipl.-Biol.

David Molnia [12347]
M.S. Elec. Eng., B.S. Physics

Sandra Pohlman [568]
B.S. Biology

Michael Eder [1234]
Dr. sc. nat., Dipl.-Chem.

Oliver Schulz [1234]
Dipl.-Phys.

Rainer Friedrich [1234]
Dr. rer. nat., Dipl.-Biochem.

Elisabeth Greiner [1234]
Dr. rer. nat., Mag. pharm.

Philipp Nordmeyer [1234]
LL.M., Dipl.-Phys.

Dominik Ho [1234]
Dr. rer. nat., MEng (Cornell)

Holger Schimmel [1234]
Dr. rer. nat., Dipl.-Ing.

Annette Hilgendorf [1234]
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Solveig Moré [1234]
Dr. rer. nat., Dipl.-Biochem.

Florian Brandt [1234]
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Andreas Brzank [1234]
Dr. rer. nat., Dipl.-Phys.

René Okoampah [1234]
Dipl.-Ing.

Julia Mössinger [1234]
Dr. rer. nat., Dipl.-Biol.

Judith Hesse [8]
LL.M.

Helena Bertelsmeier [8]
LL.M.

Barbara Englert [234]
Dr. rer. nat., Dipl.-Phys.

1 European Patent Attorney
2 European Trademark Attorney
3 European Design Attorney
4 Patentanwalt
5 Solicitor (England & Wales)
6 U.S. Patent Attorney
7 U.S. Patent Agent
8 Rechtsanwalt

Partnerschaftsregister des AG München
PR-Nr. 1278

I. Extent of the Opposition and Requests

1. The opposition is directed against the (sole) claim of European patent 2 359 834 B1 (hereinafter referred to as "the opposed patent"). It is requested that the opposed patent be revoked in its entirety pursuant to Article 101(2) EPC.

2. Oral Proceedings are requested in accordance with Article 116(1) EPC in the event that the Opposition Division does not consider the above request justified on the basis of our written submissions.

II. Grounds in Support of the Opposition

3. The opposition is based on the following grounds:

   i) The claimed subject matter of the opposed patent extends beyond the content of the earlier application as filed (Art. 100(c) EPC in connection with Art. 76(1) EPC, 123(2) EPC).
   ii) The subject matter of the opposed patent is not based on an inventive step (Art. 100(a) EPC in connection with Art. 56 EPC).
   iii) The subject matter of the opposed patent is insufficiently disclosed (Art. 100(b) EPC).

III. List of Documents

4. The following documents were cited during the prosecution of the patent EP 2 359 834 B1, hereinafter "the opposed patent"; they are enclosed herewith except for **D6** which we were unable to retrieve:

D1 US 2005/191298 A1 (Bell, Leonard *et al.*; September 1, 2005).

D2 Hillmen *et al.*, *Br. J. Hematol.* **2003**, *121 (Suppl. 1)*, 87-95.

D3 Hillmen *et al.*, *N. Engl. J. Med.* **2004**, *350(6)*, 552-559.

D4 Harding, *Drugs Future* **2004**, *29(7)*, 673-676.

D5 US 2005/221382 A1 (Rother, P. Russell; October 6, 2005)

D6 Hill, *Clin. Adv. Hematol. Oncol.* **2005**, *3(11)*, 849-50.

D7 Morris et al., *South. Med. J.* **1992**, *85(19)*, 1030-1031.

D8 Corvini *et al.*, *Ann. Allerg. Asth. Immunol.* **2004**, *93(2)*, 200-205.

D9 Sierra *et al.*, *NIPH Annals* **1991**, *14(2)*, 195-210.

5. The following additional documents are also referred to herein and submitted herewith:

P US 2006/0783070 P (Bell, Leonard *et al.*; March 15, 2006).

WO WO 2007/106585 A1.

A1 EP 2 359 834 A1.

D10 US Pharmacopeia (USP29-NF24), p. 2989;
retrieved from http://www.pharmacopeia.cn/v29240/usp29nf24s0_c1136.html
on May 17, 2017.

D11 Adis Data information, *Drugs R. D.* **2007**, *8(1)*, 61-68.

D12 Hill *et al.*, *Blood* **2005**, *106(7)*, 2559-2565.

D13 Thomas *et al.*, *Mol. Immunol.* **1996**, *33(17/18)*, 1389-1401.

D14 Mueller *et al.*, *Mol. Immunol.* **1997**, *34(6)*, 441-452.

D15 WO 97/11971 A1 (Mueller, John *et al.*, April 3, 1997).

D16 WO 2005/007809 A2 (Rother, Russell *et al.*, January 27, 2005).

D17 US 6,355,245 B1 (Evans, Mark J. *et al.*, March 12, 2002).

D18 Hillmen *et al.*, *N. Engl. J. Med.* **2006**, *355(12)*, 1233-1243.

D19 EMEA, CPMP, 20 February 2003, *Note for guidance on excipients, antioxidants and antimicrobial preservatives in the dossier for application for marketing authorization of a medicinal product*.

D20 Wang *et al.*, *J. Pharm. Sci.* **2007**, *96(1)*, 1-26. (published January 2007).

D21 Daugherty *et al.*, *Adv. Drug Del. Rev.* **2006**, *58*, 686-706.

D22 US 2005/0271660 A1 (Wang, Yi; December 8, 2005).

D23 Expert declaration by Dr. Michael Treuheit.

Samsung Bioepis Exhibit No. 1002
Page 5457

D24 Soliris® (eculizumab) *Highlights of prescribing information*.

D25 US 2012/0230982 A1 (Zhou, Xiao-Hong *et al.*, September 13, 2012).

D26 WO 2007/002571 A2 (Rother, January 4, 2007).

D27 Hill *et al.*, *Blood* **2004**, *104*;2823-2824.

IV. The Claimed Subject Matter

6. The opposed patent claims a pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein the composition is a 300 mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ml sterile, preservative free solution.

V. Inadmissible Amendment (Art. 100 (c) EPC / Art. 76 (1) EPC, 123(2) EPC)

7. The parent application as filed (**WO**), only teaches a pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein the composition is a 300 mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ml sterile, preservative free solution in the context of a *single unit dosage form*, both on page 5, lines 3-9 and page 22, lines 7-14.

8. The same applies for the corresponding passages of the divisional application **A1**, which also require a single unit dosage form (paragraphs [0018] and [0024)]. This understanding is confirmed by claim 1 of **A1**, which recites: *"[a] pharmaceutical composition comprising* ***a single unit dosage form*** *of an anti-C5 antibody"* (emphasis added).

9. It is noted in this context that the skilled person would understand a single-use dosage form to be for single use only, i.e., it cannot be used more than once. In contrast, a single unit dosage form would be understood to be synonymous to a single-unit container, which, as evidenced by the US Pharmacopeia (submitted as **D10** herewith), "*is designed to hold a quantity of drug product intended for administration as a single dose or a single finished device intended for use promptly after the container is opened*".

10. As an essential element present in the parent application as filed as well as the divisional application as filed, namely, the requirement for administration of the 300 mg dose as a single dose is missing from the claim as granted, the claim is not clearly and unambiguously derivable from the application as filed, contrary to Art. 76(1) EPC.

VI. No Entitlement to Priority

11. The opposed application claims priority of US provisional application US 783070 P (**P**). Said application does not contain any disclosure of a pharmaceutical composition comprising 300 mg of eculizumab in a volume of 30 mL, at a concentration of 10 mg/mL in a single-use dosage form of a sterile, preservative-free solution. None of these specific details, let alone their combination, is disclosed in **P**.

12. The claim of the opposed patent thus is not entitled to priority. Consequently, all documents available before the **filing date of March 15, 2007** are considered relevant state of the art.

VII. Lack of Inventive Step (Article 100(a) EPC / Article 56 EPC)

VII.1 The state of the art

13. Eculizumab, also referred to as h5G1.1 (**D11**, abstract; **D4**, abstract; **D1**, [0052]); opposed patent [0054]), is a long-acting, humanized version of the anti-C5 antibody 5G1.1 that has been under development as a therapeutic for a range of different indications since 1995 (**D11**). Indications known before the filing date of the opposed patent include rheumatic disorders, transplant rejection, adult respiratory distress syndrome, lupus nephritis and systemic lupus erythematosus, glomerulonephritis, psoriasis, dermatomyositis, pulmonary diseases, sickle cell disease, and also paroxysmal hemoglobinuria (PNH) (**D11**, Table II; **D4**, abstract and "Clinical Studies" on pages 674-675; **D22**, claims 1, 2, and 7; **D26**, e.g., claims 1 and 14; **D5** abstract, par. [0027] and [0040]).

14. The complement cascade is a part of the human immune system that binds and removes foreign particles, but can also attack and destroy endogenous cells (such as red blood cells) or tissue. The antibody eculizumab has been developed to inhibit the terminal steps of the complement cascade, in particular, eculizumab suppresses the cleavage of complement component 5 (or C5) into C5a and C5b (**D12**, p. 2559, right column, **D4**, page 673, right column, 2nd full paragraph).

15. The opposed patent at [0054] states that eculizumab is disclosed in **D17**. The variable heavy and light chain regions of eculizumab/h5G1.1 were disclosed in **D17** in the context of antibody fragments, such as an scFv (**D17**, SEQ ID NO: 20, "5G1.1 scFv CO12 (humanized)"). This document states that preferred constant regions for forming full length antibodies would be "IgG constant regions, which may be unaltered or constructed of a mixture of constant domains from IgGs of various subtypes, e.g., IgG1 and IgG4." (**D17**, col. 45, li. 24-33).

16. The first humanized 5G1.1 antibody (h5G1.1) was published in 1996 (**D13**, p. 1392, Fig. 1 A and B).

17. An advantageous combination of h5G1.1, and in particular h5G1.1 CO12, with chimeric human IgG2/IgG4 heavy chain constant regions is shown and/or suggested in **D14, D15** and **D16**: **D14** shows optimization of the antibody by the introduction of a chimeric human IgG2/IgG4 heavy chain constant region (**D14**, Fig. 7, Fig. 9, and paragraph bridging pages 450/451 and 2nd paragraph on p. 451; see also **D15**, e.g., p. 42-43; and **D16**, Fig. 2 and claim 3), which was shown to advantageously avoid undesired immune responses, FcR binding and complement activation (**D14**, paragraph bridging pages 450/451; **D15**, Fig. 15 and p. 12, li. 37; **D16,** Fig. 2, paragraph bridging pages 5 and 6).

18. The mechanism of action of eculizumab and preferred antibody structures were thus known prior to the filing date of the opposed patent, and eculizumab had been used as a therapeutic agent for a number of indications aside from PNH.

19. PNH is a hematologic disorder, symptomatically characterized by red blood cell destruction, aenemia, hemoglobinuria and thrombosis. The symptoms result from somatically mutated hematopoietic blood cells, causing a deficiency of glycosylphosphatidylinositol (GPI) anchored cell surface proteins. Among these proteins are complement inhibitors that normally protect red blood cells from complement-mediated lysis (**D12**).

20. The treatment of patients suffering from PNH with anti-C5 antibodies, such as eculizumab or pexelizumab was also widely known before the filing date. In the case of PNH, the blockade of the terminal steps of the complement cascade by eculizumab serves to block the undesired destruction of red blood cells; however, it preserves the ability of the complement system to clear microorganisms and immune complexes (**D12**, p. 2559, right column).

21. A treatment with eculizumab in a dosage of 600 mg over the first four weeks, followed by an administration of 900 mg as the fifth dose one week later, and subsequent administration of 900 mg on a bi-weekly basis is disclosed, e.g., in **D1** (par. [0082]). The route of administration was intravenous infusion (ibid.). This corresponds exactly to the dosage regimen described in the opposed patent ([0057]).

22. Further successful clinical trials of eculizumab are described in detail, in e.g. **D2** (in 2003), **D3** (in 2004), **D4** (in 2004), **D12** (in 2005), **D18** (in 2006) and **D11** (in 2007). **D27** (from 2004, abstract) teaches that treatment of PNH by eculizumab has been shown to be safe and effective for over two years of therapy. Dosage regimens requiring administration of both 600 and 900 mg eculizumab for treatment of PNH are explicitly disclosed in **D2** (li. 7 and 8), **D3** (Abstract), **D4** (page 674, last full paragraph), **D11** (last paragraph on page 66) and **D18** (page 1233, Methods), so they can be considered to be conventional.

23. **D18** in fact discloses the clinical trial that serves as the sole example in the opposed patent (the TRIUMPH trial). This includes the vaccination of all treated patients against *Neisseria meningitides* (**D18**, p. 1234, right column, 1st full paragraph), a feature that was brought forward by the applicant in the course of the prosecution in an attempt to distinguish the alleged invention from the state of the art, and later dropped again. The vaccination serves to

avoid side effects of the inhibition of complement formation in the treated patients leading to a higher susceptibility to *Neisseria* infections. This problem and its solution were well known in the art, as pointed out by the examiner with reference to **D6**, **D7** and **D8**.

24. The skilled person was consequently aware of suitable formulations for the intravenous administration of eculizumab, and these had already been successfully used in several clinical trials for PNH, as well as for treatment of numerous other diseases.

VII.2 *The closest prior art*

25. **D1** – or any of the reports of intravenous treatment of PNH with eculizumab at doses of 600 or 900 mg/ml, i.e., **D2** to **D4**, **D11** and **D18** - can be considered the closest prior art document, as all of them are highly promising springboards for the claimed subject matter.

26. We submit that it is an implicit feature of all compositions for intravenous administration that they are sterile. **D1** not only discloses the treatment of PNH with eculizumab and dosing regimen, in particular doses of 600 and 900 mg in par. [0082], as mentioned above, but also explicitly teaches that suitable 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."* (**D1**, par. [0062], emphasis added)

D1 thus also teaches that in contrast to the strict requirement for sterility, the presence of preservatives is optional. We thus suggest **D1** as the closest prior art document.

VII.3 *The subject matter of the claim*

27. The opposed patent claims a pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein the composition is a 300 mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ml sterile, preservative free solution.

28. **D1** does not explicitly teach that eculizumab is in a single-use dosage form of 300 mg comprising 30 ml of a concentration of 10 mg/ml, or that the composition is preservative free. It is noted that the amount of antibody and the concentration thereof define the volume of the composition, which consequently cannot be considered an independent technical feature.

Samsung Bioepis Exhibit No. 1002
Page 5461

VII.4 The technical effect and the problem solved

29. Notably, the opposed patent is silent about supposed technical effects or advantages of any one of these features.

30. As the treatment of patients suffering from PNH with eculizumab was well known, and a suitable pharmaceutical formulation for the antibody thus clearly already existed prior to the filing of the opposed patent, it is not apparent which technical problem is solved by the alleged invention.

31. Submissions by the applicant in the communications dated February 6, 2016 (p. 6, item 3.2) and January 13, 2016 (p. 5, item 4.3.1) imply that the technical problem to be solved was the provision of a stable and easy-to-use solution of eculizumab. This is nowhere mentioned in the opposed patent itself. The only evidence brought forward by the applicant with regard to the alleged technical effect is the package leaflet of Soliris® (eculizumab). This is supposed to prove that the marketed concentration is in fact the "*ideal and clinically approved*" concentration for the antibody (communication dated January 13, 2016, item 4.3.1). While it obviously is the clinically approved concentration, this does not represent evidence for 10 mg/mL being the "ideal" concentration. It merely shows that formulating eculizumab at 10 mg/mL is feasible. Accordingly, there is no evidence for an allegedly ideal increased stability.

32. **D1** does not teach if the administered composition comprises preservatives.

33. **D19**, a note for guidance to industry from EMEA, which can be considered to represent common general knowledge, states that the properties of antioxidants and antimicrobial preservatives are due to certain chemical groups which are usually aggressive towards living cells and which lead to certain risks when used in man. Thus, if it is not absolutely necessary to add these substances in medicinal products, they must be avoided (**D19**, page 1/9, paragraphs 4-6 of introduction).

34. Accordingly, the fact that the claimed compositions do not contain preservatives has the technical effect that potential issues connected to the use of preservatives, both from the medical and the regulatory viewpoint, are avoided.

35. Consequently, the problem solved by the invention can be considered to be provision of a composition which avoids potential medical and/or the regulatory issues connected to the use of preservatives.

Samsung Bioepis Exhibit No. 1002
Page 5462

VII.5 *The subject matter of the claim lacks inventive step in light of **D1** in combination with **D22** and common general knowledge, represented by **D19** and **D20-D21**.*

36. It has to be noted that the opposed patent teaches no advantageous effect of not using **preservatives**. In contrast, it states that among other excipients, preservatives can be present in the pharmaceutical compositions of the alleged invention (par. [0058] and [0064]).

37. Nevertheless, the skilled person who intends to produce eculizumab compositions for therapeutic application, as in **D1** (or in the opposed patent), is motivated to exclude preservatives for regulatory reasons. The German Bundesgerichtshof in "Anthocyanverbindung" (X ZR 4/11, 10.12.2013) recently confirmed that legal (including regulatory) considerations are to be taken into account by the skilled person:

> "*The skilled person to which provision of a compound for a certain application is assigned has occasion to check according to the relevant legal provisions which solutions have adequate prospects of success with regard to legal aspects*" (headnote; our translation).

38. **D19**, as cited above, not only provides such a relevant legal provision, i.e., a motivation to avoid preservatives (**D19**, page 1/9, paragraphs 4-6 of introduction), but also teaches that multi-dose preparations are most problematic with regard to the risk of contamination by micro-organisms (pages 8-9/9, section "Antimicrobial Preservatives", In the last sentence on page 9/9, the document clearly teaches:

> "*If products do not contain a preservative and do not have inherent preservative efficacy they must not be packaged in multidose presentations without a sound justification.*"

39. Accordingly, **D19**, which we submit is representative of common general knowledge, can be summarized to teach away from the use of preservatives and, accordingly, multidose presentations. Following this clear teaching, the skilled person would turn to **single-use** packaging to avoid the requirement for preservatives.

40. The skilled person would also not have expected any difficulties with this approach, as it was well known to the person of skill that antibody formulations for intravenous delivery can be prepared without using preservatives, as **D20** (p. 2-4, Table 1) and **D21** (p. 697, Table 1) show. In fact, the majority of antibody formulations reviewed in these documents do not contain preservatives. In addition, **D22** specifically discloses a formulation of eculizumab that does not contain preservatives (**D22**, Fig. 10). As a consequence, the person of skill would have readily assumed, based on the state of the art, that a single-use pharmaceutical formulation comprising eculizumab would not require the use of preservatives.

41. The skilled person was consequently faced with the selection of an appropriate **amount of antibody** to include in the single-use package.

42. As discussed above, e.g., **D1** (par. [0082]) teaches that the administration of either 600 mg, or 900 mg is the established dosing regimen for eculizumab. In full agreement with the examiner (communication dated July 21, 2011), we note that 300 mg is the obvious and most convenient single dose that enables supplying both therapeutic doses of 600 and 900 mg, while avoiding a waste of active agent. We note that the applicant did not argue that there is any unexpected technical effect or advantage associated with this feature. The amount of antibody included is thus rendered obvious by **D1** itself.

43. This leaves the antibody **concentration of 10 mg/mL** as a distinguishing feature. The applicant in his communications dated February 6, 2014 and January 13, 2016 alleged that the concentration was an inventive feature:

> *"At a minimum, in order to arrive at the instantly-claimed dosage forms at all, the inventors had to know that eculizumab was soluble. In order to produce a solution of an antibody (e.g., at 10 mg/mL), the inventors had to know the solubility of eculizumab and that eculizumab was sufficiently soluble for a 10 mg/mL formulation. Moreover, in order for a pharmaceutical solution to be commercially viable, the inventors had to know the shelf-life stability of eculizumab in solution; for if its stability was too low, a pharmaceutical solution would not have been an option. Thus, even assuming arguendo that the instantly claimed compositions are convenient, it is clear that the skilled person reading the cited references could not have arrived at the particular instantly claimed compositions by convenience. They would have required the knowledge supporting the instant invention. And none of that knowledge is disclosed or even suggested by the cited references."* (Communication submitted by applicant on February 6, 2014, p. 6, item 3.2, emphasis added)

and

> *"As indicated in section 3.2 of our petition of February 6, 2014 it was to be established at which concentration eculizumab can be solved and it was found that 10 mg eculizumab can solved in 1 ml of a sterile, preservative free solution. In addition it was to be determined whether eculizumab is storage-stable at a concentration of 10 mg/ml."* (Communication submitted by applicant on January 13, 2016, p. 5, item 4.3.1, emphasis added)

44. In contrast, we submit that the concentration of 10 mg/ml is an arbitrary choice made by the skilled person in the context of routine experimentation. Determining solubility and stability furthermore is part of routine experimentation in protein formulation and does not involve any inventive activity.

45. In a highly similar case, concerned with liquid stable protein formulations (**T 1241/03**), the Board of Appeals ruled that despite the understandable uncertainties, which are associated

with developing liquid stable protein formulations, obviousness is not only at hand when the results are clearly predictable, but also when there is a reasonable expectation of success (**T 149/93**). This does not require certainty (**T 338/97**). Even in the absence of a reasonable expectation of success, the person of skill would adopt a "try and see" attitude (**T 333/97**).

46. **D5**, a document concerned with use of eculizumab for treatment of rheumatoid arthritis, filed by a co-inventor of the opposed patent, already teaches that possible formulations for complement inhibiting antibodies such as 5G1.1-mAb (claim 4), including those for intravenous infusion, that *"may conveniently be presented in unit-dosage form [and] may be prepared according to conventional techniques well known in the pharmaceutical industry"* (**D5**, par. [0043]). Paragraph [0046] similarly refers the skilled person to guidance in the literature and common general knowledge regarding particular dosages and methods of delivery. This confirms that the skilled person did not consider formulation of eculizumab as requiring anything more than common general knowledge and routine experimentation.

47. We agree with **D5** that there certainly was guidance in the prior art for comparable antibody formulations, e.g., **D20**, **D21** and **D22,** so that the skilled person had good reasons to assume that 10 mg/mL would represent a suitable concentration. The review articles **D20** (p. 2-4, Table 1) and **D21** (p. 697, Table 1) show that it was common general knowledge that 10 mg/mL was a common concentration for antibody formulations for intravenous delivery. The formulations listed in these documents comprise antibody concentrations ranging from 0.25 mg/mL to 100 mg/mL for intravenous (IV) delivery. **D21** lists 15 different antibody formulations for IV delivery (Table 1), wherein 10 mg/mL is one of the three most common. **D20** lists 18 formulations for IV delivery, of which 10 mg/mL is the most common (Table 1, entries 3, 14, 16 and 20).

48. At the same time, **D22** indicates that the very same antibody as presently claimed, eculizumab, can be formulated in concentrations ranging from 1-30 mg/mL (**D22**, Fig. 10 and par. [0170] to [0172]). A formulation of eculizumab at 30 mg/mL is shown to be suitable for nebulization. While **D22** is concerned with antibody formulations for a different route of delivery, this does not affect the need for both stability and solubility of the antibody. Based on **D22**, there would thus have been no uncertainties left for the skilled person that 10 mg/mL were stable and soluble, and thus a suitable concentration. At least, the formulation disclosed in said document would have provided the skilled person with a reasonable expectation of success when formulating eculizumab at 10 mg/mL.

49. We furthermore submit that the specific concentration of 10 mg/mL is an arbitrary choice, and, consequently, this feature cannot contribute to inventiveness, as established in **T 206/91** (item 5.5) and **T 37/82** (item 3). We herewith provide the results of a study of the solubility and the long-term stability of eculizumab at various concentrations in the form of a declaration submitted as **D23**.

50. The eculizumab used in the study described in **D23** was recombinantly expressed. The sequence of the heavy and light chains were confirmed to be correct by comparison to peptide

mapping of commercially acquired eculizumab in Soliris® and to the sequences provided within publically available documents the patentee filed with government agencies.

51. For the analysis, the antibody was dissolved in phosphate buffered saline (pH = 7), supplemented with polysorbate 80. These buffer conditions are identical to those used for the pharmaceutical composition of the opposed patent (see prescribing information for Soliris® (eculizumab), **D24**). The stability of solutions containing 2 mg/ml, 5 mg/ml, 10 mg/mL, 30 mg/mL and 50 mg/mL was monitored over a time course of 13 weeks at storage conditions (4 °C) and at elevated temperature (25 °C). Physical and chemical stability were analyzed by size-exclusion ultra high-performance liquid chromatography (SE-UHPLC), anion exchange high-performance liquid chromatography (AEX-HPLC), and reduce sodium dodecyl sulfate capillary electrophoresis (rCE-SDS).

52. Aggregation of the antibody results in the formation of high molecular weight (HMW) species that can be visualized by SE-UHPLC. A large degree of HMW formation is a sign of low long-term stability. All of the eculizumab solutions stored at 4°C and 25°C were sufficiently stable throughout the study, i.e., there was no significant loss in main peak for any of the protein concentrations examined. (**D23**, Appendix A)

53. The storage of antibodies in solution can result in chemical degradation, leading to the formation of acidic species (e.g. by deamidation of asparagine residues), which can be detected by AEX-HPLC. A high degree of acidic species is a sign of low chemical stability. Analysis of the eculizumab solutions (**D23**, Appendix B) shows that all tested samples suffer from some chemical degradation under the tested conditions and the degradation increases over time upon storage at the elevated temperature. There is no significant difference in chemical stability between 10 mg/mL and the other concentrations.

54. rCE-SDS offers high resolution separation of most types of chemically or structurally altered species from the main (intact) species for both heavy chain (HC) and light chain (LC) of the antibody. Analysis of the eculizumab solutions by rCE-SDS (**D23**, Appendix C) shows that a detectable decrease of the main species (HC+LC) occurs at both tested temperatures for all samples. However, in agreement with the AEX-HPLC measurements, there is no significant difference across protein concentrations.

55. Taken together, this shows that solutions of eculizumab possess similar long-term stability at concentrations of 2 mg/ml, 5 mg/ml, 10 mg/mL, 30 mg/mL or 50 mg/mL. The 10 mg/mL concentration offers no apparent advantage over the other tested concentrations.

56. Document **D25** also discloses formulations of anti-C5 antibodies and specifically eculizumab (**D25**, e.g., par. [0019], [0030], [0042], [0074], Fig. 1) at concentrations ranging from 10 mg/mL to more than 200 mg/mL (**D25**, e.g., par. [0061], [0062], [0076]). Formulations of eculizumab at various concentrations were found to be stable over a period of two years (**D25**, par. [0304]). While **D25** was published after the filing date of the opposed patent, it confirms that eculizumab can be stably formulated in various buffers in a wide range of concentrations.

Samsung Bioepis Exhibit No. 1002
Page 5466

57. In summary, in light of **D1**,

- the skilled person was motivated to formulate eculizumab for treatment of PNH as a single-use dosage form avoiding the use of preservatives at least due to regulatory aspects and common general knowledge (as evidenced by **D19**);
- the amount of antibody of 300 mg included in the single-use dosage form is rendered obvious by **D1** itself;
- it was common general knowledge (**D20**, **D21**) that 10 mg/mL is a conventional concentration for antibody formulations for intravenous delivery; and there was a reasonable expectation of success for this concentration of eculizumab to be stable and soluble in light of **D22**; furthermore, as confirmed by **D25** and **D23**, the concentration of 10 mg/mL was an arbitrary choice.

58. Accordingly, the claimed subject matter lacks an inventive step in light of **D1** in combination with **D22** and common general knowledge (**D19**, **D20**, **D21**), contrary to Article 56 EPC.

VIII. Insufficient Disclosure (Article 100(b) EPC)

59. The opposed patent claims a pharmaceutical composition, wherein the composition is a solution of "eculizumab". While we hold that eculizumab is clearly and unambiguously disclosed by the cited prior art (e.g., **D1** to **D5**, **D11** to **D15**, **D17**, **D18**, **D22**, **D26**, **D27**), we submit that the skilled person may be confused in this regard by the information contained in the opposed patent, to the extent that the skilled person may no longer be able to carry out the teaching of the patent. In particular, the description offers divergent definitions of the antibody eculizumab and fails to provide a correct sequence of the light chain of the antibody.

60. First, SEQ ID NOs 1 to 4 of the opposed patent supposedly define the variable heavy chain (SEQ ID NO: 1) and light chain (SEQ ID NO: 3) segments and the full length heavy chain (SEQ ID NO: 2) and light chain (SEQ ID NO: 4) of eculizumab. We note that the sequence given for the full length light chain of eculizumab is not identical to the sequence of eculizumab in the post-published document **D25** (par. [0094]), which has a length of 214 amino acids. This length of 214 amino acids is in accordance with the prescribing information for Soliris® (**D24**, item 11). To the contrary, SEQ ID NO: 4 of the opposed patent contains 22 additional N-terminal amino acids and has a length of 236 amino acids.

61. It may be concluded that the antibody described in the opposed patent, comprising heavy chains with a sequence of SEQ ID NO: 2 and light chains with a sequence of SEQ ID NO: 4 is in fact not eculizumab.

62. In conclusion, the skilled person seeking to reproduce the alleged invention would be misguided by the opposed patent into using an antibody that may not be eculizumab. Use of the antibody eculizumab for treatment of PNH can thus be considered insufficiently disclosed by the opposed patent.

63. The claim of the opposed patent violates Article 83 EPC.

IX. Conclusion

64. As the subject matter of the claim of the opposed patent

- is not clearly and unambiguously derivable form the parent application as filed, contrary to Art. 76(1) EPC and Art. 123(2) EPC,
- lacks inventive step over **D1** in combination with **D22** and common general knowledge, as evidenced by **D19** and **D20**-**D21**,
- is insufficiently disclosed,

revocation of European patent EP 2 359 834 B1 in its entirety is justified.

Respectfully submitted,

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UD/MO/SI/KS

Enclosures
Documents P, WO, A1, D1-D5 to D7-D27

Samsung Bioepis Exhibit No. 1002
Page 5468

WEDNESDAY, 9 APRIL 2003

Oral Communication

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257

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.

258

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 *in vitro*, 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.

259

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

Samsung Bioepis Exhibit No. 1002
Page 5469

high shear, it inhibits platelet adhesion, aggregation and activation, and in a dog model, inhibits arterial thrombus formation, without prolonging the bleeding time. In a randomized, double blind study, placebo, 0.01, 0.03, or 0.05 mg/kg AJW200, were infused i.v., into 24 healthy men. No clinically significant adverse events were recorded and there was no evidence of immunogenicity. Mean PK results were: C_{max} 204.8, 586.2, 833.2 ng/ml; T_{max} 1.0, 0.92, 0.57 h, T/2 23.5, 24.3, 27.2 h, for ascending doses. The maximum vWF occupancy by AJW200 was 19.4, 51.0 and 62.4%, showing correlation with plasma AJW200 concentration. Ristocetin cofactor (RiCof) was reduced 1 h post-infusion compared to baseline: mean (SD) 58 (22) and 110 (25)%, $P < 0.0005$ and 34 (16) and 116 (41)%, $P < 0.005$, at 0.03 and 0.05 mg/kg respectively, for up to 12 h. vWF : Ag, FVIII, vWF multimers and cleaving protease activity were unchanged. Template bleeding time (BT) was normal at all AJW200 doses, but the PFA-100 showed prolonged closure times 1 h post-infusion, with both CADP: 146 (22) and 82 (10) and 202 (78) and 80 (19) s, $P < 0.01$; and CEPI cartridges: 182 (28) and 108 (16) and 233 (56) and 107 (19) s, $P < 0.01$, at 0.03 and 0.05 mg/kg AJW200, respectively. This effect lasted for 3–6 h with the lower and up to 12 h for the higher dose. The RiCof and PFA-100 responses showed dose dependent inhibition and the magnitude of the effect appeared to be related to the baseline vWF level. AJW200 showed inhibition of vWF : RiCof activity and PFA-100 responses, without prolonging the skin bleeding time, and merits further clinical studies for thrombotic disorders. This is the first study demonstrating the effect of a monoclonal antibody that blocks GPIb/vWF in humans.

260

Phase I study of the adoptive transfer of tetramer selected cytomegalovirus-specific cytotoxic T cells after allogeneic stem cell transplantation

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Cytomegalovirus (CMV) remains an important cause of morbidity and mortality following stem cell transplantation (SCT). Adoptive transfer of donor CMV-specific CD8+ T cell clones has been shown to protect against CMV reactivation in previous studies. However, this procedure has not been widely used due to the need for extensive *ex vivo* culture of T cells prior to infusion. We have combined HLA-peptide tetramer staining with the use of magnetic beads against phycoerythrin to facilitate the rapid selection of CMV-specific CTL from donor peripheral blood using a CliniMACS machine. In this study we infused tetramer selected donor CMV-CTL to six allogeneic stem cell transplant (SCT) recipients following CMV reactivation. An additional patient had undergone transplantation from a matched unrelated donor and CTL were infused for treatment of CMV reactivation refractory to Foscarnet, Ganciclovir and Cidofovir. In the 6 sibling allografts between 10^5 and 1.6×10^6 (mean 6×10^5) CMV-specific CD8+ T cells were infused from 250 mL of donor peripheral blood. The unrelated donor transplant patient received 2×10^6 CMV-CTL isolated from a cryopreserved lymphopheresis product. No infusion reactions were noted. One patient developed grade II GvHD which responded to treatment. CMV-CTL were not detectable prior to infusion in three of six patients but were detected in all patients following infusion. CMV-CTL expanded to represent up to 22.1% of the CD8 compartment. This expansion occurred in the first 14 days followed by a contraction to a basal level. Four patients did not require antiviral therapy. In the unrelated transplant recipient CMV-specific CTL reached a peak of 0.6% of the CD8 compartment and CMV antigenaemia became negative 6 days following CTL infusion. This novel approach to the adoptive transfer of antigen-specific CTL is rapid and relatively straightforward. It has considerable potential in the development of antigen-specific T cell therapy after allogeneic transplantation.

261

Bone marrow mesenchymal stromal cells protect chronic lymphocytic leukaemia (CLL) from spontaneous and drug-induced apoptosis

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CLL cells are able to survive for prolonged periods *in vitro* and yet in most instances undergo rapid spontaneous apoptosis when cultured *in vitro*. Sustained viability *in vitro* is probably maintained by contact with 'nurse-like' cells in the peripheral blood (PB) and the bone marrow (BM) microenvironment. An *in vitro* model to mimic *in vitro* conditions was developed using adherent BM mesenchymal stromal cells (MSCs) from the iliac crest of normal donors. This model was used to test protection of CLL PB mononuclear cells both from apoptosis and from the effects of CD20 (Rituximab) and CD52 (CAMPATH-1H) therapeutic monoclonal antibodies, the proteasomal inhibitor MG132 and Bis9, an inhibitor of protein kinase C. Levels of apoptosis were determined by flow cytometry, using AnnexinV-FITC and propidium iodide. BM MSCs abrogated spontaneous apoptosis of CLL cells over a 5-day period of culture (80–90% viability, $n = 9$). Protection required CLL cells to be in close contact with the MSCs; separation by a microporous membrane induced apoptosis, comparable to that seen in control samples cultured without MSCs (30–40% viability). Rituximab treatment did not have any obvious apoptotic effect on CLL cells *in vitro* but CAMPATH-1H was able to induce apoptosis in a subpopulation of cells even in the presence of MSCs. Treatment with Bis9 and MG132 caused substantial and rapid apoptosis of CLL cells (0–15% viability $n = 3$). At lower doses of both drugs (200 nM), this apoptosis was prevented by MSC co-culture (70–90% viability). MSCs can therefore maintain CLL viability *in vitro* and can influence drug-induced apoptosis. The critical cell–cell interactions remain unknown, but are likely to include the beta-integrin, TNFSF13B and the CXCR4/CXCRL12 pathways. The expression of these molecules and their possible interactions in maintaining CLL viability will be investigated to assess if any might be targeted therapeutically.

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Samsung Bioepis Exhibit No. 1002
Page 5470

262

Targeting FGFR3 in multiple myeloma: the use of SU5402 and PD173074 to inhibit t(4;14) positive cells

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The t(4;14)(p16.3;q32), associated with 10–20% of cases of multiple myeloma (MM), deregulates the expression of two genes, MMSET and FGFR3. Most t(4;14) positive myeloma patients express FGFR3 and constitutive activation of this receptor is seen in some cases. To assess the potential of FGFR3 as a drug target we evaluated the effects of three FGFR inhibitors (SU6668, SU5402 and PD173074) on control and MM cell lines. Activity was assessed initially against Ba/F3 cells transformed to IL-3 independence by ZNF198-FGFR1, a constitutively active fusion gene associated with the 8p11 myeloproliferative syndrome (EMS), and compared to activity against control BaF3 cells grown with IL-3. Three methods were employed: MTS to assess proliferation/metabolic activity, trypan blue staining to determine cell viability and TUNEL to assess apoptosis. SU6668 inhibited the growth of both transformed and control cells lines at concentrations of >20 μM and therefore was not pursued further. SU5402 and PD173074 specifically inhibited the growth of ZNF198-FGFR1 transformed cells in a dose-dependent fashion with an IC50 of 5 μM and 5 nM, respectively. Both compounds also caused decreased cell viability and increased apoptosis. These compounds were then tested against two t(4;14) positive MM lines, KMS-11 and OPM-2, which express FGFR3 and harbour activating mutations of this gene. Dose-dependent growth inhibition was observed in both lines with IC50s similar to those in ZNF198-FGFR1 transformed cells. Importantly, inhibition was still observed in the presence of IL-6. Both compounds induced a dose-dependent reduction in cell viability and an increase in apoptosis, accompanied by a decrease in FGFR3 and ERK phosphorylation. In contrast, no inhibition was seen with either compound against the t(4;14) negative lines U266 and HS-Sultan, or the t(4;14) positive, FGFR3 negative cell line KMS-18. FGFR3 is thus a plausible candidate for targeted therapy in a subset of MM patients.

Oral Communication

Myeloid Malignancy

263

Decreased expression of a novel growth regulatory protein, Nov, in chronic myeloid leukaemia

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Chronic myeloid leukaemia (CML) is characterized by the constitutively active Bcr-Abl tyrosine kinase. We have used a murine multipotent haematopoietic stem cell line, FDCP-MIX, expressing a temperature-sensitive mutant Bcr-Abl (ts Bcr-Abl FDCP-MIX), to investigate changes in gene expression using DNA microarray analysis. RNA was prepared from cells expressing the Bcr-Abl tyrosine kinase and from control cells, labelled with biotin and hybridized to an Affymetrix Mu6500 array (Affymetrix, USA). The Bcr-Abl kinase activity resulted in differential expression of 300 genes, the most significant being a 25-fold decrease in expression of a novel gene called nephroblastoma overexpressed (Nov). Northern blot analysis detected a 4-fold reduction in Nov expression in the Bcr-Abl positive cells in comparison with the control cells. Western blot analysis of immunoaffinity-purified Nov detected a two-fold reduction in expression as a result of the Bcr-Abl kinase activity. Confocal microscopy revealed strong vesicular cytoplasmic staining for Nov in FDCP-MIX control cells whilst fluorescence was barely detectable in cells expressing Bcr-Abl. A similar pattern of Nov expression was observed in primary human cells (five CML, five normal controls). Normal CD34+ progenitor cells uniformly expressed an intense fluorescence signal for Nov expression. In contrast, the majority of CD34+ cells within the CML patient samples were negative ($P < 0.001$). Nov expression has previously been associated with cell differentiation, quiescence and has been shown to promote cell adhesion. Loss of these properties is consistent with the CML phenotype.

264

Reduced hTR expression in CML CD34+ cells at diagnosis: a novel mechanism for telomere loss in leukaemia

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CML is characterized by massive myeloid expansion, and significant telomere loss has been documented at diagnosis and during disease progression. Critical telomere shortening results in chromosomal instability, which can lead to disease progression. The regulatory mechanisms and expression levels of the key telomerase components (hTERT and hTR, the reverse transcriptase and RNA template respectively) in the CML stem cell compartment have not been described; however studies in solid tumours and cell lines generally show increased expression of both components. We selected CD34+ cells from 16 CML diagnostic leucapheresis specimens and 12 non-CML mobilized PBSC (control population) (median purity 94.1, 96.3% respectively; BCR-ABL positive in CML group). High-resolution cell cycle analysis demonstrated increased cycling in the CML CD34+ fraction. Q-RT-PCR for full-length (functional) hTERT showed an approximately two-fold rise in CML expression compared with control cells. RT-PCR with primers designed to detect the full-length hTERT transcript plus the three non-functional splice variants (with quantitative analysis) demonstrated a shift away from splicing in the CML samples. By combining these two complementary techniques we determined that expression of all (i.e. full length plus splice variants) transcripts are not different between CML and control cells: the increased levels of functional hTERT mRNA appear to be accounted for mainly by a reduction in the splicing out of the active sites. Surprisingly, Q-RT-PCR for hTR demonstrated a five-fold reduction in hTR levels in CML compared with control cells. Even more striking was the difference in hTERT/hTR ratio: 1 : 180 in CML cells versus 1 : 1800 in non-CML cells. We therefore hypothesize that despite increased hTERT levels there is a disassociated reduction in hTR, which in conjunction with increased cell cycling and replication

Samsung Bioepis Exhibit No. 1002
Page 5471

induced telomere loss, could alter telomere homeostasis in CML. This could contribute to disease evolution via chromosomal instability.

265

Reduced-intensity allogeneic haematopoietic stem cell transplantation (HSCT) using fludarabine, busulphan and alemtuzumab conditioning in 63 patients with myelodysplastic syndromes (MDS)

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We report the largest series of patients with MDS who have received a reduced-intensity HSCT using Fludarabine 30 mg/m^2 i.v. (day −9 to −5); busulphan 4 mg/kg/day orally (day −3 to −2) and alemtuzumab 20 mg i.v. (day −5 to −1). Immunosuppression comprised ciclosporin from day −1 titrated to plasma troughs of 150–200 ng/L. 63 patients with MDS (RA = 16, RAEB-I = 9, RAEB-II = 11, CMML = 5, TLD-AML = 21, aCML = 1) have received transplants from matched sibling (MSD) (24) or volunteer unrelated donors (VUD) (39). Except for RA, all had received at least one course of chemotherapy. Twenty-two of 24 MSD utilized PBSCs compared with 19 of 39 VUD HSCTs, with a median CD34+ cells of 4.6×10^6/kg (1.83–17.5) and 3.9×10^6/kg (0.44–16.7) respectively. There was no difference in engraftment latency, with the median time to neutrophil engraftment of 15 days (4–57) and platelets 18 days (7–105). 85% achieved full donor chimerism with a 6% incidence of grade III/IV aGvHD (43% grade I–IV) and 3% extensive cGvHD. At a median follow-up of 343 days (50–1213) for MSD, and 248 days (37–1316) for VUD recipients, 63 and 62% were alive respectively. The median age of sibling allografts recipients was 54 years (41–70). At day +200, the TRM was 5% and DFS 68%. The median age for VUD recipients was 49 years (25–58). The TRM at day +200 was 21% with DFS 65%. At 1 year, the estimated OS was 66% for VUD and 77% for MSD, with a DFS of 57 and 53% respectively. Twenty-one received DLI at a median of day +273 (126–1290); 11 for relapse and 10 for declining donor chimerism. A graft versus leukaemia effect was demonstrable. Although longer follow-up is required, RIC allogeneic HSCT appears to be safe and feasible in this high-risk group of patients.

266

Combined clofibric acid and medroxyprogesterone acetate exert multiple anti-leukaemic actions against acute myeloid leukaemia cells and cell lines

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Poor survival rates in acute myeloid leukaemia (AML) relate to both the failure of cytotoxic chemotherapy and inability of older patients to tolerate that therapy. Thus non-toxic drugs that specifically target the differentiation and apoptotic pathways of AML cells are urgently required. We and others have demonstrated that fibrates such as clofibric acid (CA) and inhibitors of the aldo-keto reductase AKR1C3, including medroxyprogesterone acetate (MPA), potentiate all trans-retinoic acid (ATRA) induced HL-60 cell differentiation. Fibrates are pharmacological PPARalpha ligands commonly used in the treatment of hyperlipidaemia whereas MPA is used to treat breast carcinoma. We recently demonstrated that, potentiation of HL-60 differentiation by AKR1C3 inhibitors occurs via the consequent activation of PPARgamma. Thus combined CA and MPA (CA/MPA) provide the means to simultaneously target PPARalpha and gamma using established drugs with low systemic toxicities. Here we demonstrate that CA/MPA killed cultured primary AML blasts in the presence or absence of IL3 and SCF ($P = 0.0001$) but not normal CD34+ve myeloid blasts. CA/MPA also sensitized AML cell killing by suboptimal doses of daunorubicin and Ara-C ($P < 0.003$) highlighting the drugs' potential as chemosensitizing agents. Although ATRA did not enhance AML cell killing by CA/MPA, all three drugs together resulted in the differentiation of the surviving cells from three of seven non-acute promyelocytic AML samples. Finally, high serum Nm23-H1 protein concentrations have been associated with the poorest prognosis in AML. Here we provide the rationale for this by demonstrating that Nm23-H1 acts as an autocrine survival factor. Importantly we also demonstrate that CA/MPA rapidly down-regulated Nm23-H1 expression in those AMLs that had highest initial expression ($P = 0.001$). We conclude that CA/MPA exert multiple antileukemic actions against AML cells and should be considered as adjuncts in the treatment of elderly and relapsed AML.

267

The utility of *in vitro* testing for imatinib sensitivity in BCR-ABL negative myeloproliferative disorders

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Imatinib mesylate (STI571, Glivec) inhibits the tyrosine kinase activity of the protein tyrosine kinases ABL, ARG, PDGFRA, PDGFRB and KIT, and has proven clinical efficacy in the management of patients with BCR-ABL positive CML and MPDs with PDGFRB rearrangements. However, it is not known to what extent patients with BCR-ABL negative MPDs may harbour activating rearrangements or point mutations of imatinib-responsive genes. To test for imatinib responsiveness we have employed two *in vitro* assays. First, blood or marrow mononuclear cells (MNC) were grown in methylcellulose supplemented with growth factors and treated with 0, 1 or 5 μM imatinib. Colonies were counted on days 7 and 14. Secondly, blood or marrow MNCs were placed in liquid culture with 0, 1 and 5 μM imatinib and cell numbers were enumerated over 2–3 weeks. Imatinib-treated cells from CML patients showed a reduction in colony and cell numbers over time compared with untreated cells and treated cells from normal individuals. We have obtained results using one or both methods in 38 BCR-ABL, TEL-PDGFRB negative patients who had been diagnosed with atypical CML, BCR-ABL negative MPD, CMML, mastocytosis, myelofibrosis or hypereosinophilic syndrome (HES). Eleven patients showed a positive response, four of whom had a PDGFRB rearrangement as determined by FISH and one had TEL-ABL. The remaining six patients had HES ($n = 2$), secondary MDS with eosinophilia following treatment for AML ($n = 1$), aCML ($n = 1$), MF ($n = 1$) or mastocytosis ($n = 1$). Six

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Samsung Bioepis Exhibit No. 1002
Page 5472

patients (five with PDGFRB or ABL rearranged MPD and one with HES) were treated and all showed a cytogenetic and/or haematological response. We conclude that *in vitro* cultures are useful for predicting patients who might respond clinically to imatinib. Evidence to date suggests that most BCR-ABL negative patients who are imatinib-responsive have elevated eosinophil counts.

268

Identification of a novel gene, GEMS, that is fused to FGFR1 in the 8p11 myeloproliferative syndrome

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The 8p11 myeloproliferative syndrome (EMS) is an atypical stem-cell myeloproliferative disorder which is caused by activation of the transmembrane receptor tyrosine kinase FGFR1. This typically occurs as a result of fusion of the tyrosine kinase domain of FGFR1 to an unrelated partner gene, which promotes dimerisation and consequent activation of the kinase in the absence of an appropriate ligand. To date, five FGFR1 partner genes have been identified: ZNF198 at 13q12, FOP at 6q27, CEP110 at 9q33, BCR at 22q11 and HERV-K at 19q13. In this study, we describe a patient with a clinical picture typical of EMS, who was found to have an acquired ins(12;8)(p11;?p11p22) by cytogenetics. Southern blotting and FISH were used to confirm that FGFR1 was disrupted and RT-PCR analysis indicated that the patient did not harbour any of the known FGFR1 fusion mRNAs or BCR-ABL. To identify the partner gene in this patient we employed 5′-RACE PCR, exploiting the fact that all known FGFR1 translocations generate mRNA fusions in which the partner gene is spliced to FGFR1 exon 9. A 629-bp product was obtained which was found to consist of sequence derived from chromosome band 12p11 fused to exon 9 of FGFR1. Analysis of ESTs and other clones indicated that the sequence was derived from a widely expressed novel gene that we have called GEMS (gene disrupted in eight p11 myeloproliferative syndrome). GEMS is predicted to be translated into a 29-kDa protein that contains an N-terminal coiled-coil domain but no other recognisable motifs. RT-PCR confirmed the presence of the GEMS-FGFR1 fusion but the reciprocal chimaeric mRNA was not detected. Experiments to determine the transforming ability of this novel fusion gene are underway.

Oral Communication

Haemostasis and Thrombosis 2

269

Venous thromboembolism (VTE) associated with the management of acute thrombotic thrombocytopenic purpura (TTP)

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VTE is reported to occur in 3% of TTP/HUS cases following plasma exchange (PEX) therapy. From May 1997 to May 2002, 68 patients with acute TTP were treated. Eight documented VTE episodes (six DVTs and three PEs) were identified in seven patients (all female, mean age 31 years) during therapy for acute TTP (five primary presentation and three relapses). All six DVTs were associated with central lines at the site of thrombosis. Other precipitating factors included pregnancy (one), immobility (eight), obesity (three) and FV Leiden heterozygosity (one). VTE occurred at a mean of 53 days following the first PEX. Solvent detergent plasma (Octaplas, Octapharma, Austria) was the last plasma to be used in PEX prior to the VTE in seven of eight events. Octaplas was used as the sole plasma for three TTP episodes and in the other episodes was substituted for cryosupernatant or FFP owing to poor response (four) or allergy (one). The mean volume of Octaplas replacement prior to the VTE was 100 L (range 22–206). The mean platelet count at VTE was 161 (range 12–232) $\times 10^9$/L. The platelet count had been in the normal range for a mean of 18 days prior to the VTE in those with a normal platelet count (six of eight). One patient received prophylactic LMW heparin from initial TTP diagnosis and seven of eight received aspirin. In conclusion, we identified VTE in 12% of TTP cases and 88% of these were associated with Octaplas replacement in PEX. VTE is a multifactorial disease and several known precipitating factors are present in TTP patients. The use of large volumes of Octaplas in PEX may be an additional risk factor, possibly associated with protein S (PS) deficiency, particularly as the median functional PS in Octaplas is 58 IU/dL compared with 91 IU/dL and 102 IU/dL in FFP and cryosupernatant respectively.

270

Results of D-dimer in the UK NEQAS (Blood Coagulation) proficiency testing programme: urgent need for improvements in standardization of testing and reporting

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We report the proficiency testing of D-dimer in diagnostic departments. Two lyophilised samples were tested for D-dimer by over 240 centres using different methods. Around 30% used semi-quantitative tile-based latex agglutination assays and 70% used automated techniques including 20% who reported in fibrinogen equivalent units (FEU). Six automated methods were used in 10 or more centres and the median and range of results obtained with these methods are shown below in ng/mL. For sample 1, 70% of centres reported a result above the cut-off for DVT. For one method the median test result was within the normal range, whereas for other methods the median was 40–80% above the normal range depending on the technique. It was a major cause for concern that 24% reported a normal result for this sample. There appeared to be a poor understanding of the difference between the upper limit of normal and the cut-off for DVT diagnosis. For the 103 centres who reported using a cut-off for DVT diagnosis the value in use ranged from 80 to 1000 ng/mL. For sample 2, a pooled normal plasma, six centres reported their result as positive, abnormal or consistent with DVT. As reconstituted lyophilised plasma is unsuitable for the Nycocard system a different test sample was tested by 67 users of the Nycocard system who obtained results in the range of 80 to 1300 ng/mL for

Samsung Bioepis Exhibit No. 1002
Page 5473

a liquid control sample having a median result of 500 ng/mL. We conclude that standardization of D-dimer testing and reporting is currently poor and that there is an urgent need for international reference preparations and recommendations.

		Sample 1		Sample 2		
	n	Median	Range	Median	Range	Median upper limit normal
Biomerieux VIDAS*	10	872	798–983	169	149–250	500
Dade-Behring DD Plus	10	170	120–219	100	60–170	190
Instrumentation Lab	68	352	240–848	103	10–280	260
Organon (FEU)*	18	915	770–4320	200	80–830	500
Organon (other units)	10	433	400–850	80	30–100	300
Stago*	15	890	800–1360	220	130–640	500

* FEU, fibrinogen equivalent units.

271

Progression of liver disease in hepatitis C infected haemophiliacs assessed by serial liver biopsy

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More than 90% of haemophiliacs treated with plasma-derived factor concentrates prior to 1984 are infected with the hepatitis C virus. Although the timescale for progression to cirrhosis is many years, most patients will develop chronic liver disease. It has been suggested that liver biopsy in haemophiliacs is particularly hazardous and should be avoided. However, current treatment guidelines use liver biopsy to determine those who require therapy with ribavirin/interferon. We have previously reported our experience of liver biopsies in 35 patients with haemophilia/von Willebrand's disease and hepatitis C. We now report follow-up data on repeat biopsies on the same patient group 3–5 years following initial biopsy to assess progression of liver disease in this group. Patients who were HCV-PCR negative and those with inhibitors were excluded. To date, 25 patients have undergone repeat biopsy. Biopsies were graded according to fibrosis and inflammatory scores. Ten patients have progressed by one fibrosis grade. Three patients have progressed from grade 2 or less to grade 3 thereby qualifying for combination therapy. One patient has progressed from grade 0 to grade 3 and another progressed from grade 0 to grade 5. Both these patients had a history of excessive alcohol consumption. Thirteen patients showed no evidence of progression. Over a time course of at least 18 years only four patients have developed cirrhosis. There have been no bleeding complications related to the biopsies. We have performed 70 biopsies in patients with haemophilia/severe VWD and shown it to be a safe procedure. We have demonstrated the slow progression of liver disease in this group. The majority of our patients do not qualify for combination therapy according to national guidelines.

272

UK surveillance of acquired haemophilia A: May 2001–April 2002

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Reports on acquired haemophilia (AH) are exclusively from referral centres which may have lead to publication bias. This study aims to establish the incidence and clinical characteristics of AH in all UK patients. In the first 12 months 256 of 257 UK Trusts circularised reported 98 cases (incidence 1.7/million/year). Clinical details are available on 68 patients. Median age is 80 (range 32–97). 39% had died at 6 months: 10% infection (pneumonia/sepsis), 4% bleeding, 7% malignancy, 7% cardiac and 11% others. Clinical associations are malignancy 29%, inflammation 20%, pregnancy 3%, pemphigoid 3% and none 59%. Median (range) factor VIII and anti-human inhibitor levels were 3 IU/dL (<1–25) and 13 BU/mL (<0.4–717). Bleeding manifestations are subcutaneous 78%, muscular 37%, gastrointestinal 22%, retroperitoneal 9%, haemarthrosis 7%, haematuria 7% and intracranial 3%. Subcutaneous bleeding was the only manifestation in 28%. Bleeding manifestations did not vary between CCC, haemophilia centres (HC) and other centres. Haemostatic treatment: none 38%, FEIBA 34%, rFVIIa 24%, human FVIII 22% and porcine FVIII 4%. No haemostatic treatment was required in 26% of patients at CCC, 55% at HC and 45% at other centres. Immunosuppression induced remission in 68% but only 44% of the cohort was alive and inhibitor-free at 6 months. There was no difference in remission rate between steroids alone 73% or cytotoxics and steroids 69% (IVIG made no significant difference); however, these results must be interpreted with caution as they are retrospective and not randomized. The remission rate at CCC, HC and other centres was 63, 65 and 91% respectively. These interim data suggest that overall survival is worse than previously reported, more patients died of infection than bleeding and many patients required no haemostatic treatment. Patients treated at non-haemophilia centres require less haemostatic treatment and are more likely to achieve remission suggesting that previously published data may be biased to a more severe phenotype.

273

Procoagulant changes in human macrophages induced by environmental microparticles [PM10]: a possible pathogenetic mechanism in cardiovascular disease

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In epidemiological studies a strong relationship has been confirmed between increasing concentrations of environmental particles [PM10 and PM25] and acute cardiovascular events including myocardial infarction and stroke. No plausible pathogenetic mechanism

Samsung Bioepis Exhibit No. 1002
Page 5474

has been demonstrated to explain this association. Recently it has been demonstrated that inhaled particles pass into the systemic circulation in humans (1). Therefore we hypothesized that vascular events may be triggered by coagulation activation on cell surfaces induced by contact with environmental particles. Human peripheral blood monocyte-derived macrophages and a human bronchial epithelial cell line were exposed to PM10 and supernatants examined for procoagulant activity in a plasma recalcification time assay. Control experiments employed culture medium only or hydrogen peroxide. Both PM10 and hydrogen peroxide induced an increase of procoagulant activity in culture supernatants from both cell types [shortening of clotting time versus control with PM10 was 31% with macrophages and 19% with epithelial cells; mean of three experiments, $P < 0.001$ and < 0.01 respectively]. In order to characterise the procoagulant activity from macrophages induced by PM10 we went on to determine the effects of these particles on surface expression of negatively charged phospholipid by annexin V binding, and tissue factor mRNA expression by RT-PCR. Finally we measured macrophage interleukin 8 (IL8) mRNA expression and release in response to PM10. Environmental particles induced macrophage tissue factor mRNA expression and significantly increased bound annexin V. Furthermore IL8 message and release were significantly enhanced also. We have demonstrated, for the first time, that environmental particles associated with increased cardiovascular risk can activate macrophages *in vitro* with transformation to a procoagulant phenotype which may be mediated through apoptosis/negatively charged phospholipid exposure and tissue factor expression. This may represent a novel prothrombotic mechanism triggered when environmental particles reach the systemic circulation.

Reference

1 Nemmar *et al. Circulation* 2002; **105**: 411–4.

274

Is early activation of the clotting cascade involved in the pathogenesis of verotoxin-producing *Escerichia coli*-associated haemolytic uraemic syndrome?

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In most cases, haemolytic uraemic syndrome (HUS) is a thrombotic complication of infection with *Escherichia coli* O157 : H7. It usually follows bloody diarrhoea caused by verotoxin secreted by the bacteria, and comprises acute renal failure, non-immune, microangiopathic haemolytic anaemia and thrombocytopaenia. HUS is primarily a disorder of the vascular endothelium, with platelet aggregation at sites of endothelial cell damage in the renal microvasculature. Several *in vitro* studies demonstrate changes in endothelial cell anticoagulant properties when exposed to cytokines and under apoptotic conditions. Of interest are changes in tissue factor (TF) expression and activity, TF being crucial in initiating the coagulation cascade. Additionally, the expression of antithrombotic membrane components, notably thrombomodulin, has been shown to be downgraded. We examined the changes in TF and thrombomodulin on endothelial cells exposed to the *E. coli* products, verotoxin and lipopolysaccharide (LPS), and the inflammatory cytokines, interferon-gamma (IFNgamma) and tumour necrosis factor-alpha (TNFalpha), and their relationship to apoptosis. Macrovascular [primary culture of human umbilical vein endothelial cells (HUVEC)] and microvascular [human microvascular endothelial cells (HMEC)] endothelial cells were cultured with LPS and verotoxin in the presence and absence of cytokines. Surface TF and thrombomodulin expression and apoptosis were assessed using flow cytometry. Increases in TF expression were observed in activated HUVECs; changes were less apparent in HMECs which constitutively express TF. Thrombomodulin was consistently reduced on HMECs under similar conditions. It appeared not to be the apoptotic population that changed their expression of membrane proteins. HUVECs cultured with LPS and TNFalpha demonstrated the greatest increase in TF. Decreases in thrombomodulin were greatest on HMECs exposed to verotoxin. We demonstrate an up-regulation of TF and a down-regulation of thrombomodulin on endothelial cells stimulated with LPS, verotoxin and inflammatory cytokines. These cells are rendered more thrombogenic, mechanisms that may be involved in the pathogenesis of HUS.

Oral Communication

Paediatric and General Haematology

275

Revealed: D-dimers are being inappropriately requested. Results of audit to influence subsequent requesting

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D-dimers, cross-linked fibrin degradation products, are extremely sensitive, but non-specific markers for coagulation activation; a process that complicates many conditions. They have been shown to be useful in negatively predicting for deep vein thrombosis (DVT), particularly in combination with a clinical assessment score for DVT. Junior medical staff and GPs are increasingly using the D-dimer test to screen for the presence of DVT. Radiology departments use a positive D-dimer to 'gate-keep' their Doppler service. Haematology departments have therefore identified a dramatic increase in D-dimer, requesting and associated cost (£26,000 in 2002 at Derriford Hospital). Over-investigation may lead to unnecessary, and potentially dangerous, further investigation and anticoagulation. This audit was conducted to address these concerns. The case-notes were reviewed from 50 consecutive patients presenting to/within Derriford Hospital with suspected DVT (34%) or pulmonary embolism (66%) in 2002. Not surprisingly, junior-most of the doctors requested the bulk of these investigations, 11% by registrars and none by the consultants. The reasons for requesting were documented in all but seven patients. The Biomerieux MDA D-dimer assay kit was previously evaluated locally, giving a positive cut-off level of 0.5 mg/mL for DVT with >95% sensitivity. In the current group of patients, 28% fell below the cut-off value. Of the positive results, only 61% were investigated further. The management was not affected by the D-dimer result in 45% of cases. Of the original cohort, only four DVTs and two PEs were confirmed. One PE was diagnosed in spite of negative D-dimer. These data support the theory regarding over-requesting of D-dimers. The low specificity is highlighted by only six confirmed DVT/PEs of 72% patients having a raised D-dimer. Locally, the co-importance of validated clinical assessment score is emphasized and the futility/danger of performing D-dimers on

Samsung Bioepis Exhibit No. 1002
Page 5475

in-patients and those with suspected PE until a specific cut-off value has been established.

276

Anti-D immunoglobulin in the treatment of immune thrombocytopenic purpura in adults in the United Kingdom: an evaluation of clinical outcomes and resource utilization

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Immune thrombocytopenic purpura [ITP] is an autoimmune disorder distinguished by a low platelet count and mucocutaneous bleeding. Estimated incidence is 10 per 100,000 persons per year. ITP is classified as primary, or secondary to an underlying disorder, and acute (of 6 months or less in duration), or chronic. In 70% of children, resolution occurs in 6 months. By contrast, ITP in adults has an insidious onset, and is chronic in nature. Given comparable efficacy of anti-D immunoglobulin and IVIg in the treatment of ITP, this inquiry was designed to contrast health resource utilization patterns stemming from the use of each agent in the treatment of ITP in adults. Stemming from the schematic developed by Cines and Blanchette (1), and information contained in the report of the British Committee for Standards in Haematology, General Haematology Task Force (Guidelines for the investigation and management of idiopathic thrombocytopenic purpura in adults, children, and in pregnancy) we employed Monte Carlo simulation to evaluate the utility (cost implications) of various treatment schema (intent-to-treat format; steroids; IVIg; anti-D immunoglobulin) for the treatment of ITP. Utilizing 100,000 replicate simulations per year of age examined [15 through 80 years (5-year intervals); quadratic regression equations] we discerned a significant ($P < 0.05$) efficacy/health service utilization-based saving (percent reduction) for the 'steroids to anti-D immunoglobulin' arm, relative to the 'steroids to IVIg' arm [34.0% (SE = 0.1%) for males; 33.4% (SE = 0.1%) for females]. In the United Kingdom, use of anti-D immunoglobulin in the management of ITP is estimated to result in reduced health service expenditures relative to IVIg. Specifically, use of anti-D immunoglobulin reduces the time required (cost) for medication administration, patient monitoring, and occupancy of health care facilities. The effect on patient's quality-of-life warrants further research.

Reference
1 Cines and Blanchett. *NEJM* 2002; 346: 995–1008.

277

Reduced numbers and antigen unresponsiveness of natural killer T-cells in idiopathic (autoimmune) thrombocytopenia (ITP)

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Natural killer T cells (NKT cells) are a rare cell subset implicated in the control of autoimmunity. Here, untreated ITP patients and healthy controls were compared for the number of circulating NKT cells and their proliferative response to the NKT cell antigen alpha-galactosylceramide. The cells were identified as Valpha24 Vbeta11 double-positive (CD4 positive or CD4 CD8 double-negative) lymphocytes using flow cytometry. In controls, NKT cell frequency ranged from 0.008 to 0.351% of total lymphocytes (mean 0.13%, $n = 9$). In the ITP group, a low NKT cell frequency was much more common, resulting in a mean of 0.04% (range 0.002–0.336%, $n = 14$). The capacity for alpha-galactosylceramide-driven NKT cell expansion was variable in the ITP group, which could be divided into responders (55% of tested donors, $n = 13$) and non-responders (45%). In comparison, 92% of controls ($n = 13$) responded. Interestingly, repeated testing of a limited number of ITP patients showed that proliferative response to the antigen or unresponsiveness was not constant over time, thus one and the same patient may respond at one time but not another. Furthermore, while IL-2 increased antigen-induced NKT cell proliferation in the majority of controls, this was not observed in the ITP group. In conclusion, the number of circulating NKT cells is reduced in ITP and a proportion of ITP patients have both antigen and IL-2 unresponsive NKT cells. The lack of proliferative response after antigen exposure and the IL-2 unresponsiveness may be due to adaptive tolerance, or *in vivo* anergy. This is believed to be caused by a prior incomplete antigen activation *in vivo*. These observations therefore suggest that NKT cells from ITP patients are anergic and this may contribute to the autoimmune process. Future research is planned to assess NKT cell function and involvement in autoimmune thrombocytopenia.

278

Outcome heterogeneity in childhood high hyperdiploid acute lymphoblastic leukaemia

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High hyperdiploid (HeH) karyotypes (51–65 chromosomes) are found in approximately one-third of children with acute lymphoblastic leukaemia (ALL). Generally, these patients respond well to standard therapy and enjoy a superior outcome compared with other children. It has been suggested that specific cytogenetic features might further refine this prognosis and provide a method for identifying those with a poorer outlook, however no consensual picture has yet emerged. We analysed outcome data from 700 HeH ALL children enrolled on two MRC trials (UKALLXI and ALL97) to examine the effect of sex, age, white blood cell (WBC) count, individual trisomies, modal number and structural abnormalities on survival. Univariate analysis showed that age (1–9 years), sex (female), trisomies of chromosomes 4, 10 and 18, and a modal number of 54–65 chromosomes were all associated with improved survival, whereas the presence or absence of structural abnormalities and WBC had no effect on outcome. Multivariate analysis revealed that only age, sex, trisomy 4 (+4) and trisomy 18 (+18) were independent indicators of outcome. These results enabled the construction of two hazard scores for predicting relapse and mortality. Three risk groups were found using age, sex and +18, with event-free survival (EFS) rates of 86, 75 and 50% at 5 years ($P < 0.0001$). The group with the highest risk of relapse comprised:

Samsung Bioepis Exhibit No. 1002
Page 5476

boys aged >9 years, boys aged 1–9 years without +18 and girls >9 years without +18. While girls aged 1–9 years with +18 made up the group with the best EFS. In terms of mortality, those aged <10 years with both +4 and +18 had a significantly improved survival (96 versus 84% at 5 years, $P < 0.0001$). These findings confirm that the outcome of children with HeH ALL is heterogeneous and information regarding specific trisomies can help identify those with a greater risk of treatment failure.

279

Correction of the anaemia of chronic disease (ACD) with small doses of recombinant erythropoietin

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The anaemia associated with chronic disease such as infectious, inflammatory or neoplastic conditions, contributes significantly to morbidity. It has been recognized recently that a similar anaemia is also seen in congestive cardiac failure (CCF) and diabetes without significant renal failure. Pharmacological doses of recombinant erythropoietin (EPO) correct ACD associated with rheumatoid arthritis, inflammatory bowel disease, and AIDS. Good responses to EPO have also been seen in cardiac failure, and type 1 diabetes. Large doses of EPO are used in some centres to treat anaemia associated with cancer and its treatment. We corrected ACD with small doses of EPO in 10 patients: six males and four females, age range 58–88 years. Three patients had inflammatory disease, three were diabetic, and four had CCF. Six had either normal or reactive bone marrow histology, normal cytogenetics, and normal or increased iron stores. Six were transfusion dependent prior to EPO therapy. The dose of subcutaneous recombinant human erythropoietin (Eprex) was titrated according to the haemoglobin. Nine patients were evaluated, one having died from his underlying disease. The mean dose required was 9000 units/week (range 3000–14,000). The average maximum increase in haemoglobin was 4.1 g/dL (range 1.3–6.8). Few side effects were encountered. One patient suffered transient cerebro-vascular ischaemia, possibly related to a rapidly rising haemoglobin. All patients became transfusion independent. All reported improved general health without the peak and trough effect associated with blood transfusion. Avoidance of red cell transfusion is desirable in view of the possible infective and non-infective complications of transfusion and concern over the availability and cost of blood products. Although EPO is expensive, the doses required were comparable with those used in chronic renal failure, and much smaller than usually required to treat anaemia associated with cancer and its treatment. The cost therefore, compared favourably with blood transfusion.

280

Epidemiological evidence for an infectious origin for childhood leukaemia

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The aim of the study was to interpret the results of several analyses of childhood leukaemia data from the Manchester Children's Tumour Registry (MCTR), in view of current hypotheses regarding an infectious origin. The MCTR is population-based and has been collecting case data from a geographically defined region of North West England since 1954. Trend tests, space-time clustering tests (Knox and K-function), ecological regression and geographical mapping were used to study the data on the leukaemias, broken down by sub-type (ALL, AML). For the most recent period (since 1980), ALL was further examined by immunophenotypic sub-type. There was an increasing incidence of ALL, due mainly to an increase of the precursor B-cell sub-type in the childhood peak. Space-time clustering was evident for cases of ALL, aged 0–4 years, for the time period 1954–1985, mainly based on date of diagnosis and place of birth. For the time period 1980–2001, space-time clustering was confined to cases of precursor B-cell ALL, aged 18–54 months, based on date and place of birth. The incidence of ALL was greater in more densely populated wards, largely because of cases of non-precursor B-cell ALL. The studies present a complex picture, but are supportive of an infectious aetiology, particularly for precursor B-cell ALL. It is concluded that there may be at least two infectious mechanisms operating, with a different mechanism predominating in the earlier years from that in the later years.

Samsung Bioepis Exhibit No. 1002
Page 5477

The European Agency for the Evaluation of Medicinal Products
Evaluation of Medicines for Human Use

London, 20 February 2003
CPMP/QWP/419/03

COMMITTEE FOR PROPRIETARY MEDICINAL PRODUCTS (CPMP)

<DRAFT>

NOTE FOR GUIDANCE ON EXCIPIENTS, ANTIOXIDANTS AND ANTIMICROBIAL PRESERVATIVES IN THE DOSSIER FOR APPLICATION FOR MARKETING AUTHORISATION OF A MEDICINAL PRODUCT

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Note:
This Note for Guidance replaces 3AQ9a Excipients in the Dossier for Application for Marketing Authorisation of a Medicinal Products and CPMP/CVMP/QWP/115/95 Note for Guidance on Inclusion of Antioxidants and Antimicrobial Preservatives in Medicinal Products

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Samsung Bioepis Exhibit No. 1002
Page 5478

NOTE FOR GUIDANCE ON EXCIPIENTS, ANTIOXIDANTS AND ANTIMICROBIAL PRESERVATIVES IN THE DOSSIER FOR APPLICATION FOR MARKETING AUTHORISATION OF A MEDICINAL PRODUCT

INTRODUCTION

This note for guidance is concerned with the application to excipients, antioxidants and antimicrobial preservatives of Module 3, sections P.1, P.2, P.4, P.5, P.8 of the Common Technical Document with a view to granting a marketing authorisation for a new medicinal product.

The data should be presented according to the standard format described in the CTD Module 3, part P.

Antioxidants and Antimicrobial Preservatives are substances which are used to extend the shelf-life of medicines by respectively retarding the oxidation of active substances and excipients, and by reducing microbial proliferation.

The properties of these substances are due to certain chemical groups which are usually aggressive towards living cells and which lead to certain risks when used in man.

If it is not absolutely necessary to add these substances in medicinal products they must be avoided.

The purpose of this note for guidance is to describe the information that needs to appear in application for marketing authorisations with regards to the addition of any antioxidants or antimicrobial preservatives.

For each antioxidants and antimicrobial preservative the application should contain :

- reason for inclusion
- proof of efficacy
- the method of control in finished product
- details of the labelling of the finished product
- safety information

Several guidelines should be also taken into account :

- ICH Topic Q 6 A : Specifications : Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products : Chemical Substances (3.3.2.2. Oral liquids : d) Antimicrobial preservative content, e) Antioxidants preservative content ; 3.3.2.3. Parenteral Drug Products : g) h) and Decision Tree 8 : Microbiological Attributes of Non-sterile drug products).
- Note for Guidance on Development Pharmaceutics : 3.3. Liquid and Semi-solid Formulations.
- 3BC7A : Excipients in the Label and Package leaflet of Medicinal Products for Human Use
- Note for guidance on maximum shelf-life for sterile products after first opening or following reconstitution.

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Samsung Bioepis Exhibit No. 1002
Page 5479

SECTION P.1 Description and Composition of the Drug Product

Excipients must be listed, specifying their common name, their quantity and the use and reference to any relevant standard. When the common name is not sufficient to indicate functional specifications, the brand name with commercial grade should be specified. In the case of excipients presented as a mixture of compounds, details as to the composition should be provided in qualitative and quantitative terms. However, for flavouring agents and aromatic substances, it is permitted to give the qualitative composition only.

Antimicrobial preservatives and antioxidants should be chemically defined (reference to existing European Pharmacopoeia monographs may be used) and designated by the Chemical Abstract Service Registry Number (RN-CAS).

The purpose for the inclusion of any antioxidant or microbial preservative should be stated (antioxidant for the benefit of active substance or excipient or both, or antimicrobial preservative).

SECTION P.2 Pharmaceutical development

SECTION P.2.1 Components of the Drug Product

The section P.2.1.2 should comprise an explanation of the choice of the excipient (and grade where necessary) and the level of excipient according to the note for guidance "Development pharmaceutics and process validation".

For antimicrobial preservatives and antioxidants, during the pharmaceutical development of the product the applicant should demonstrate:

- the necessity to add an antioxidant or preservative to the finished product at the chosen level
- the physical and chemical compatibility of the antioxidant and of the preservative with other constituents of the finished product, the container and the closures.

The used concentration must be justified in terms of efficacy and safety, such that the minimum concentration of preservative is used which gives the required level of efficacy. The appropriate test method for efficacy of antimicrobial preservation is that of the European Pharmacopoeia. This should be used to determine whether the required level of activity is achieved.

In the case of antioxidants, these should only be used once it has been shown that their use cannot be avoided, even if the manufacturing process is optimised to minimise the potential for oxidation, for instance by manufacturing and filling products under an inert headspace gas.

The safety of the antioxidant or preservative should be supported by bibliographic and /or experimental data.

Some antioxidants or antimicrobial preservatives may be undesirable under certain circumstances :

- mercury containing preservatives : see the CPMP Position Paper on Thiomersal, Implementation of the Warning Statement Relating to Sensitisation (CPMP/2612/99) and EMEA Position Statement on recent developments concerning thiomersal in vaccines (EMEA/CPMP/1578/00). This kind of preservative should be strictly avoided except if no other possibility may be considered but in this case the choice should be fully justified.

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Samsung Bioepis Exhibit No. 1002
Page 5480

- benzyl alcohol : when used in parenteral products for children under the age of two years. Its degradation product and metabolite is the benzaldehyde, toxic for the CNS.
- Benzoic acid esters (Para hydroxy benzoate and their derivates etc) : when used in any dosage-form for parenteral use.
- Sulphites and Metabisulphites.

Parenteral infusions do not contain any added antimicrobial preservatives and no antimicrobial preservatives are added when the medicinal product is intended for administration by routes where for medical reasons an antimicrobial preservative is unacceptable, such as intercisternally or by any other route of administration which gives access to the cerebrospinal fluid or retro-ocularly.

SECTION P.4 Control of Excipients

Examples of different kinds of excipients are given in the annex.

1. Specifications (P.4.1), Analytical Procedures (P.4.2), Validation of Analytical Procedures (P.4.3) and Justification of Specifications (P.4.4) :

1.1 Excipients described in the European Pharmacopoeia or, if not described in the European Pharmacopoeia, pharmacopoeia of a Member State

The routine tests which are to be carried out on each batch of starting materials must be stated in the application for marketing authorisation. If tests other than those mentioned in the pharmacopoeia are used, proof must be supplied that the test methods used are suitable to establish that the starting materials meet the quality requirements of that pharmacopoeia. When the monograph covers a family of related products, the particular specifications chosen for the excipients must be submitted. In addition and when necessary, the test used to determine the quality of the excipient should be shown to be in relation to the function that it fulfils in the medicinal product.

Data on microbiological contamination of the excipients used in the manufacture of sterile products should always be given where membrane filtration is used to achieve sterility.

Antimicrobial preservatives and antioxidants are defined as excipients and as such should be controlled following the rules governing medicinal products in the European Union. These data should be provided in part P.4.

1.2 Excipients not described in the European Pharmacopoeia or in the pharmacopoeia of a Member State

An appropriate specification of the excipient must be established, based on the following types of tests:

* Physical characteristics
* Identification tests
* Purity tests, including limits for total or individual impurities, which should be named. Purity tests may be physical, chemical, biological and, if appropriate, immunological.

  Where sterile filtration is used in the manufacture of a parental medicinal product, data and routine tests on microbiological contamination of excipients should always be given.
* Other relevant tests including, e.g. the tests on parameters which may influence the performance of the dosage form.

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Samsung Bioepis Exhibit No. 1002
Page 5481

* Assay or limit tests if necessary.

When an excipient is not described in the European Pharmacopoeia or in the pharmacopoeia of a Member State or in an other compendium of established use (e.g. USNF Pharmacopoeia and Japanese Pharmacopoeia), validation data of the test methods used should be presented, where appropriate.

1.3 Justification of Specifications

Justifications of specifications takes into account the choice and use of an excipient which is used for a particular purpose: it will determine the properties which must be checked during the routine tests and which will be the subject of certain specifications in connection with the bioavailability of the product (see Note for Guidance "Specifications and control tests on the finished product").

Nevertheless, justification of specifications are not systematically required for well-known excipients. For example, they are not required for excipients which have been used in similar medicinal products for a long period of time and when their characteristics and properties have not changed significantly.

For solid and semi-solid dosage forms, the justification of specifications should, if necessary, provide information on the relevant characteristics of the excipient. Special tests are often necessary (e.g. to verify the capacity of the excipient to emulsify and disperse, or to measure the viscosity...).

Appropriate data are needed for excipients used in a new route of administration.

Justification of specifications on excipients already included in the European Pharmacopoeia, or if not included in the European Pharmacopoeia, in the pharmacopoeia of a Member State and other well-known excipients already used in a medicinal product.

For these excipients, justification of specifications will normally not be required. However, any particular specification concerning the characteristics, as defined in Section P.2.1.2, should be justified (e.g. sieve analysis, in relation to microcrystallinity).

Excipients of Human or Animal Origin (P.4.5)

Viral Safety and TSE Risk should be documented in accordance with the relevant directives.

Novel Excipients (P.4.6)

For novel excipients : a dossier should be established containing the same data as required for new active substances:

a) A strict definition of the excipient, its function and its conditions of use. If the excipient is complex or is made of a mixture of compounds, the composition must be specified in qualitative and quantitative terms.

b) For new excipients and for excipients presented as a mixture of compounds the following should be taken into consideration:

   i. Any bibliographical data on the chemistry and on the toxicology and the field in which the product is already used.

   ii. The Community provisions concerning additives in foodstuff: any criteria which are based on the toxicological data, with cross-references to these data.

   The quality specifications which have been laid down in the directives are satisfactory as long as the routine control tests used are validated.

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Samsung Bioepis Exhibit No. 1002
Page 5482

iii. The international specifications (FAO/WHO/JECFA), and other publications such as the Food Chemical Codex.

iv. For medicinal products for topical use, data on the starting material in cosmetic products (Directive 76/768/EEC).

v. Data concerning the toxicology of the new excipient should be presented according to the dosage form and the route of administration of the medicinal product (if applicable).

c) Documentation on chemistry of excipients is required for all new excipients, taking as its basis the Note for Guidance "Chemistry of Active Substances".

* The origin of the excipient, including the name and address of manufacturer.
* A general outline of the synthesis (manufacture and purification).
* Structure.
* Physical, chemical properties, identification and purity tests.
* Validated methods of analysis with a presentation of batch results.
* Miscellaneous information (microbiological tests, etc).
* Contamination, presence of foreign substances, residual solvents, etc.
* In the case of an excipient obtained from a mixture of several components, the quality of each component and the physico-chemical tests for the mixture should be described.

The routine test procedures and limits should be established on the basis of the documentation given in the dossier.

SECTION P.5 Control of Drug Product

Apart from those situations envisaged in the note for guidance "Specifications and control tests on the finished product" it is not usually necessary to carry out identity testing and an assay of the excipients in the finished product at release.

Nevertheless, the finished product release specifications should include an identification test and limits for any antioxidants and antimicrobial preservatives present in the formulation. The finished product specification against which the product is tested throughout its shelf-life should also include limits for the antimicrobial preservatives present.

Where antioxidants are used up during the manufacture of the product, the release limits should be justified by batch data. The adequacy of specified limits should be justified on the basis of controlled conditions and in-use stability testing to ensure that sufficient antioxidant remains to protect the product throughout its entire shelf-life and during the proposed in-use period.

The control of antioxidants and antimicrobial preservatives should comply with the requirements identified in the guideline : ICH Topic Q 6 A : Specifications : Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products : Chemical Substances .

The antimicrobial preservative and antioxidants must be routinely identified and quantified at release.

P.8 Stability

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Samsung Bioepis Exhibit No. 1002
Page 5483

The maintenance of the physico-chemical properties of the finished product depend on the properties and the stability of the excipients (see note for guidance "Specifications and control tests on the finished product").

For new excipients, stability data should be provided as required for new active substances.

The application should follow the current QWP/CPMP guidelines on the stability of new dosage forms and should ensure that antimicrobial preservative and antioxidants levels are quantified periodically throughout the shelf-life of the finished product. In addition the efficacy of the preservatives should be established using the European Pharmacopoeia test for efficacy of antimicrobial preservation. This should be performed on the finished product at the end of the shelf-life and at the lower preservative limit in the end of shelf-life specification. The former is necessary, even if no evidence of degradation of the antimicrobial preservative and of the antioxidant is observed on storage, as other chemical and physical changes in the finished product may influence the efficacy of the antimicrobial preservative and the antioxidant.

In the case of products presented in multidose containers, the efficacy of the antimicrobial preservative under simulated in-use conditions must be established. The tests should be performed under the same condition as it is expected to be used by the final user. It may also be appropriate to examine the efficacy of the antimicrobial preservative following storage of opened or used containers for the proposed in use shelf-life.

R LABELLING

- Labelling must in accordance with relevant EC directive 2001/83/EC, Note for Guidance : Excipients in the Label and Package leaflet of Medicinal Products for Human Use and CPMP Position Paper on Thiomersal, Implementation of the Warning Statement Relating to Sensitisation (CPMP/2612/99).

However, if a product is presented in a multidose container without a preservative because :

a) it is intended for single use only (e.g. cytotoxic),

b) the product is self-preserving,

c) the product is oils based,

the labelling and product literature should indicate the absence of a preservative. This would not only emphasise the increased risk associated with the use of such products, but also aid the physician to specifically identify a product without preservative.

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Samsung Bioepis Exhibit No. 1002
Page 5484

ANNEX

EXAMPLES OF REQUIREMENTS CONCERNING DIFFERENT KINDS OF EXCIPIENTS

1. Excipients, which are a single chemical entity, include, for example, organic and inorganic acids and their salts, sugars and alcohols.

   They may have undergone physical treatments, which gave them special technological characteristics (e.g. micronisation).

2. Chemically transformed excipients include excipients which have undergone a special chemical treatment in order to confer certain technological characteristics (e.g. modified starch). The name and quality of such excipients should be defined in such a way as to avoid confusion with an unmodified excipient.

3. Mixtures of chemically related components include, for example, polyol esters (mixture of mono, di and tri esters), hydrogenated glucose syrup, maltitolsyrup. For these products the dossier should specify the following characteristics of the excipient:

   * the nature and content of each component with a statement of its acceptable limits;
   * technological criteria (appropriate criteria to the performance of dosage form);
   * any additives which may be present.

4. Mixed excipients are ready-for-use preparations, for example for direct compression or film coating.

   * The qualitative and quantitative composition of the mixed excipient should be submitted, the specifications of the product as a whole and of each component must be stated.

5. Excipients of natural origin, so called "natural" products have often undergone some kind of chemical treatment. In general and if relevant for the quality control of the product, data should give an outline of the operations carried out to obtain and to purify the product, and any special characteristics: decomposition products, specific impurities, chemical substances used during the treatment with residual limits, methods of sterilisation or decontamination, with a description of the effect of these processes on the excipient (e.g. modification of the physical structure).

6. For biological excipients of animal or human origin, the risk of transmitting adventitious agents should be considered and appropriate documentation submitted (e.g.; method of preparation and control of tissues and body fluids used as starting material). In addition, the name of the manufacturer and site of manufacture should be specified.

7. Flavouring agents (flavours and aromatic substances) are either natural products and/or products obtained by chemical synthesis. Because of the complexity of their composition, it is only necessary to describe the general qualitative composition mentioning the main constituents with an appropriate process of identification to ensure the consistency of the composition (in particular, identification of the main constituents and if necessary carriers).

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Samsung Bioepis Exhibit No. 1002
Page 5485

Most constituents of artificial flavours have internationally accepted purity criteria in food use (FAO/WHO). Reference to these standards is acceptable for medicinal products.

8. Colouring matters: - Community legislation on colouring matters in medicinal products is applicable.

ANTIOXIDANTS

Antioxidants are used to reduce the oxidation of active substances and excipients in the finished product. Antioxidants should not be used to disguise poorly formulated products or inadequate packaging. The need to include an antioxidant should be explained and fully justified. Oxidative degradation can be accelerated by light and by the presence of mineral or metallic impurities, due to the formation of free radicals.

There are three types of antioxidants :

Type	Definition	Example
True antioxidants	These are thought to block chain reactions by reacting with free radicals	Butylated hydroxytoluene (BHT)
Reducing agents	These have a lower redox potential than the drug or excipient they are protecting	Ascorbic acid
Antioxidants synergists	These enhance the effects of antioxidants	Sodium edetate

The efficacy obtained for an antioxidants depends on its nature, the stage at which it is incorporated within the finished product, the nature of the container and the formulation.

The efficacy of antioxidants must be assessed in the finished product in conditions, which simulate actual use by measuring the extent of degradation in the finished product, with and without the antioxidant.

Antioxidants should only be included in a formulation if it has been proven hat their use cannot be avoided. This applies to cases where the manufacturing process is optimised to minimise the potential for oxidation.

ANTIMICROBIAL PRESERVATIVES

Antimicrobial Preservatives are used to prevent or inhibit the growth of micro-organisms which could present a risk of infection or degradation of the medicinal product. These micro-organisms may proliferate during normal conditions of use of the product by the patient, particularly in multidose preparations.

On no account should preservatives be used as an alternative to Good Manufacturing Practice (GMP).

Preparations at greatest risk of contamination are those which contain water such as solutions, suspensions and emulsions to be taken orally, solution for external use, creams and sterile preparations used repeatedly (e.g. injectable multidose preparations and eye-drops).

The level of efficacy will vary according to the chemical structure of the preservative, its concentration, the physical and chemical characteristics of the medicinal product (especially pH) and the type and level of initial microbial contamination. The design of the pack and the

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Samsung Bioepis Exhibit No. 1002
Page 5486

temperature at which the product is stored will also affect of activity of any antimicrobial preservatives present.

The antimicrobial efficacy of the preservative in the finished should be assessed during product development, particularly during stability studies and at the end of the proposed shelf-life, using Pharmacopoeia European test.

If products do not contain a preservative and do not have inherent preservative efficacy they must not be packaged in multidose presentations without a sound justification.

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Samsung Bioepis Exhibit No. 1002
Page 5487

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Formulation and delivery issues for monoclonal antibody therapeutics☆

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Abstract

Antibodies can have exquisite specificity of target recognition and thus generate highly selective outcomes following their systemic administration. While antibodies can have high specificity, the doses required to treat patients, particularly for a chronic condition, are typically large. Fortunately, advances in production and purification capacities have allowed for the exceptionally large amounts of highly purified monoclonal antibodies to be produced. Additionally, genetic engineering of antibodies has provided a stable of antibody-like proteins that can be easier to prepare. Together, these advances have made antibody-based therapies one of the most commonly pursued pharmaceuticals in biotechnology pipelines. With this success, however, has come a series of technical challenges in the formulation of antibody-based materials to maintain sufficient stability in a variety of configurations and sometimes at particularly high concentrations. This review focuses on issues related to identifying and verifying stable antibody-based formulations.
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Keywords: Antibody; Stability; Delivery; Formulation

Contents

1. Introduction 687
2. Antibody characteristics relevant for formulation and delivery 688
3. General concerns for antibody formulations 689

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Samsung Bioepis Exhibit No. 1002
Page 5488

4. Stability issues for antibody formulations . . . 690
4.1. Oxidation . . . 690
4.2. Deamidation . . . 691
4.3. Aggregation . . . 692
4.4. Fragmentation . . . 694
5. Strategies to stabilize antibody formulations . . . 694
5.1. Lyophilization . . . 694
5.2. Polymer delivery systems . . . 695
6. Formulation issues for alternative forms of antibody delivery . . . 696
6.1. Oral . . . 698
6.2. Respiratory tract . . . 698
6.3. Miscellaneous delivery sites of the body . . . 699
6.4. Intracellular targeting . . . 699
7. Other delivery issues . . . 699
7.1. Antibody fragments . . . 699
7.2. Immunoconjugates . . . 700
8. Conclusions — challenges and opportunities . . . 701
Acknowledgements . . . 702
References . . . 702

1. Introduction

Therapeutic and diagnostic antibodies have become the fastest growing area of biopharmaceutical applications. There are now 18 monoclonal antibodies on the market and over 100 in clinical trials, which highlights the significance of these therapeutics and the advances made in antibody engineering. Further, by 2008, engineered antibodies are projected to be the source of over a third of the revenues from biotechnology [1,2].

A few seminal events have led to the current and projected prominence of antibodies as biopharmaceuticals include the identification of methods to generate murine monoclonal versions of antibodies [3], the cloning of human antibody sequences to allow for humanization of murine monoclonal antibodies through complementary-determining region (CDR) grafting [4] and even the establishment of fully humanized systems to generate monoclonal antibodies [5]. With the sequential identification of these technological advances, antibodies for therapeutic and prophylactic indications have moved from fully murine, to partially murine (mostly human) to completely human constructions. Added to these events, dramatic advances in production have led to the ability to prepare monoclonal antibodies at scales that can provide sufficient material at costs that make this area appealing to pharmaceutical companies. One important outcome of these various advances is a greater potential to use antibody drugs in chronic settings, tremendously expanding their biopharmaceutical applications.

The majority of currently approved antibody drugs address previously unmet medical needs in the areas of cancer and inflammation. These indications came from practical considerations related to earlier forms of murine and partially murine antibody therapeutics; the first monoclonal to be approved (OKT-3®, muromonab-CD3) was approved in 1986 for a single treatment regimen to protect from life-threatening tissue rejection following kidney transplant [6]. Adverse reactions due to acute immunogenicity problems due to delivery of a non-self protein limited the use of OKT-3® to only acute application [7,8]. Subsequent partially murine antibodies such as Panorex® (17-1A), Zevalin® (ibritumomab tiuxetan) and Bexxar® (tositumomab) were approved for a limited number of administrations to cancer patients who had failed frontline therapies [2]. With the advent of fully humanized antibodies, chronic therapeutic or prophylactic applications for infectious agents have become a realistic clinical option [2]. Another critical aspect to the success of antibodies as therapeutic agents involves improved methods to express, purify and characterize these proteins [9]. In general, antibody therapeutics are

Samsung Bioepis Exhibit No. 1002
Page 5489

large (typically >150 kDa), complex in nature (most are glycosylated) and must be administered in stoichiometric rather than catalytic quantities (nearly a gram per dose is required for many antibodies to be effective). Production and purification scales have thus reached levels of production that were previously assumed impossible in biotech companies [10]. Novel concerns then related to the development of stable formulations and delivery strategies for such large amounts of a complex molecule. Understanding product instability with regard to physicochemical and thermodynamic events becomes more and more critical to ensure market success [11]. The required high concentrations of antibodies in these formulations reduced the need to add a carrier protein, as is the case where serum albumin is commonly added in formulations of highly potent proteins such as erythropoietin alpha (Epogen®) and Factor VIII (Kogenate®) to enhance stability and reduce loss to vial surfaces. Simultaneously, the large amounts of an antibody protein used in these formulations allowed degradation products to be more readily detectable particularly when using the highly sensitive methods designed for evaluating hormone biotherapeutics.

Finally, the ability to prepare large quantities of an antibody that could be administered for chronic therapy resulted in a plethora of new and novel applications [9,10] involving the delivery of chemotherapeutics, radionuclides and imaging agents — to name a few. New issues, related to identifying stable formulations such as high concentration formulations that might reduce the volume of antibody administrations, arose in the move from intravenous infusions to the realm of subcutaneous injection strategies. In some cases the combination of agents with antibodies produced conflicting stability profiles. Additionally, potential new indications for antibody drugs increased the desire to find novel methods for sustained release, intracellular targeting [12] or delivery via methods not involving an injection. Thus, as is the case in most scientific advances, technological advances opened up possibilities for therapeutic opportunities that were not possible previously as well as a totally new set of problems that required solving. In this review we have attempted to identify the major issues associated with formulating and delivering antibody drugs, to recognize challenges already identified accompanied when possible by solutions, as well as to discuss concerns that will need to be addressed in the future as the use of antibody drugs continues to evolve.

2. Antibody characteristics relevant for formulation and delivery

The human body produces several classes of antibodies that appear to perform specific functions under different conditions and at discrete anatomic sites. Most of the currently approved antibody-based drugs are of the IgG1 class [13] and are glycosylated on the Fc (fragment crystallizable) domain. Glycosylation has been shown to participate in specific cell response events mediated by antibodies [14–16]: antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cell-mediated cytotoxicity (CDCC) through receptor recognition events. Individual glycoforms of antibodies can provide improved efficacies for certain clinical outcomes [17]. In general, IgG1 and IgG3 antibodies are effective at initiating ADCC while IgG2 readily fixes complement but is poor at inducing ADCC. IgG4 appears to be inefficient at both ADCC and CDCC [13]. Interestingly, antibodies can be re-engineered to increase their capacity to recruit complement [18] or to adjust their affinities to the various classes of Fc receptors and thus ADCC function through mutation of specific amino acids within the Fc domain [19]. Molecular engineering has also provided a spectrum of new antibodies (Fig. 1). Other chapters in this issue delve deeply into the many characteristics of antibodies that make them remarkable and diversely applicable treatment agents: ligand binding properties, effector functions and potential for modification in their design. Other chapters will also address applications of newly engineered antibody-like molecules that move beyond the realm of intact, full-length IgG antibodies. In the current review, attention will be focused primarily on IgG1 since this is where the majority of published information exists for antibody drug formulation and delivery.

Antibodies, due to their large size (being greater than the filtration cut-off size of the kidney) and their ability to be sequestered for periods of time from the blood, via interactions with FcRn receptors present on

Samsung Bioepis Exhibit No. 1002
Page 5490

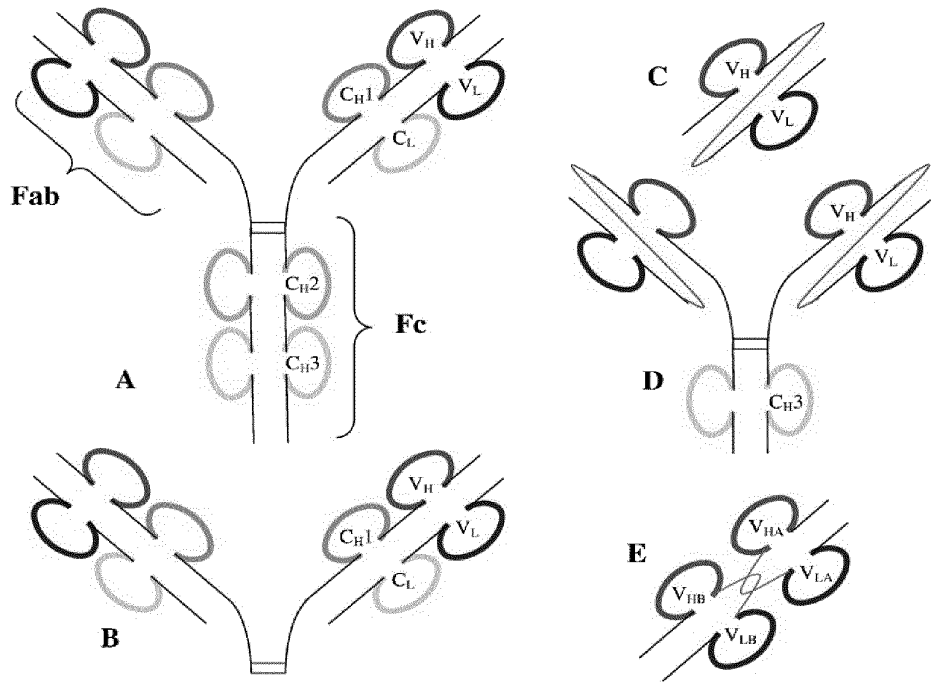


Fig. 1. General antibody structure and some antibody-based protein constructions. A) Human antibodies have a common framework that can be described as Fc (fragment crystallizable) and Fab (fragment antigen binding) domains that are derived from the folded structure of heavy (H) and light (L) chain proteins. Multiple constant (C) regions on both the H and L chain proteins are donated as are the variable (V) segments involved in antigen-specific recognition. B) Removal of the Fc domain produces a F(ab)2 structure. C) Single chain structures of the antigen binding region (scFv) can be prepared by themselves or D) in a construct that includes Fc domain elements. E) Bi-specific antibody elements (diabodies) can be prepared to bring two different antigens into close proximity. C_H, heavy chain constant regions; C_L, light chain constant region; V_H, heavy chain variable region; V_L, light chain variable region.

endothelial cells, can have extended serum profiles in the range of weeks. A number of engineered antibody-like molecules are now being examined for therapeutic applications [20]. 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 [21] and methods to prepare antibodies using non-mammalian cell systems, such as plants [22], from the eggs of chimeric chickens [23] and from transgenic animals [24], 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.

3. General concerns for antibody formulations

Due to their proteinacous nature, antibodies present generic formulation issues that are similar for most protein therapeutics [11]. Proteins, in general, can be degraded under conditions when they are exposed to heat, freezing, light, pH extremes, agitation, sheer-stress, some metals and organic solvents. With ever advancing analytical technologies, the ability to assess the physicochemical and thermodynamic instability of antibody drugs has also led to the identification of several events that are more specific to the unique nature of this particular class of proteins: variations in

Samsung Bioepis Exhibit No. 1002
Page 5491

Fc glycosylation, partial heavy chain C-terminal Lys processing, Fc methionine oxidation, hinge-region cleavage and glycation of Lys residues [25]. In addition to these issues is the difficulty of preparing dosing materials that might have protein concentrations in the range of 100 mg/ml or greater. Since early preclinical assessment is so critical to successful identification of a new biotherapeutic product, general pre-formulation strategies are usually employed for initial studies with new antibody drugs. An initial formulation effort will likely employ an isotonic, non-hemolytic, slightly acidic solution that can be administered by intravenous infusion or subcutaneous injection in studies employing a relevant animal model. In some cases, some stability information for the antibody drug can be obtained from initial in silico and in vivo testing during this pre-clinical phase that aids in the selection of a formulation that is used in clinical trials [26].

Since most early pre-clinical studies attempt to emulate the delivery strategy that will be used in the clinic, initial studies frequently identify problems with either the initial pre-formulation approach and/or stability issues with the antibody drug itself. In the case of the formulation, protein precipitation can occur with refrigerated storage or with repeated freeze–thaw cycles; events that may be facilitated by an inappropriate pH or buffer selection. Analysis of antibody drugs stored in this initial pre-formulation system may also identify unstable sites within the protein or its linkage to attached molecules (chemotherapeutics, imagining agents, etc.). In the case of the antibody, this information is frequently evaluated to determine if re-engineering of the protein could be performed, for example, to provide greater thermodynamic stability or folding efficiency [27]. Such changes in the Fc region are typically conservative, but might act to modify Fc receptor binding properties to provide a different biological outcome [28,29]. Amino acid substitutions can also be made within the Fv region to improve thermal stability [30], but such changes might also alter antigen binding specificity [31].

4. Stability issues for antibody formulations

If one examines the amino acid sequences for currently approved IgG1-based antibody drugs, it becomes quite clear that only a small segment of these proteins are dramatically different from one another, being the Fv segments that are involved in antigen binding. It should be pointed out, however, that there are some minor differences in conserved regions as well. A priori, one might assume that by finding a stable formulation for one of these antibody drugs, that such a formulation would be good for most if not all, similar antibodies. If this were borne out by experience, there would be no need for a review such as this. Instead, each antibody seems to have a unique personality related to its requirements for 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.

Probably the most important aspect of identifying a successful antibody drug formulation is in the identification of truly detrimental alterations rather than all of the modifications that might occur. Although, from a regulatory standpoint, it may be beneficial to understand all of the potential degradation events associated with an antibody formulation, it is the recognition of a stability-indicating parameter (or several parameters) that allows the formulation scientist to focus in on what is critical to development of a successful product. Here, a series of physicochemical and bioanalytical assays can be used to identify these parameters [32]. Although in vivo bioassays may ultimately be required to validate a stability-indicating formulation parameter, the most critical tools at the disposal of the formulation scientist are rapid and reproducible assays to measure protein changes due to oxidation, deamidation, aggregation, fragmentation and other forms of chemical modification that could lead to a loss of function of the antibody drug.

4.1. Oxidation

Methionine and cysteine residues are frequently a site of oxidation in protein drugs and this is also the case for antibody drugs [33]. In the case of antibody-based drugs, specific methionine residues within the Fc domain may be prone to oxidation, resulting in the

Samsung Bioepis Exhibit No. 1002
Page 5492

production of methionine sulfoxide [25]. While cysteines are present in the Fc framework as disulfide pairs, unpaired cysteines in the variable region may also be sites of oxidation. Besides methionine and cysteine residues, oxidation of histidine, tyrosine, tryptophan and phenylalanine residues can also occur [34]. A number of methods can be used to monitor oxidation of amino acid residues. As a starting point, the number of reactive protein thiols can be monitored using reagents such as 5,5′-dithionitrobenzoic acid or iodoacethyl dansylcadaverine [35], providing a simple readout on the number of protein thiols per protein. Total amino acid analysis can also be used to monitor reductions in methionine, tyrosine and phenylalanine residues. Tryptic digestion and peptide separation by liquid chromatography followed by mass spectroscopy analysis (LC-MS) can be used to identify specific sites of amino acid oxidation.

A careful study of methionine oxidation has been performed for the humanized monoclonal antibody that recognizes the her2/neu gene product. For this protein it was determined that a methionine at position 255 in at heavy chain of the Fc region was the primary site of oxidation and that a methionine at position 431 could also oxidize under more aggressive conditions. In this study, a chromatography assay was used to detect oxidation events following papain digestion to separate Fab and Fc segments of the antibody [36]. Another study on the stability of this same protein provides a valuable template for dealing with antibody oxidation: identification of a critical oxidation event, validation of a robust assay for monitoring that event and comparison of formulation parameters to determine conditions that facilitate oxidation. Addition of antioxidants or stoichiometric levels of free methionine or thiosulfate were found to reduce the extent of oxidation in formulations of this antibody [37].

Issues associated with administration of oxidized forms of an antibody may extend beyond concerns of reduced efficacy. Systemic lupus erythematosus (SLE) is a disease characterized by the induction of anti-double-stranded DNA antibodies. Studies have shown that increased levels of markers of protein oxidation, including those of methionine sulfoxide and 3-nitrotyrosine, correlated with SLE disease activity [38]. Additionally, oxidative modifications of antibodies isolated from synovial fluid samples collected from patients with rheumatoid arthritis suggested alteration of histidine, methionine, tyrosine and cysteine residues [39]. While no direct causal association is shown or implied, these observations bring attention to the issue that we do not fully understand the clinical impact of antibody oxidation events.

4.2. Deamidation

Glutamine and asparagine residues show a propensity for deamidation [40]. Both light and heavy chains of antibodies can undergo deamidation of these amino acids [41] and deamidation events are considered to be one of the major sources of charge heterogeneity of monoclonal antibodies [42]. Deamidation events appear to be highly selective events for individual antibodies. For example, analysis of several monoclonal antibodies that bind the her2/neu gene product demonstrated asparagine 30 deamidation on one or both light chains and asparagine 55 on the heavy chain [43]. Deamidation events lead to more acidic forms of the antibody through the acquisition of additional carboxylic acid groups. Conversely, aspartic acid residues can also undergo modification to a succinimide moiety that results in a basic antibody isoform of the antibody by removal of a carboxylic acid group [43]. In general, deamidation events appear to be relatively slow but are accelerated by unfolding of the antibody [44]. Initial detection of deamidation in antibody preparations is typically identified by differences in charge distribution or content using methods such as isoelectric focusing (IEF) or high-performance cation-exchange chromatography [43].

High performance chromatography can then be used to separate succinimide, isoaspartic and aspartic acid isoforms of tryptic digest fragments of the antibody which can be unambiguously characterized by tandem mass spectroscopy (MS). Correlation between tryptic digest tandem MS results and some other more readily accessible method, such as IEF, can provide a rapid format to follow antibody deamidation events. Using these techniques, asparagine deamidation events residues appears to occur more frequently than glutamine deamidation and specific asparagine residues appear to be selectively targeted for this event [33]. Deamidation of asparagine is frequently associated with rearrangement

Samsung Bioepis Exhibit No. 1002
Page 5493

events that lead to the formation of isoaspartic acid [42] and this outcome is correlated to amino acids adjacent to the asparagine prone for deamidation [44] that provide a method for prediction of potential deamidation sites (BOX). In particular, asparagine–glycine sequences can demonstrate accelerated deamidation rates since glycine residues lack a side-chain structure that would otherwise act to decreased conformation interference for backbone-associated isoaspartyl formation [45].

Box 1

Amino acid* sequences predicted to deamidate
SNG, LNG, LNN and ENN
(Note these have a following amino acid of G or N)
and those that are less prone to deamidation
GNT, GNS, GNV, TNY, SNY, SNF, SNT, SNK, SNL,
WNS, WNS, FNW, FNS, FNR HNA, HNH, LNW,
CNV, NNF, DNA, and YNP
*standard single amino acid letter nomenclature.

Non-enzymatic parameters of asparagine and glutamine deamidation in proteins have been defined in detail for factors such as pH, temperature and ionic strength [46,47]. The primary sequence dependence of these deamidation events can be explained by a simple steric and catalytic model [46,47]. Prediction of deamidation events based upon three-dimensional structures have also been described but the data set for these studies is not yet sufficient to produce a general model [48]. That asparagine and glutamine deamidation appear to be a constant phenomenon of proteins and correlate with their turnover provides the basis for the hypothesis that the presence and distribution of these amino acids within a protein can act as a molecular clock for its biological turnover [49,50]. For the formulation scientist, empiric assessment of factors such as pH and ionic strength provide the best strategy to identify a formulation that minimizes deamidation events. In general, deamidation does not result in a decrease in potency and deamidation may be hard to detect as the charge difference is small. At times, however, there can be a detrimental effect on potency through the introduction of an unfavorable negative charge that can have further ramification on biological function, particularly when deamidation occurs in the binding regions [51]. As particularly reactive residues are identified, it may be possible to have the antibody engineered to remove the problematic amino acid or to change adjacent residues to reduce its reactivity.

4.3. Aggregation

Liquid formulations of protein therapeutics frequently exhibit a decrease in concentration due to adsorptivity to container walls at low concentration and can show aggregation events at high concentrations. Initial preparations of antibody drugs were administered in acute situations by IV infusion that could allow for medium protein concentration (1–10 mg/ml) formulations not dramatically affected by adherence or aggregation losses. As antibody-based drugs became more widely used in chronic care, high concentration formulations that would allow for clinically acceptable SC injection volumes became desirable and aggregation became an important concern for designing formulations. Along with aggregation events, protein solutions at concentrations > 50 mg/ml may become sufficiently viscous as to no longer readily pass through the gauge of a needle that would be comfortable for patients' use. Some antibodies demonstrate high concentration viscosity issues while others do not. For example, the anti-respiratory syncytial viral antibody Synagis® is formulated at 100 mg/ml protein and is administered successfully by IM injection. Thus, addressing viscosity, adsorptive loss and aggregation in any specific antibody formulation will likely be performed on a case-by-case basis.

As protein–protein contact frequency increases at high concentrations, the opportunity for stable aggregate formation increases proportionally. Proteins are folded in such a way as to internalize hydrophobic domains and surface-expose more hydrophilic domains in a thermodynamically stable situation. As folded proteins undergo normal molecular motion transient surface exposure of hydrophobic domains can lead to protein–protein contacts that hide these

Samsung Bioepis Exhibit No. 1002
Page 5494

surfaces in a more energetically favorable manner. The outcome of these events is the stabilization of these protein–protein contacts with the ultimate outcome of dimerization, trimerization and so on — leading to extended aggregation events [52]. As protein concentration and temperature goes up, so does the probability of energetically favorable contacts leading to aggregation with increased frequency and duration of reversible protein–protein contacts leading to a greater extent of stable (even irreversible) aggregation events. Aggregation (covalent, non-covalent, dissociable, non-dissociable) must be considered a likely degradation event for high concentration antibody formulations.

For some antibodies reduced temperatures can incite reversible self-associations; these molecules are known as cryoimmunoglobulins [53]. IgG3 cryoimmunoglobulins have been shown to self-associate through sugar structures of the Fc domain [54]. Self-association events involving IgG1 antibodies seem to occur primarily through weak ionic and hydrophobic interactions [55]. More recent studies have examined kinetic and thermodynamic aspects of antibody solution dimerization using a recombinant human monoclonal antibody that recognizes vascular endothelial growth factor (rhMab-VEGF) as a model [56]. 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. Not surprisingly, self-association events were accelerated as the storage temperature was increased. 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.

A number of additives have been identified that can reduce the rate of protein aggregation [52,57]. A rather wide spectrum of agents can reduce protein aggregation rates: urea, guanidinium chloride, amino acids (in particular glycine and arginine), various sugars, polyalcohols, polymers (including polyethylene glycol and dextrans), surfactants and even antibodies themselves. Studies have shown that different proteins benefit from different types of anti-aggregating factors, consistent with the notion that the interfacial surfaces that drive interactions leading to aggregation are protein-specific. Conceptually, anti-aggregating agents might fall into one of two categories: small agents that fit into channels or grooves and large agents that might interact with lower-curvature surfaces of the protein. In essence, the small agents would act to stabilize a protein from acquiring a conformation that might be more reactive toward aggregation while a large agent might act to reduce the frequency of surface contacts that lead to an aggregation event. In the case of antibody formulation, either or both may be important. As we will discuss, agents found to effectively protect an antibody from aggregation may differ for various stress conditions; antibodies in solution that are undergoing agitation and thermal stress may differ from those of the same antibody as it undergoes lyophilization and reconstitution.

Protection from aggregation can be limited in antibody formulations by the addition of surface-active agents such as detergents, both charged and uncharged. As an interesting aside, such surface-active agents can help reduce protein loss due to adherence to surfaces that can occur with low concentration protein formulations. Polysorbate 20 and 80 (now available from a vegetal source) are examples of detergents that are widely used to reduce the rate of protein aggregation in high concentration antibody drug formulations. A precautionary note must be sounded, however, as detergents perform this function optimally at low concentrations (0.01% to 0.05%) and can act detrimentally to participate in protein unfolding at higher concentrations, e.g., 1%.

That antibody aggregation can be accelerated by several physical conditions provides the potential for multiple methods to determine if a particular antibody drug is prone to aggregation and to rapidly test formulations for their ability to impede aggregation. Agitation studies are done, in addition to thermal stability studies, to determine if aggregation can occur in a given formulation. Different test methods are used, employing a shaker or a stirring apparatus. In one study done on an IgG1 antibody found sizes of aggregates produced by the two methods were comparable [58]. They concluded that a higher throughput test could be constructed using stirring analyzed by absorbance measurement. In their work,

Samsung Bioepis Exhibit No. 1002
Page 5495

the stirred samples exhibited much higher absorbance and second particle species in DLS, indicating that stirring stress produces a higher amount of smaller protein aggregates. While Mahler found polysorbate 80 protects against aggregation, the stirred samples had an increase in turbidity and more aggregate products were detected by DLS as compared to a surfactant-free formulation. Polysorbate 80 stabilizes small aggregates and prevents further progress in the aggregation process.

4.4. Fragmentation

Intact, full-length antibodies are composed of several segments that have natural flexure sites. One site, in particular, exists at the flexure point between the Fc and Fv domain. Cleavage at this hinge region is a common concern when assessing antibody drug stability [25]. Molecular sizing methods such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and size-exclusion high performance liquid chromatography (SE-HPLC) can be used to rapidly identify fragmentation events in antibody drugs [59]. Fragmentation of full-length antibody drugs is a common occurrence and invariably affects their function. In general, cleaved forms are present in such low amounts that it is likely that little change in efficacy would be seen [60]. At the very least, the antibody drug may have some small reduction in efficacy following fragmentation or may be more prone to proteases, degradation and clearance events that would limit the duration of its efficacy. At the worst, a fragmented antibody preparation may result in a different biodistribution and thus a different safety profile. This can be of particular concern if the antibody is being used to delivery a cytotoxic chemotherapeutic agent. Fortunately, the extent of fragmentation in an antibody can be easily limited in the formulated product.

5. Strategies to stabilize antibody formulations

While liquid antibody formulations are less expensive, faster to develop and generally easier to prepare for administration than alternative formulation approaches, liquid antibody formulations are prone to oxidation, deamidation, aggregation and fragmentation as discussed above. In each of these events, water is the common culprit: water mediates electron transfer during oxidation and deamidation events and its required addition across a peptide bond can be a critical step in fragmentation. The thermodynamic stresses that lead to protein aggregation are caused by hydrophobic protein surfaces being exposed to water and trying to find a lower energy state by non-native protein–protein interactions. Thus, water is a big problem for stabilizing antibody-based drugs. Lyophilization or introduction into hydrophobic polymer systems can reduce the impact of water on antibody drug formulations.

5.1. Lyophilization

Although some antibodies have demonstrated a propensity to undergo precipitation as they are cooled from 37 °C [53], antibodies are typically robust to freeze/thaw cycles [61]. Early studies reported that antibodies were stable to the freeze-drying (lyophilization) process in the absence of excipients that function as cryoprotectants [62], however, with the technical advancement of certain analytical methodologies, became evident that antibodies are generally damaged in some way as a result of lyophilization as demonstrated by extensive formation of insoluble aggregates [61]. More recently, studies have shown that freeze-drying antibodies to a specific percent of residual water (usually between 1 and 8%) allows for optimal stabilization in the dry state and stability during reconstitution [63]. Higher moisture content in lyophilized antibody preparations correlates with increased aggregation (as seen by SEC) and asparagine deamidation and isomerization (as measured by HIC). Studies have also been performed to identify optimized lyophilization schedules where the secondary drying phase is performed at an elevated temperature [64]. This technique allows the lyophylized cake to achieve optimum moisture content, allowing the final product to be conformationally stable (as measured by DSC) during extended storage. Solid-state structural studies can be used to monitor conformational changes and aggregation events during the storage of a lyophilized monoclonal antibody preparation [65]. Improved native-like structure and a reduction in aggregation can be obtained by incorporation of a carbohydrate excipient in sufficient

Samsung Bioepis Exhibit No. 1002
Page 5496

quantities to fulfill the hydrogen bonding requirements on the protein surface, suggesting a critical role for non-water molecules to act as placeholders for water molecules during drying and in the dry state. That the addition of excess carbohydrate failed to improve the stabilizing function of this excipient, reaffirms the conclusion that antibodies have a limited number of sites that must be protected in this fashion [65]. A variety of carbohydrates or polyol compounds such as sucrose, trehalose and mannitol have been shown to provide this stabilizing function [66,67].

The rate of rehydration when water is reintroduced during reconstitution of lyophilized formulations is a critical parameter. As water is removed during the different drying stages of the lyophilization cycle and replaced by a shell of non-water excipients, the antibody drug can undergo structural modifications that result in a non-native conformation as may be seen by differential scanning calorimetry [63]. If the rate of rehydration is sufficiently slow to allow recovery of native conformation as water replaces non-water excipient molecules, reconstitution will typically provide a satisfactory outcome for the antibody. If, however, the antibody does not have sufficient time or capacity to recover its native state during reconstitution, extensive aggregation can result. Efforts to protect monoclonal antibodies from the rigors of freeze-drying and reconstitution have been aided by a number of analytical methods that can examine protein modifications. In the solid state, circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy may be used to evaluate the protein; after reconstitution in the liquid phase, SEC or SDS-PAGE have been utilized to gather information from lyophilization studies [68,69]. A microscopic apparatus to visually monitor freeze-drying events has even been described [70]. Excipients that have been reported to slow the reconstitution rate and thereby preventing such changes in the rehydrated antibody are glycerol [71,72] and polymeric materials such as polyethylene glycol [73].

Pharmaceutical scientists frequently use increased temperatures for accelerated formulation stability studies. Although such studies can be used to assess formulation parameters such as pH and ionic strength, antibodies lyophilized in the presence of a cryoprotective sugar excipient provide a more complicated picture. Lyophilized amorphous solids composed of a monoclonal antibody and a sugar cryoprotectant material such as sucrose or trehalose form glassy structures, liquids that are too viscous to flow. That antibody-excipient complexes exhibit a glass transition temperature (Tg) provides the basis for observations showing that these materials have unique properties above and below this value. Thus, in predicting shelf life in accelerated degradation studies, it is important to use testing temperatures that do not exceed the Tg threshold of the complex [67]. In general, lyophilized formulations containing trehalose may likely show less tendency toward antibody aggregation than those with sucrose, since trehalose preparations have a higher Tg [67]. With this in mind, a promising approach to assess secondary structural parameters in evaluating antibody formulation stability is the use of amide I band Raman spectroscopy. This method demonstrates a good correlation between structural protein perturbations immediately after lyophilization and the rate of aggregation during long-term storage under accelerated conditions [74].

5.2. Polymer delivery systems

Removal of water from antibody preparations, either by lyophilization or spray freeze-drying, provides a provides a starting point for incorporation of this dried material into a hydrophobic polymer matrix such as polylactide-co-glycolide (PLGA) that can be made into microspheres generated through a solid-in-oil-in-water (S/O/W) encapsulation process [75]. Although both mannitol and trehalose can stabilize human IgG during spray freeze-drying, the double-emulsion solvent evaporation method used to load spray freeze-dried human IgG into PLGA microspheres has been shown to produce extensive antibody aggregation [75]. Such an outcome is likely the result of thermodynamic stress experienced at the water/organic solvent interface and a variety of process methods and excipients can be use to minimize this concern [76].

One feature of PLGA microparticle preparations is their recognition by immune elements and their capacity to stimulate immunization against incorporated proteins [77–79]. While this has clear advantages for vaccines, it poses a serious concern for therapeutic protein delivery. Hydrophobic microparticles have been reported to incite local inflammation

Samsung Bioepis Exhibit No. 1002
Page 5497

at subcutaneous injection sites [80], events that may be partially mediated through macrophage recruitment [81]. Not only is the potential for antibody formation increased with local inflammation, the resulting injection site reaction may be clinically unacceptable.

In spite of some serious caveats, polymeric microspheres and delivery systems can be successful antibody formulations if administered to the appropriate sites, using certain types of polymers or preparing microspheres or polymeric formulations by methods that are more protective of the antibody. An example of a certain delivery site that may have less proclivity towards an inflammatory response is reported, where PLGA microspheres loaded with an anti-VEGF antibody were injected into the intravitreal humor of the eye, an immune-privileged site, for the treatment of age related macular degeneration (AMD) [82]. Resorbable polymers that show less potential to incite inflammation, e.g., have smoother surface characteristics, might be used to deliver antibodies in a sustained manner. Direct, local delivery of antibodies using carboxymethylcellulose aqueous gels can be an effective post-surgical anti-infective strategy [83]. Antibodies covalently coupled to a biodegradable hyaluronic acid hydrogel through a labile hydrazone linkage have been used for the sustained delivery of antibodies to sites within the central nervous system [84]. Introduction of dry powder IgG into ethylene–vinyl acetate copolymer (EVAc) has also been examined as a potential polymer-based antibody delivery system [85]. Similar to PLGA systems, EVAc microspheres can extend the time course of IgG release. This property makes polymers loaded with antibodies interesting opportunities for injectable sustained release delivery formats but also for topical application at mucosal surfaces. For example, long-term vaginal delivery of an antibody can be achieved in a mouse model using polymer vaginal rings [86,87]. Formulations of a biomedical grade polyurethane hydrogel with an antibody coating have also been prepared and shown to release bioactive protein [88]. It is important to note that one complicating aspect of many of these approaches is the requirement of a polymer solubilization step using a solvent, such as methylene chloride or isopropanol, which can compromise protein drug stability. Sustained release formulation approaches such as microspheres prepared using water, such as the PROMAXX microspheres (Epic Therapeutics) or crystallization of proteins [89] can bypass the use of solvents that are incompatible with proteins and antibodies.

6. Formulation issues for alternative forms of antibody delivery

To date, antibody development has focused primarily on injectable routes of administration: intravenous (IV), intramuscular (IM) and subcutaneous (SC). A long history of IV antibody infusions exists [90] and the advantages are clear for antibody-based drugs that are used in cancer treatments in order to reach tumor sites that may be diffuse or not otherwise accessible. In fact, the majority of the approved antibody-based drugs are administered by IV infusion (Table 1) with the total dose administered approaching one gram of protein per treatment in some cases. For many antibody-based therapies a critical safety concern involves the onset of acute or delayed systemic reactions following infusion [91,92]. Parameters and characteristics of the "infusion reaction" as well as treatment protocols have been well described with acute events commonly occurring within 10 min to 4 h and delayed events occurring 24 h to 4 days post dosing [93]. Although formation of antibodies to the administered antibody drug appear to be a risk factor for infusion reaction events [94], infusion reactions can occur following the initial exposure [93] and these reactions do not appear to be due to IgE responses or mast cell activation [95]. Most importantly for the patient, there appear to be treatment protocols to provide re-treatment of patients even after an infusion reaction event [93] with concomitant administration of immunomodulation molecules [94]. There are many unanswered questions about the mechanism and prevention of adverse infusion reaction events associated with IV infusion of antibody drugs. Certainly, one method is to explore IM and SC delivery when possible, although these delivery approaches may also have confounding aspects of tissue response and volume limitations.

Other routes are also amenable to antibody delivery. The increased number of antibody therapeutics on the market and the greater exploration of non-life threatening indications being treated by antibodies, drive efforts to deliver these agents by routes other than IV

Samsung Bioepis Exhibit No. 1002
Page 5498

Table 1
Formulations of approved products

Antibody product; company	Generic name; description	Delivery route	Formulation concentration	Buffer components; pH[a]	*Excipients*
Othroclone OKT3®; J & J-Ortho Biotech	Muromonab-CD3; Murine IgG2a, anti-CD3	IV	1 mg/ml	Na,K phosphate; pH 7	NaCl, polysorbate 80
Reopro®; Centocor-Lilly	Abciximab; Chimeric IgG1, anti-GPIIb/IIIa, Fab	IV	2 mg/ml	Na phosphate; pH 7.2	NaCl, polysorbate 80
Rituxan®; Idec-Genentech	Rituximab; Chimeric IgG1, anti-CD20	IV	10 mg/ml	Na citrate; pH 6.5	NaCl, polysorbate 80
Zenapax®; Roche	Daclizimab; Humanized IgG1, anti-CD25	IV	5 mg/ml	Na phosphate; pH 6.9	NaCl, polysorbate 80
Simulect; Novartis[b]	Basilixamab; Chimeric IgG, anti-CD25	IV	4 mg/ml	Na,K phosphate, glycine	NaCl, sucrose, mannitol, polysorbate 80
Synagis®; MedImmune[b]	Palivizumab; Humanized IgG1, anti-RSV	IM	100 mg/ml	Histidine, glycine	Mannitol
Remicade®; Centocor[b]	Infliximab; Chimeric IgG1, anti-TNFα	IV	10 mg/ml	Na phosphate; pH 7.2	Sucrose, polysorbate 80
Herceptin®; Genectech[b]	Trastuzumab; Humanized IgG1, anti-HER2	IV	21 mg/ml	Histidine; pH 6	Trehalose, polysorbate 20, benzyl alcohol[c]
Mylotarg®; Wyeth[b]	Gemtuzumab ozogamicin; Humanized IgG4, anti-CD33, immunotoxin	IV	4 mg/ml	Na phosphate	NaCl, sucrose, dextran 40
Campath®-1H; Millennium-ILEX	Alemtuzumab; Humanized IgG1, anti-CD52	IV	Unclear — 30 mg /vial	Na,K-phosphate pH 7	NaCl, KCl, NaEDTA, polysorbate 80
Zevalin®; Biogen Idec	Ibritumomab tiuxetan; Murine IgG1, anti-CD20; radiolabeled (^{90}Y or ^{111}In)	IV[d]	1.6 mg/ml	Na Acetate, Na, K-phosphate pH 7	NaCl, human albumin
Humira®; Abbott	Adalimumab; Human IgG1, anti-TNFα	IV	50 mg/ml	Na phosphate, Na Citrate; pH 5.2	Mannitol, polysorbate 80
Xolair®; Genentech[b]	Omalizumab; Humanized IgG1, anti-IgE	SC	125 mg/ml	Histidine	Sucrose, polysorbate 20
Bexxar®; Corixam-GSK	Tositumomab-I131; Murine IgG2a, anti-CD20, radiolabeled (^{131}I)	IV	14 mg/ml	Na phosphate; pH 7.2	NaCl, maltose
Raptiva®; Genentech	Efalizumab; Humanized IgG1, anti-CD11a	SC	100 mg/ml	Histidine; pH 6.2	Sucrose, polysorbate 20
Erbitux®; Imclone Systems	Cetuximab; Chimeric IgG1, anti-EGFR	IV	2 mg/ml	Na phosphate; pH 7.2	NaCl
Avasatin®; Genentech	Bevacizumab; Humanized IgG1, anti-VEGF	IV	25 mg/ml	Na phosphate; pH 6.4	Trehalose, polysorbate 20
Tysabri; Biogen Idec	Natalizumab; Humanized IgG4, anti-4α-integrin	IV	20 g/ml	Na phosphate; pH 6.1	NaCl; polysorbate 80

Ordered by approval dates in the US.
Abbreviations: RSV=respiratory syncytial virus; TNFα=tumor necrosis factor alpha; EGFR=epidermal growth factor receptor; VEGF=vascular endothelial growth factor; NaEDTA=sodium edentate.
[a] Some lyophilized formulations do not stipulate a pH (or range) following reconstitution.
[b] Lyophlized product, concentration, pH, etc. values are obtained after reconstitution.
[c] Added as a preservative to generate a multi-dose vial configuration.
[d] Kit where components are mixed just prior to administation.

infusion. These routes may be more effective, less invasive, provide easier delivery, permit self-administration and thereby be more convenient and possibly have lower costs if less active agent can be used, as in the case where the alternate route of administration delivers the therapeutic agent to the intended site directly. Interestingly, some studies have suggested that antibodies administered by IV infusion are more

Samsung Bioepis Exhibit No. 1002
Page 5499

susceptible to proteolysis than via IM injection [59] and sucrose-stabilized immunoglobulins have been associated with acute renal failure following IV infusion [96]. The antibody used to protect neonates from respiratory syncytial viral (RSV) infection (Synagis®) is one example of an antibody administered by IM injection. For reasons discussed above, the majority of antibody-based drugs have moved toward SC delivery with formulations efforts being focused to this end. Based upon the desired actions for a number of antibody-based drugs, however, routes of administration other than IV, IM or SC may be desirable. These alternative routes can provide unique formulation and stability issues.

6.1. Oral

Uptake of antibodies from the intestinal lumen can occur through fetal Fc receptors (FcRn) expressed at the apical surface of enterocytes [97]. This natural antibody uptake mechanism was initially identified in the gastrointestinal (GI) tract of neonatal rodents and its expression shown to vanish following "closure" of the intestine after weaning. It was only recently that the continued expression of this receptor in the adult intestine was demonstrated and this finding has prompted efforts to examine this receptor system as a means of delivery antibodies after oral administration. Although this pathway appears to be a viable route for the delivery of therapeutic antibodies, issues associated with antibody stability in the GI tract [98] and competition from endogenous antibodies can dramatically reduce the efficiency of uptake after oral delivery. Outside of this specific mechanism, no other transcellular mechanism of efficient antibody transport across epithelial cell of the GI tract has been demonstrated to date. Topical application of antibodies to produce a desired clinical outcome have been shown, however, with the oral delivery of a chicken-derived (IgY) antibodies for treating and preventing infectious diseases of the GI tract [98–100]. It should be noted that IgY antibodies have been shown to not bind to (or be transported by) FcRn [97].

6.2. Respiratory tract

Intranasal and pulmonary administration of protein therapeutics has shown more promising clinical outcomes than oral delivery. While the surfaces of both the respiratory tract and the gastrointestinal tracts are readily accessible, the respiratory tract is a far less hostile environment for an administered protein therapeutic. Nonetheless there are physical clearance mechanisms in place to remove foreign particles which act to reduce residence time dramatically following administration. In the case of nasal administration the use of a cream emulsion and a polymer delivery system has been shown to significantly decrease the clearance rate for a monoclonal antibody when applied to the nasal mucosa [101]. This study provides one approach for the topical application of a therapeutic antibody designed to provide clinical benefit at the mucosal surface. It does not, however, suggest that an appreciable amount of an antibody might be absorbed into the systemic circulation from this site. The nasal mucosa affords only a small absorptive surface area compared to the epithelial linings of the lung.

Aerosol delivery of antibodies to the lung has been explored as either a liquid or dry powder formulation, where in both cases the droplets or particles must be of sufficiently small size that they are respirable perhaps to the level of the deep lung but large enough that they are not readily exhaled or just impact the back of the throat [102]. The sizes of particles that provide this performance have a mass mean aerodynamic diameter and density such that they enter the alveoli and remain in the lung. This size window has been reported to be between 1 to 5 μm depending on the formulation used and in one case, far larger, 20 μm where the particles are dense (<0.4 g/cm^3) and highly porous [102].

Formulation efforts were made for producing fine dry powder particles of an anti-IgE monoclonal antibody (E25) in the size range desired for delivery to the lung [103]. These studies showed that sodium phosphate was capable of extending the usefulness of mannitol as a protective excipient in spray-dried antibody formulations and that mannitol can play a role both in maintaining protein stability as well as producing a suitable aerosol preparation. Further studies to assess carbohydrate excipients for spray-dried aerosol E25 formulations showed that excipient to protein ratios were critical. Degradation issues involved not only protein aggregation but also protein glycation or lactosylation, a protein modification

Samsung Bioepis Exhibit No. 1002
Page 5500

event where a reducing sugar can add covalently at a free amine [104]. The ability to formulate and delivery an antibody-based drug to the lung provides the potential for systemic delivery of that protein via uptake through the FcRn expressed at this mucosal surface. Elegant studies showing the distribution and function as well as the application of this transport route for systemic deliver of an Fc-containing protein chimera has been demonstrated [105,106]. Thus, the potential or systemic delivery of antibody-based drugs via the respiratory tract looks promising.

6.3. Miscellaneous delivery sites of the body

Unique aspects of antibody–polymer formulations designed for intravitreal injection [82] or topical vaginal delivery [86,87] have been discussed above. Additionally, antibody formulations using hyaluronic acid hydrogels for sustained brain delivery have been noted [84]. These methods focus on strategies to administer large amounts of an antibody at a specific site in order to provide a sustained level to improve efficacy and/or to minimize injection frequency. Other strategies have been described to deliver antibodies to miscellaneous sites throughout the body, For example, an anti-transferrin antibody (OX-26) has been described as a carrier for delivery of covalently attached therapeutic molecules across the blood–brain barrier following IV injection [107]. An anti-tumor necrosis factor antibody has been adsorbed onto cardiovascular stent wires for local neutralization of this cytokine in order to limit restenosis events following stent placement [108]. A CMC gel containing antibodies to prevent post-surgical infections has been administered to the intraperitoneal cavity [83].

6.4. Intracellular targeting

Some promising antibody drug targets are intracellular. Although techniques exist where a cell can synthesize an antibody and secrete it into the cell cytoplasm, successful use of this technology requires gene therapy. Instead, it is possible to chemically couple an antibody to a pH-sensitive polymer that functioned to disrupt membranes at acidic pH. Many antibodies are internalized by cells through a receptor-mediated uptake process that leads to their delivery into early and then late endosomes. During endosomal maturation after receptor–antibody internalization the pH of these vesicles will drop from near neutral to below pH 6. This approach was demonstrated in a format where the pH-responsive polymer poly(propylacrylic acid) was covalently attached to a monoclonal antibody to facilitate its delivery to the cytoplasm of target cells [12].

7. Other delivery issues

A wide range of novel drugs and drug conjugates are being developed using monoclonal antibodies and engineered antibody fragments [20]. For some of these novel therapeutic approaches to be successful, they require additional or non-traditional delivery strategies. A cursory discussion of some of these approaches as they relate to intracellular targeting, site placement and engineered antibodies fragments follows.

7.1. Antibody fragments

An entire spectrum of antibody fragments have been identified through protein engineering studies with many of these showing remarkable potential for unique and novel clinical opportunities [109]. These smaller recombinant antibody fragments, the monovalent antibody fragments, Fab and single chain Fv and the engineered variants, diabodies, triabodies, minibodies and single-domain antibodies are an emerging form of antibody therapy that are less costly to manufacture while still retaining the targeting specificity of full length antibodies. In fact some may have greater efficacy and more applications than whole monoclonal antibodies. It may be anticipated that this new subset of antibodies are posing new formulation challenges, however, to date, publications detailing stability studies and formulation development for these molecules are rare in the scientific literature so formulation data on antibody fragments is scant. The single V domains have been found to be poorly soluble and have a tendency to aggregate [109], but these problems are being overcome by the identification of mutants that minimize the hydrophobic interface and selected by phage display [110]. Five Fab molecules have been approved as of this writing with the one humanized

Samsung Bioepis Exhibit No. 1002
Page 5501

Fab (ranibizumab rhFab) awaiting approval in the very near future, but many are in early clinical trials and most in preclinical development. One study has been described that focused on Fab fragments. In general, the binding of an antibody to an effector protein does not necessarily inactivate that target's function [111]. When an antibody is effectively inactivating, however, the Fab fragments in the absence of an Fc domain can frequently function as highly potent binding agents capable of masking binding sites or enzymatic elements on macromolecules. For example, Fab fragments prepared from sheep anti-snake venom antibodies (Digibind, from GSK) can be an effective anti-venom. Storage of liquid formulations of such anti-venoms in sub-tropical countries where refrigeration may not be possible provides a significant formulation challenge. Although lyophilized products can perform well under these conditions, a pH 4.0 (acetate buffer) formulation was found to also be stable and potent for at least one year at room temperature [112], presumably room temperatures characteristic of the tropical and subtropical regions where the material would be used. Strikingly, this stable formulation did not require the presence of a protective carbohydrate excipient. To improve drug delivery, antibody fragments have been fused to many types of molecules, such as radionuclides, cytotoxic drugs, toxins, peptides and proteins, enzymes and liposomes [113]. An immunoliposome made with a Mab fragment is reported to deliver drugs to the brain as such a molecule is able to cross the formidable barrier of the blood brain barrier [114].

7.2. *Immunoconjugates*

Arming antibodies with materials useful for detection or cell killing has become a prominent approach to increase the utility of these proteins, reviewed by Wu and Senter, [20]. Antibodies provide a useful format for the production of radiopharmaceuticals for site- or tissue-specific localization using gamma scintigraphy [10,115] or for enhanced cell killing. There are currently three approved antibody–radionuclide drugs: two are murine radiolabeled Ab for B cell lymphomas ibritumomab tuixetan (^{90}Y, Zevalin) and tositumomab (^{131}I Bexxar) and the third is a humanized, gemtuzumab ozogamicin (calicheamicin, Mylotarg) for leukemia. The presence of a high energy emitting radionuclide, however, will compromise long-term stability of any antibody formulation; not only would the released energies act to damage the protein but there is strong preference to use short half-life isotopes for these procedures [116]. Thus, in these instances, a stable formulation based upon the principles discussed above can provide a product that can be radiolabeled and then used soon after this step. One example of this approach is a lyophilized antibody formulation that is reconstituted and prepared by a kit method prior to patient dosing [117].

Antibodies coupled with cytotoxins for targeted cell killing [118] are particularly useful in antibody-based cancer therapies [119]. Beyond the identification of a stable formulation for the antibody, such materials have several additional problems for the formulation scientist to solve: stability issues of the attached agent and stability of the linker used to join the attached agent to the antibody. In the case of a monoclonal antibody-vinca alkaloid conjugate, a pH range of 4.5–5.5 in a phosphate buffer showed improved solution stability compared to material stored at pH 6.5–7.4 as evidenced by antibody aggregation and hydrolysis of the chemical linker used to conjoin the alkaloid to the antibody [120]. In most cases, cytotoxic materials are coupled to antibodies using reversible chemistries to provide a release mechanism for maximal activity at the target site. Many of these antibody conjugates use a disulfide bond linkage that can be reduced by a novel redox enzyme present in endosomes and lysosomes of target cells [121]. Slightly oxidizing conditions that would provide added stability of this linker in the formulation could, over time, act to cause detrimental oxidation events in the antibody and/or the linked compound. Other conjugates use an acid-labile linker [122]. In this case, formulation pH must be restricted to either neutral or alkaline conditions. Enzymatic separation of antibody and a cytotoxic material could also be achieved by coupling the two through an amino acid linker that is recognized as a substrate by a specific protease or peptidase [123]. Formulation instability of this linker sequences can be corrected to some extent by selective changes of the amino acids used.

Thus, the challenges of immunoconjugates include maintaining both drug and antibody potency and stability. While some antibodies are efficacious by

Samsung Bioepis Exhibit No. 1002
Page 5502

themselves, many are much more therapeutically active after being armed with a toxin, drug and radionuclide [124]. These changes make the resulting product more difficult to prepare, stabilize and deliver. Yet, there is tremendous enthusiasm for these approaches as they provide the promise for some companies to transform their antibody into a potentially curative drug. For this reason many antibodies are combined with a generic delivery system, such as a liposome, that can be used to delivery a variety or even multiple agents simultaneously through antibody-directed targeting [125]. Examples of such an approach include studies performed using an antibody directed against the Her2/neu antigen to delivery liposomes containing a chemotherapeutic have been described [126], antibodies used to deliver cationic liposomes for the administration of nucleic acid material for gene therapy [127] and antibodies used in the targeted delivery of modified gelatin nanoparticles [128] that can be coupled through a disulfide linkage [129].

8. Conclusions — challenges and opportunities

As antibody-based drugs come to the forefront of therapeutics derived from biotechnology, they provide a range of new and exciting opportunities to treat a number of currently unmet medical needs. Successful clinical application of these novel agents requires the development of stable formulations that can be used for specific delivery methods. Antibodies, because of their endogenous nature, have built-in features that may pose problems for stability as biotherapeutics, that function in the normal clearance of these proteins from the body [40]. Thus, despite our best efforts to prepare stable antibody-based drug formulations, the ultimate stability of these products may be limited to inherent mechanisms of degradation pre-designed for the rate of clearance optimal for their function and turnover in the body. Protein engineering has led to significant advances in improving some of these parameters and the trend would predict that additional advances will be made in the near future (reviewed in [109]). Protein modifications can be engineered such that stability and efficacy can be improved in ways that cannot be achieved solely, if at all, with formulation alterations. While it is clear that more research is needed in the relatively new area of engineered antibody fragments for clinical use [109], they have great potential for diagnostic and therapeutic application.

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. Added to this is the potential use of a surface active agent to help with liquid stability such as a non-ionic detergent at a low concentration [58] as well as the possible addition of preservatives required for multi-use systems [130].

The future of antibody based drugs is likely to include more complex systems than those currently approved for use in man. Possibly the most critical aspect of generating a pharmaceutically successful formulation for antibody based drugs is an understanding the critical aspects of stability for the protein or the materials associated with the protein. It has been known for some time that even a single amino acid change in the Fv region of an antibody can result in dramatic differences in antigen-binding specificity [31]. Also, alterations at specific amino acids in the Fc domain can dramatically change functional aspects of an antibody. Thus, these kinds of changes must be considered more important in the search for a successful formulation than those changes that do not appear to affect function. In the case of antibody or antibody-like agents being used to delivery a payload such as a toxin or chemotherapeutic, the stability of the material to be delivered and its continued association with the delivery antibody is crucial.

Samsung Bioepis Exhibit No. 1002
Page 5503

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Samsung Bioepis Exhibit No. 1002
Page 5507

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Samsung Bioepis Exhibit No. 1002
Page 5508

MINIREVIEW

Antibody Structure, Instability, and Formulation

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ABSTRACT: The number of therapeutic monoclonal antibody in development has increased tremendously over the last several years and this trend continues. At present there are more than 23 approved antibodies on the US market and an estimated 200 or more are in development. Although antibodies share certain structural similarities, development of commercially viable antibody pharmaceuticals has not been straightforward because of their unique and somewhat unpredictable solution behavior. This article reviews the structure and function of antibodies and the mechanisms of physical and chemical instabilities. Various aspects of formulation development have been examined to identify the critical attributes for the stabilization of antibodies. © 2006 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 96:1–26, 2007

Keywords: biotechnology; stabilization; protein formulation; protein aggregation; freeze drying/lyophilization

INTRODUCTION

Protein therapies are entering a new era with the influx of a significant number of antibody pharmaceuticals. Generally, protein drugs are effective at low concentrations with less side effects relative to small molecule drugs, even though, in rare cases, protein-induced antibody formation could be serious.[1] Therefore, this category of therapeutics is gaining tremendous momentum and widespread recognition both in small and large drug firms. Among protein drug therapies, antibodies play a major role in controlling many types of diseases such as cancer, infectious diseases, allergy, autoimmune diseases, and inflammation. Since the approval of the first monoclonal antibody (MAb) product -OKT-3 in 1986, more than 23 MAb drug products have entered the market (Tab. 1). The estimated number of antibodies and antibody derivatives constitute 20% of biopharmaceutical products currently in development (about 200).[2] The global therapeutic antibody market was predicted to reach $16.7 billion in 2008.[3]

There are several reasons for the increasing popularity of antibodies for commercial development. First, their action is specific, generally leading to fewer side effects. Second, antibodies may be conjugated to another therapeutic entity for efficient delivery of this entity to a target site, thus reducing potential side effects. For instance, Mylotarg is an approved chemotherapy agent composed of calicheamicin conjugated to humanized IgG4, which binds specifically to CD33 for the treatment of CD33-positive acute myeloid leukemia. Another example is the conjugation of immunotoxic barnase with the light chain of the anti-human ferritin monoclonal antibody F11 as potential targeting agents for cancer immunotherapy.[4] Third, antibodies may be conjugated to radioisotopes for specific diagnostic purposes. Examples include CEA-Scan for detection of colorectal cancer and ProstaScint for detection of prostate cancer. Lastly, technology advancement has made complete human MAb available, which are less immunogenic.

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Samsung Bioepis Exhibit No. 1002
Page 5509

Table 1. Commercial Monoclonal Antibody Products

#	Brand name	Molecule	MAb	Year	Company	Route	Indication	MAb Conc	Buffer	Excipients	Surfactant	pH
1	Avastin	Bevacizumab	Humanized IgG1, 149 kDa	2004	Genetech and BioOncology	IV infusion	Metastatic carcinoma of colon or rectum, binds VEGF	100 mg and 400 mg/vial (25 mg/mL) solution	5.8 mg/mL monobasic NaPhos H_2O; 1.2 mg/mL dibasic NaPhos anhydrous (4 mL, 16 mL fill in vial)	60 mg/mL α-Trehalose dihydrate (4 mL, 16 mL fill in vial)	0.4 mg/mL PS20 (4 mL, 16 mL fill in vial)	6.2
2	Bexxar	Tositumomab and I-131Tositumab	Murine IgG2λ	2003	Corixa and GSK	IV Infusion	CD20 positive follicular nonHodgkins lymphoma	Kit: 14 mg/mL MAb solution in 35 mg and 225 mg vials; 1.1 mg/mL *I131-MAb* solution	10 mM phosphate (MAb vial)	145 mM NaCl, 10 % w/v Maltose; *I131-MAb:* 5–6% Povidone, 1–2, 9–15 mg/mL Maltose, 0.9 mg/mL NaCl, 0.9–1.3 mg/mL Ascorbic acid		7.2
3	Campath	Alemtuzumab	Humanized, IgG1k, 150 kDa	2001	Ilex Oncology; Millenium and Berlex	IV infusion	B-cell chronic lymphocytic leukemia, CD52-antigen	30 mg/3 mL solution	3.5 mg/3 mL dibasic NaPhos, 0.6 mg/3 mL monobasic KPhos	24 mg/3 mL NaCl, 0.6 mg/3 mL KCl, 0.056 mg/3 mL Na2EDTA	0.3 mg/3 mL PS80	6.8–7.4
4	CEA-Scan (lyo)	Arcituomab; Tc-99	Murine Fab, 50 kDa	1996	Immunomedics	IV injection or infusion	Imaging agent for colorectal cancer	1.25 mg/vial Lyophilized MAb. Reconstitute w 1 mL Saline w Tc99m	0.29 mg/vial Stannous chloride, potassium sodium tartrate tetrahydrate, NaAcetate.$3H_2O$, NaCl, glacial acetic acid, HCl	Sucrose		5.7
5	Erbitux	Cetuximab	Chimeric human/ mouse IgG1k, 152 kDa	2004	ImClone and BMS	IV infusion	Treatment of EGFR-expressing colorectal carcinoma	100 mg MAb in 50 mL; 2 mg/ mL solution	1.88 mg/mL DibasicNaPhos · $7H_2O$; 0.42 mg/mL MonobasicNaPhos · H_2O	8.48 mg/mL NaCl		7.0–7.4
6	Herceptin (lyo)	Trastuzumab	Humanized IgG1k	1998	Genetech	IV infusion	Metastatic breast cancer whose tumor overexpress HER2 protein	440 mg /vial, 21 mg/mL after reconstitution	9.9 mg/20 mL L-HistidineHCl, 6.4 mg/20 mL L-Histidine	400 mg/20 mL α-Trehalose Dihydrate	1.8 mg/20 mL PS20	6
7	Humira	Adalimumab	Human IgG1k, 148 kDa	2002	CAT and Abbott	SC	RA patients not responding to DMARDs. Blocks TNF-alpha	40 mg/0.8 mL solution (50 mg/mL)	0.69 mg/0.8 mL Monobasic NaPhos · $2H_2O$; 1.22 mg/0.8 mL DibasicNaPhos · $2H_2O$; 0.24 mg/0.8 mL NaCitrate, 1.04 mg/ 0.8 mL Citric acid · H_2O	4.93 mg/0.8 mL NaCl; 9.6 mg/0.8 mL Mannnitol	0.8 mg/0.8 mL PS80	5.2
8	Lucentis	Ranibizumab	Humanized IgG1k fragment	2006	Genentech	Intravitreal injection	Age-related macular degeneration (wet)	10 mg/mL solution	10 mM HistidineHCl	10% α-Trehalose-Dihydrate	0.01% PS20	5.5

DOI 10.1002/jps

Samsung Bioepis Exhibit No. 1002
Page 5510

9	Mylotarg (lyo)	Gemtuzumab ozogamicin	Humanized IgG4k conjugated with calicheamicin	2000	Celltech and Wyeth	IV infusion	Humanized Ab linked to calicheamicin for treatment of CD33 positive acute myeloid leukemia	5 mg protein-equivalent lyophilized powder/20-mL vial	Monobasic and dibasic NaPhosphate	Dextran 40, Sucrose, NaCl		
10	OncoScint	Satumomab pendetide	Murine IgG1k conjugated to GYK-DTPA	1992	Cytogen	IV injection	Imaging agent for colorectal and ovarian cancer	0.5 mg conjugate/mL solution (2 mL per vial)	Phosphate buffer saline			6.0
11	Orthoclone OKT	Muromomab-CD3	Murine, IgG2a, 170 kDa	1986	Ortho Biotech	IV injection	Reversal of acute kidney transplant rejection (anti CD3-antigen)	1 mg/mL solution	2.25 mg/5 mL monobasic NaPhos, 9.0 mg/5 mL dibasic NaPhos	43 mg/5 mL NaCl	1 mg/mL PS80	7 ± 0.5
12	ProstaScint	Indium-111 capromab pendetide	Murine IgG1k-conjugated to GYK-DTPA	1996	Cytogen	IV injection	Imaging agent for prostate cancer	0.5 mg conjugate/mL solution (1 mL per vial)	Phosphate buffer saline			5–7
13	Raptiva (lyo)	Efalizumab	Humanized IgG1k	2003	Xoma and Genentech	SC	Chronic moderate to severe plaque psoriasis, binds to CD11a subunit of LFA-1	150 mg MAb/vial; 125 mg/ 1.25 mL (100 mg/mL) after reconstitution with 1.3 mL SWFI	6.8 mg/vial L-HistidineHCl · H_2O; 4.3 mg/vial L-Histidine	123.2 mg/vial Sucrose	3 mg/vial PS20	6.2
14	Remicade (lyo)	Infliximab	Chimeric human/ murine MAb against TNFalpha (app. 30% murine, 70% corresponds to human IgG1 heavy chain and human kappa light chain constant regions)	1998	Centocor	IV infusion	RA and Crohn's disease (anti TNFalpha)	100 mg / 20-mL Vial, 10 mg/mL on reconstitution	2.2 mg/10 mL MonobasicNaPhos H_2O, 6.1 mg/10 mL DibasicNaPhos · $2H_2O$	500 mg/10 mL Sucrose	0.5 mg/10 mL PS80	7.2
15	ReoPro	Abciximab	Fab, Chimeric human-murine, 48 kDa	1994	Centocor / Lilly	IV injection and infusion	Reduction of acute blood clot related complications	2 mg/mL solution	0.01 M NaPhosphate	0.15 M NaCl	0.001% (0.01 mg/ mL) PS80	7.2
16	Rituxan	Rituximab	Chimeric mouse/ human IgG1k with murine light and heavy chain variable region (Fab domain), 145 kDa	1997	IDEC and Genentech	IV infusion	NonHodgkin's lymphoma. (anti CD20-antigen)	10 mg/mL solution	7.35 mg/mL NaCitrate · $2H_2O$	9 mg/mL NaCl	0.7 mg/mL PS80	6.5

(*Continued*)

Samsung Bioepis Exhibit No. 1002
Page 5511

Table 1. (*Continued*)

#	Brand name	Molecule	MAb	Year	Company	Route	Indication	MAb Conc	Buffer	Excipients	Surfactant	pH
17	Simulect (lyo)	Basiliximab	Chimaric IgG1k, 144 kDa	1998	Novartis	IV injection and infusion	Prevention of acute kidney transplant rejection, IL-2 receptor antagonist	10 mg and 20 mg/ vial, 4 mg/mL on reconstitution	3.61 mg, 7.21 mg Monobasic KPhos; 0.50 mg, 0.99 mg Na_2HPO_4	0.8 mg, 1.61 mg NaCl; 10 mg, 20 mg Sucrose; 40 mg, 80 mg Mannitol; 20 mg 40 mg Glycine		
18	Synagis (lyo)	Palivizumab	Humanized IgG1k, CDR of murine MAb 1129, 148 kDa	1998	MedImmune	IM injection	Prevent replication of the Respiratory syncytial virus (RSV)	50 mg and 100 mg / vial, 100 mg/mL on reconstitution	47 mM Histidine, 3.0 mM Glycine	5.6 % Mannitol		
19	Tysabri	Natalizumab	Humainzed IgG4k	2004	Biogen IDEC	IV Infusion	MS relapse	300 mg/15 mL solution	17.0 mg MonobasicNa Phos·H_2O, 7.24 mg diBasicNaPhos·$7H_2O$ for 15 mL	123 mg/15 mL NaCl	3.0 mg/15 mL PS80	6.1
20	Verluma	Nofetumomab	Murine Fab	1996	Boehringer Ingelheim and DuPont Merck	IV injection	Imaging agent for lung cancer	10 mg/mL solution	Phosphate buffer saline			?
21	Xolair (lyo)	Omalizumab	Humanized IgG1k, 149 kDa		Genentech w Novartis and Tanox	SC	Asthma, inhibits binding of IgE to IgE receptor FCeRI	202.5 mg/vial, Deliver 150 mg/ 1.2 mL on reconstitution with 1.4 mL SWFI	2.8 mg LHistidineHCl·H_2O; 1.8 mg LHistidine	145.5 mg Sucrose	0.5 mg PS20	
22	Zenapax	Daclizumab	Humanized IgG1, 144 kDa	1997	Roche	IV infusion	Prophylaxis of acute organ rejection in patients receiving renal transplants. Inhibits IL-2 binding to the Tac subunit of IL-2 receptor complex	25 mg/5 mL MAb Solution	3.6 mg/mL MonobasicNa Phos·H_2O; 11 mg/mL DibasicNaPhos·$7H_2O$	4.6 mg/mL NaCl	0.2 mg/mL PS80	6.9
23	Zevalin	Ibritumomab-Tiuxetan	Murine IgG1k-thiourea covalent linkage to Tiuxetan		IDEC	IV infusion	CD20 antigen. (Kit with Ytterium-90 induces cellular damage by beta emission)	3.2 mg/2 mL solution		09% NaCl		7.1

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Samsung Bioepis Exhibit No. 1002
Page 5512

Development of commercially viable antibody pharmaceuticals has, however, not been straightforward. This is because the behavior of antibodies seems to vary, even though they have similar structures. In attempting to address some of the challenges in developing antibody therapeutics, Harris et al.[5] reviewed the commercial-scale formulation and characterization of therapeutic recombinant antibodies. In a different review, antibody production and purification have been discussed.[2] Nevertheless, the overall instability and stabilization of antibody drug candidates have not been carefully examined in the literature. This article, not meant to be exhaustive, intends to review the structure and functions of antibodies, discuss their instabilities, and summarize the methods for stabilizing/formulating antibodies.

ANTIBODY STRUCTURE

Antibodies (immunoglobulins) are roughly Y-shaped molecules or combination of such molecules (Fig. 1). Their structures are divided into two regions—the variable (V) region (top of the Y) defining antigen-binding properties and the constant (C) region (stem of the Y), interacting with effector cells and molecules. Immunoglobulins can be divided into five different classes —IgA, IgD, IgE, IgM, and IgG based on their C regions, respectively designated as α, δ, ε, μ, and γ (five main heavy-chain classes).[6] Most IgGs are monomers, but IgA and IgM are respectively, dimmers and pentamers linked by J chains. IgGs are the most abundant, widely used for therapeutic purposes, and their structures will be discussed as antibody examples in detail.

Primary Structure

The structure of IgGs have been thoroughly reviewed.[6] The features of the primary structure of antibodies include heavy and light chains, glycosylation, disulfide bond, and heterogeneity.

Heavy and Light Chains

IgGs contain two identical heavy (H, 50 kDa) and two identical light (L, 25 kDa) chains (Fig. 1). Therefore, the total molecular weight is approximately 150 kDa. There are several disulfide bonds linking the two heavy chains, linking the heavy and light chains, and residing inside the chains (also see next section). IgGs are further divided

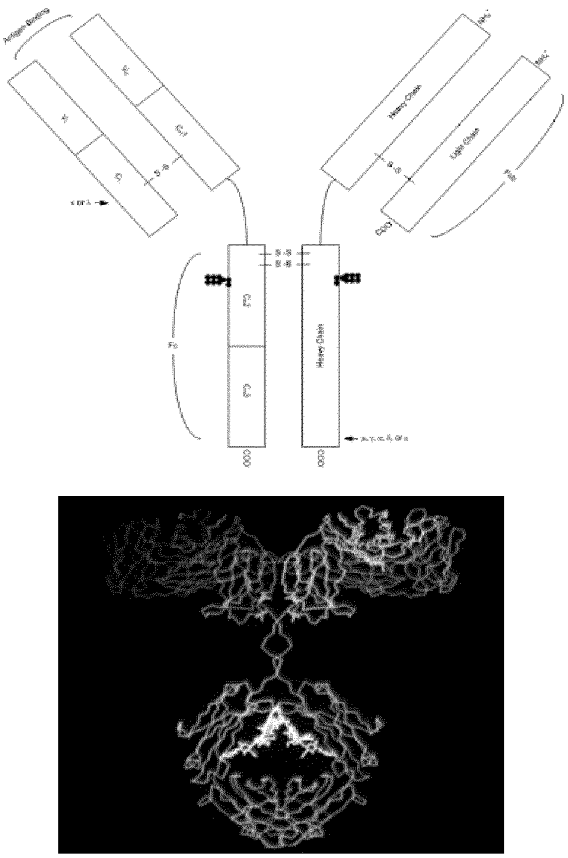


Figure 1. Linear (upper panel) and steric (lower panel) structures of immunoglobulins (IgG).

into several subclasses—IgG1, IgG2, IgG3, and IgG4 (in order of relative abundance in human plasma), with different heavy chains, named γ1, γ2, γ3, and γ4, respectively. The structural differences among these subtypes are the number and location of interchain disulfide bonds and the length of the hinge region. The light chains consist of two types—lambda (λ) and kappa (κ). In mice, the average of κ to λ ratio is 20:1, whereas it is 2:1 in humans.[6] The variable (V) regions of both chains cover approximately the first 110 amino acids, forming the antigen-binding (Fab) regions, whereas the remaining sequences are constant (C) regions, forming Fc (fragment crystallizable) regions for effector recognition and binding.[6] The N-terminal sequences of both the heavy and light chains vary greatly between different antibodies. It was suggested that the conserved sequences in human IgG1 antibodies

are approximately 95% and the remaining 5% is variable and creates their antigen-binding specificity.[5]

The V regions are further divided into three hypervariable sequences (HV1, HV2, and HV3) on both H and L chains. In the light chains, these are roughly from residues 28 to 35, from 49 to 59, and from 92 to 103, respectively.[6] Other regions are the framework regions (FR1, FR2, FR3, and FR4). The HV regions are also called the complementarity determining regions (CDR1, CDR2, and CDR3). While the framework regions form the β-sheets, the HV sequences form three loops at the outer edge of the β barrel (also see Section 2.2).

Disulfide Bonds

Most IgGs have four interchain disulfide bonds—two connecting the two H chains at the hinge region and the other two connecting the two L chains to the H chains.[6] Exceptions do exist. Two disulfide bonds were found in IgG1 and IgG4 linking the two heavy chain in the hinge region but four in IgG2.[7] In IgG1 MAb, HC is linked to the LC between the fifth Cys (C217) of HC and C213 on the LC. In IgG2 and IgG4 MAbs, it is the third Cys of HC (C123) linking to the LC.[7] A disulfide bond between HC C128 and LC C214 was found for mouse catalytic monoclonal antibodies (IgG2a).[8]

IgGs have four intrachain disulfide bonds, residing in each domain of the H and L chains, stabilizing these domains. The intrachain disulfide bonds in V_H and V_L are required in functional antigen binding.[9] Native IgG MAbs should not have any free sulfhydryl groups.[7] However, detailed examination of the free sulfhydryl groups in recombinant MAbs (one IgG1, two IgG2, and one IgG4) suggests presence of a small portion of free sulfhydryl group (approximately 0.02 mol per mole of IgG2 or IgG4 MAb and 0.03 for IgG1.[7] In rare cases, a free cysteine is found. A nondisulfide-bonded Cys at residue 105 was found on the heavy chain of a mouse monoclonal antibody, OKT3 (IgG2a).[10]

Oligosaccharides

There is one oligosaccharide chain in IgGs.[6] This N-linked biantennary sugar chain resides mostly on the conserved Asn 297, which is buried between the C_H2 domains.[5,11] For example, the oligosaccharide resides on Asn-297 of the C_H2 domain of chimeric IgG1 and IgG3 molecules[12] but on Asn 299 in a monoclonal antibody, OKT3 (IgG2a).[10] The oligosaccharide, often microheterogeneous, is typically fucosylated in antibodies produced in CHO or myeloma cell lines[5] and may differ in other cell lines.[2,11] There are many factors that dictate the nature of the glycan microheterogenity on IgGs. These include cell line, the bioreactor conditions and the nature of the downstream processing. An additional oligosaccharide can be found in rare cases. A human IgG produced by a human-human-mouse heterohybridoma contains an additional oligosaccharide on Asn 75 in the variable region of its heavy chain.[13] In addition, O-linked carbohydrates could also exist in this antibody.

Proper glycosylation is critical for correct functioning of antibodies.[11] It was demonstrated that removal of the oligosaccharide in IgGs (IgG1 and IgG3) made them ineffective in binding to C1q, in binding to the human FcγRI and activating C; and generally more sensitive to most proteases than their corresponding wild-type IgGs (one exception).[12] This is because the binding site on IgG for C1q, the first component of the complement cascade, is localized in the C_H2 domains.[11] Furthermore, the glycosylation can affect the antibody conformation.[12]

Oligosaccharides in other regions can also play a critical role. Removal of an oligosaccharide in a Fv region of the CBGA1 antibody resulted in a decreased antigen-binding activity in several ELISA systems.[13] In addition, this oligosaccharide might play critical role in reducing the antigenicity of the protein.[14]

The sugar composition of the oligosaccharide is also critical in antibody functions. It has been shown that a low fucose (Fuc) content in the complex-type oligosaccharide in a humanized chimeric IgG1 is responsible for a 50-fold higher antibody-dependent cellular cytotoxicity (ADCC) compared with a high Fuc counterpart.[15]

Heterogeneity

Purified antibodies are heterogeneous in structure. This is true for all monoclonal antibodies (MAbs) due to differences in glycosylation patterns, instability during production, and terminal processing.[5] For example, five charged isoforms were found in recombinant humanized monoclonal antibody HER2 as found by capillary isoelectric focusing (cIEF) and sodium dodecyl sulfate–capillary gel electrophoresis (SDS–CGE).[16] Six separate bands were focused under

IEF for two mouse monoclonal antibodies IgG2a (κ) and IgG1 (κ).[17] A mature monoclonal antibody, OKT3 (IgG2a), contain cyclized N-terminus (pyroglutamic acid, −17 D) in both H and L chains, processed C-terminus (no Lys, −128 D) of the H chains, and a small amount of deamidated form.[10] Similar observation was also reported for a humanized IgG1 (κ).[18] In rare cases, gene cross-over may lead to formation of abnormal heavy chains. For example, a purified monoclonal anti-IgE antibody contains a small amount of a variant H chain, which had 16 fewer amino acid residues than the normal H chain (position is between Arg108 of the L chain and Ala124 of the H chain).[19]

Secondary and Higher-Order Structure

The basic secondary and higher-order structural features of IgGs have been reviewed.[6] Only a small portion of the three-dimensional structures of IgGs has been solved.[20] The antibody's secondary structure is formed as the polypeptide chains form anti-parallel β-sheets. The major type of secondary structure in IgGs is these β-sheets and its content is roughly 70% as measured by FTIR.[21] The light chain consists of two and the heavy chain contains four domains, each about 110 amino acid long.[6,20] All these domains have similar folded structures—β barrel, also called immunoglobulin fold, which is stabilized by a disulfide bond and hydrophobic interaction (primary). These individual domains (~12 kDa in size) interact with one another (V_H and V_L; C_H1 and C_L; and between two C_H3 domains except the carbohydrate-containing C_H2 domain) and fold into three equal-sized spherical shape linked by a flexible hinge region. These three spheres form a Y shape (mostly) and/or a T shape.[22]

The less globular shape of IgGs is maintained both by disulfide bonds and by strong noncovalent interactions between the two heavy chains and between each of the heavy-chain/light-chain pairs.[23] Through noncovalent interactions, a less stable domain becomes more stable, and thus, the whole molecule can be stabilized.[24] A detailed study indicates that the interaction between two CH3 domains are dominated by six contact residues, five of these residues (T366, L368, F405, Y407, and K409) forming a patch at the center of the interface.[25] These noncovalent interactions are spatially oriented such that variable domain exchange (switching V_H and V_L; inside-out IgG; ioIgG) induces noncovalent multimerization.[26]

The six hypervariable regions in CDR (L1, L2, L3, H1, H2, and H3) form loops of a few predictable main-chain conformations (or canonical forms), except H3 loop, which has too many variations in conformation to be predicted accurately.[27,28] There is a slight difference in the loop composition and shape between the two types of light chains.[20] However, no functional difference was found in antibodies having λ or κ chain.[6]

Basic Functions of Antibodies

The basic functions of antibodies have been reviewed.[6] There are two functional areas in IgGs—the V and C regions. The V regions of the two heavy and light chains offer two identical antigen-binding sites. The binding of the two sites (bivalent) can be independent of each other and does not seem to depend on the C region.[29] The exact antigen-binding sites are the CDR regions with participation of the frame work regions.[30] Binding of antigens seems through the induced-fit mechanism.[31,32] The induced-fit mechanism allows multispecificity and polyreactivity. It has been suggested that about 5–10 residues usually contribute significantly to the binding energy.[32]

The C regions of antibodies have three main effector functions (1) being recognized by receptors on immune effector cells, initiating antibody-dependent cell cytotoxicities (ADCC), (2) binding to complement, helping to recruit activated phagocytes, and (3) being transported to a variety of places, such as tears and milk.[6] In addition, C domains also modulate *in vivo* stability.[23,29,33] The function of Fc is affected by the structure of Fab. Variable domain exchange (switching V_H and V_L; inside-out IgG; ioIgG) affected Fc-associated functions such as serum half-life and binding to protein G and FcγRI.[26]

The hinge region provides flexibility in bivalent antigen binding and activation of Fc effector functions.[26] Two chimeric IgG3 antibodies lacking a genetic hinge but with Cys residues in CH2 regions was found to be deficient in their intermolecular assembly, and both IgG3 ΔH + Cys and IgG3 ΔH + 2Cys lost greatly their ability to bind FcγRI and failed to bind C1q and activate the complement cascade.[34]

Alternative Forms of Antibodies

In addition to species-specific antibodies, other antibody forms are generated to meet various needs. In the early development of antibody therapies, antibodies were made from murine

sources. However, these antibodies easily elicit formation of human anti-mouse antibody (HAMA). Therefore, humanized chimeric antibodies were generated. Chimeric monoclonal antibodies (60–70% human) are made of mouse variable regions and human constant regions.[2] Such antibodies can still induce formation of human anti-chimeric antibody (HACA). Highly humanized antibodies, CDR-grafted antibodies, are made by replacing only the human CDR with mouse CDR regions (90–95% human).[2] These antibodies are almost the same in immunogenicity potential as completely human antibodies, which may illicit formation of human anti-human antibody (HAHA).

Other alternative forms of antibodies have also been generated and these different forms have been reviewed.[35] Treatment with papain would cleave the N-terminal side of the disulfide bonds and generate two identical Fab fragments and one Fc fragment. Fab's are 50 kDa ($V_H + C_H1$)/($V_L + C_L$) heterodimers linked by a single disulfide bond. Treatment with pepsin cleaves the C-terminal side of the disulfide bonds and produces a $F(ab)'_2$ fragment. The remaining H chains were cut into several small fragments.[6] Cleavage by papain occurs at the C-terminal side of His-H228[36] or His-H227.[37] Reduction of $F(ab')_2$ will produce two Fab'.[23]

Fv fragments are noncovalent heterodimers of V_H and V_L. Stabilization of the fragment by a hydrophilic flexible peptide linker generates single-chain Fv (scFvs).[2] Fragments without constant domains can also be made into domain antibodies (dAbs). These scFvs are 25–30 kDa variable domain ($V_H + V_L$) dimers joined by polypeptide linkers of at least 12 residues. Shorter linkers (5–10 residues) do not allow pairing of the variable domains but allow association with another scFv form a bivalent dimer (diabody) (about 60 kDa, or trimer: triabody about 90 kDa).[38] Two diabodies can be further linked together to generate bispecific tandem diabody (tandab).[39] Disulfide-free scFv molecules are relatively stable and useful for intracellular applications of antibodies—"intrabodies."[38] The smallest of the antibody fragments is the minimal recognition unit (MRU) that can be derived from the peptide sequences of a single CDR.[2]

ANTIBODY INSTABILITY

Antibodies, like other proteins, are prone to a variety of physical and chemical degradation pathways, although antibodies, on the average, seem to be more stable than other proteins. Antibody instabilities can be observed in liquid, frozen, and lyophilized states. The glycosylation state of an antibody can significantly affect its degradation rate.[40] In many cases, multiple degradation pathways can occur at the same time and the degradation mechanism may change depending on the stress conditions.[41] These degradation pathways are divided into two major categories—physical and chemical instabilities. This section will explore the possible degradation pathways of antibodies and their influencing factors.

Physical Instability

Antibodies can show physical instability via two major pathways—denaturation and aggregation.

Denaturation

Antibodies can denature under a variety of conditions. These conditions include temperature change, shear, and various processing steps. Compared with other proteins, antibodies seem to be more resistant to thermal stress. They may not melt completely until temperature is raised above 70°C,[21,42,43] while most other mesophilic proteins seem to melt below 70°C.[44] Shear may cause antibody denaturation. For example, the antigen-binding activity of a recombinant scFv antibody fragment was reduced with a first-order rate constant of 0.83/h in a buffer solution at a shear of approximately 20,000/s.[45]

Lyophilization can denature a protein to various extents. An anti-idiotypic antibody (MMA 383) in a formulation containing mannitol, saccharose, NaCl, and phosphate was found to loose its *in vivo* immunogenic properties (only 10–20% of normal response rate) upon lyophilization.[46] Since the protein showed no evidence of degradation after lyophilization, no change in secondary structure by CD (29% β-sheet, 14% α-helix, and 57% "other"), the loss of activity was attributed to the conformational change. Indeed, tryptophan fluorescence properties were different between the lyophilized and unlyophilized antibodies.[46]

Aggregation

Antibody aggregation is a more common manifestation of physical instability. The concentration-dependent antibody aggregation was considered the greatest challenge to developing protein formulations at higher concentrations.[47] This is

Samsung Bioepis Exhibit No. 1002
Page 5516

because protein aggregates generally have reduced activity and more importantly, greater immunogenicity potential because of the multiplicity of epitopes and/or conformational changes.[14,48] Immunoglobulin aggregates have been shown to cause serious renal failure[49] and anaphylactoid reactions such as headache, fever, and chills.[50] Therefore, the aggregate level in commercial intravenous immunoglobulin products is limited to less than 5% based on the WHO standards.

Aggregates can form easily both in liquid and solid states under a variety of conditions (Tab. 2). Since protein aggregation is often the consequence of protein–protein interactions, a process influenced by diffusion rate and geometric constraints of the interaction sites, such factors would influence significantly the aggregation rate, including protein concentration change, viscosity, ionic strength, pH, and temperature.[51] Other conditions may also affect protein aggregation, including shaking, long-term storage, freeze-thaw process, lyophilization process, etc.

Increasing the concentration of antibodies often increases the aggregation tendency of the protein. It was demonstrated that increasing the IgG1 concentration (in 10 mM citric acid, 100 mM NaCl pH 5.5) from 2.7 mg/mL to 50 mg/mL almost linearly increased the Nephelometric Unit (NU) from 2 to 40.[52] Since EP defines a clear solution as having equal or less than three NU, most antibody solutions in the study are opalescent except those at 5 mg/mL or less. However, as the weight average molecular weight was found to be 0.9–1.3 times that expected of a monomer (about 149 kDa), minimal protein–protein association (and readily reversible) is suggested.[52] Shaking can accelerate antibody precipitation and the shape of the precipitates may depend on the sample volume in a container.[53] It appears that protein solutions exhibiting lower surface tension are more susceptible to protein denaturation and precipitation.[53]

Low-temperature treatment may induce aggregation of antibodies. The reversible low-temperature-induced aggregation (below 37°C) of serum cryoglobulins is well known.[54] Human IgM cryoglobulin preparations easily precipitate or gel at temperatures below 10–12°C and the process is reversible at a higher temperature.[20] In a recent study, aggregation of IgG1 at low temperature at above 18 mg/mL was reversible as measured by light scattering.[52] The low-temperature-induced aggregation of cryoglobulins is poorly understood and was thought to involve sites within both Fab and Fc.[20] The authors of this article believe that low temperature reduces the hydrophobic interaction, which is the major force in protein folding. Without enough hydrophobic interaction at low temperature, hydrophobic regions of antibodies become more exposed to solvent and lead to increased intermolecular hydrophobic interaction, leading to aggregation.

In close relation to the low-temperature effect, freeze-thaw process often induces protein aggregation. However, freeze-thaw-induced antibody aggregation does not seem to be a major issue, partly due to the reversibility of antibody aggregates. Freeze-thawing of a chimeric (L6) antibody solution at pH 7.2 for as many as 35 times led to formation of dimers (not larger aggregates).[41] Maximum amount of dimers (about 20%) was observed at pH 6.5 and minimum was below 5.5 or above 8.5 (less than 2%). The freeze-thaw-induced aggregation is apparently reversible after treatment at 37°C for a few hours.[41] Freeze-thawing of rhuMAb anti-CD20 three times (freezing to either −20 or −70°C, thawing to 5°C) did not lead to significant change in aggregation, no significant loss of monomer by RP-HPLC or SEC-HPLC.[55] On the other hand, isolated Fabs seems to be more prone to freeze-thaw-induced aggregation relative to full-length antibodies. For example, Lee[56] found that freeze-thawing of a scF_v (MW 27,000 d) at 1.45 mg/mL in sodium phosphate buffered saline (PBS) at pH 7.3 dropped the %monomer content (due to aggregation) from 95% to 84%, 59%, 44%, and 37%, respectively after 1–4 cycles.

Lyophilization may induce antibody aggregation to a variable degree. It was demonstrated that lyophilization of a recombinant humanized monoclonal antibody (rhuMAb HER2), in the absence of sugars, led to a slight increase in aggregate (less than 1.2%).[57] Lyophilization caused detectable aggregation of a human monoclonal antibody in PBS or physiological saline as measured by OD600,[58] and aggregation of a mouse monoclonal antibody (MN12) [IgG2a (κ)] in the absence or presence of 5% sucrose, dextran, or hydroxypropyl-beta-cyclodextrin (HP-β-CD), as measured by visual examination.[59] In a different study, lyophilization of a monoclonal antibody-vinca conjugate in PBS at pH 7.4 caused 6.2% aggregation.[60] The lyophilization-induced aggregates could be non-covalent-linked monomers and/or even disulfide-linked.[61,62]

Antibody aggregation can easily occur during storage in a liquid state. Storing of a fully human anti-IL8 monoclonal antibody (ABX-IL8; IgG2)

JOURNAL OF PHARMACEUTICAL SCIENCES, VOL. 96, NO. 1, JANUARY 2007

Table 2. Instabilities of Antibodies

Instability	Antibody	Formulation	Condition	Effect	References
Denaturation	scFv	3 μg/mL in 10 mM phosphate-buffered saline containing 0.02% sodium azide	Shaking at a mean velocity gradient approximately 20,000/s	Loss of activity at a first-order rate of 0.83/h	45
Aggregation	Chimeric mouse/human IgG1	2 mg/mL in PBS at pH 7.2	Stirring for 48 h with Teflon bar at 600 RPM	Increased turbidity Formation of >15 k particles (>10 μ)	91
	Human Anti-IL-8 (IgG2)	55 mg/mL, 5 mM His, pH 6	Freeze/thaw three times (room/−70°C)	About 0.6% aggregates (by SEC-HPLC)	21
	Anti-IL-8 (IgG2)	100 mg/mL, 200 mM Sucrose, 15 mM Arg, 15 mM His, pH 6	Incubation at 40°C for 6 months	About 11% aggregates (by SEC-HPLC)	21
	rhuMAbE25	10 mg/mL in excipient-free solution	Spray drying Tin = 105°C, Tout = 50–55°C	5–6% aggregate (by SEC-HPLC)	65
	MAb (type not specified)	MAb at 40 mg/mL, 85 mM sucrose, 5 mM His at pH 6, 0.01% Tween 20	Lyophilization	Cloudy (OD350 > 0.2)	1
	Human Anti-IL-8 (IgG2)	50 mg/mL, 20 mM Gly, 4 mM His, 0.2% mannitol, 16 mM Glu, 0.03% Tween 20 pH 5.7	Incubation of lyophilized form at 37°C for 1 month	4.8% aggregates (by SEC-HPLC)	21
	RhuMAb HER2	50 mg/mL in 100 mM trehalose, 5 mM His at pH 6.0	Incubation of lyophilized form at 40°C for 23 months	9.7% aggregates (by SEC-HPLC)	57
Deamidation	OKT3 (IgG2a)	1 mg/mL, PBS at pH 7.0 + Tween80	Incubation at 37°C for 2 months	90% deamidation of Asn386	10
	LA298 (IgG1)	0.5 mg/mL in 10 mM citrate at pH 7	Incubation at 25°C for 6 weeks	~12% increase in deamidation at Asn55	78
Isomerization	MAb	Lyophilized at 40 mg/mL, 85 mM sucrose, 5 mM His, 0.01% Tween 20, pH 6.0, containing 8.4% moisture	Incubation at 5°C	First-order rate constant $\sim 0.33 \times 10^{-4}$/day	1
Oxidation	rhuMAb HER2	5 mg/mL, 147 mM NaCl, 0.01% polysorbate 20, 5 mM sodium acetate at pH 5.0	5, 30, and 40°C for 2 weeks	10, 17, and 52% (total peak area)	81

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Samsung Bioepis Exhibit No. 1002
Page 5518

in aqueous solutions at 2–8°C or 25°C led to formation of high-molecular-weight (HMW) species.[21] Incubation of two mouse monoclonal antibodies IgG2a (κ) and IgG1 (κ) at 37°C for 32 days caused precipitation in pH 3 and 4 solutions for both antibodies.[17] Aggregation can also occur to an antibody conjugate. Storing a monoclonal antibody-vinca alkaloid conjugate (murine IgG2a) in phosphate-buffered saline solutions at 5°C between pH 4.5 and 7.4 for 1.5 months caused precipitation regardless of solution pH and aggregation was accelerated at 30°C throughout the pH range.[63]

Antibody aggregation is a common phenomenon during storage in a lyophilized state. Aggregation (OD350 > 0.2) was observed of a high concentration, lyophilized, monoclonal antibody (type not specified) by SEC under accelerated conditions.[1] So did a lyophilized chimeric monoclonal antibody during storage at 5°C (0.4% after 2 months).[64] In the absence of sugars, the extent of rhuMAb HER2 aggregation increased from about 1% to >10% after 3 months at 40°C and the rate of aggregation for these formulations was about 0.2% per month at 40°C.[57]

Antibody aggregation can readily occur in other solid forms during storage.[5] It was found that the primary degradation upon storage of a spray-dried anti-IgE (protein:mannitol = 80:20) is aggregation, and the amount of aggregates increased with increasing temperature and relative humidity (Maa et al., 1998a).[65] As much as 52% aggregate was observed by SEC-HPLC of a spray-dried anti-IgE (protein:mannitol = 80:20) after 1 year at 40°C and 38% RH (Maa et al., 1998b).[66]

Antibody aggregation is usually accelerated upon thermal treatment. In the study of chimeric (human/mouse) monoclonal antibody (BR96), Alexander and Hughes demonstrated that thermal treatment at 60°C for 166 h generated noncovalently linked aggregates.[67] In the evaluation of the relative stability of murine IgG2a (κ), chimeric IgG1(κ), trioma IgG1 (λ), and murine IgG1 (κ), Hartmann et al. demonstrated that IgG2a (κ) aggregated and precipitated after heating at 55°C for 8 h and the half-life for the loss of monomer for those four IgGs is 1, 18.1, 3.4, and 8.2 h with different aggregate species (dimer, trimer...) by SEC-HPLC.[68]

Surface Adsorption

Antibodies, like other proteins, can easily adsorb to a variety of surfaces. Surface adsorption can significantly reduce the antibody concentration in a solution. For example, a mouse monoclonal IgG1 has been shown to be adsorbed on the surface of glass shake flasks, causing a loss of protein in plant culture media.[69] Such a loss was minimized by coating the glass vessels or by addition of Pluronic F127.

Chemical Instability

A variety of chemical degradation pathways have been observed. These chemical degradants may or may not loose protein activity depending on the site of changes. For examples, deamidation of a monoclonal antibody, OKT3 (IgG2a) as measured by change in IEF pattern does not correlate with loss of antigen binding potency (90% deamidation of Asn386 vs. 80% activity in the potency assay).[10] In comparison, the activity of light chain deamidated recombinant humanized monoclonal antibody HER2 (rhuMAb HER2, Herceptin, trastuzuMAb) at Asn30 (to aspartate) is only 70% of the main species and that of the isomerized (heavy chain) asp102 species is only 9–21% of major species.[37] The major chemical degradation pathways in an antibody include cross-linking, deamidation, isomerization, oxidation, and fragmentation.

Disulfide Formation/Exchange

Disulfide bond formation/exchange is probably the most common cross-linking pathway, leading to chemical aggregation. This degradation pathway can occur readily in various processing steps. For example, lyophilization of a murine monoclonal antibody (IgG2a) without additives reduced the average number of –SH groups per molecule from 6.65 to 6.28, suggesting disulfide formation.[70] Since the thiolate ions are often the initiating species for this chemical degradation, increasing formulation pH usually leads to increased formation of disulfide formation. This is the case for an antifebrin T2G1s Fab′ (IgG1 (κ)), containing free –SH groups, where the percentage of dimers increased with increasing the pH from 5.8 to 9.5.[61]

Storage often leads to disulfide-based aggregation. Storage of a lyophilized excipient-free antibody (IgG1, containing 12 intra and 4 interchain disulfides) formulation for 1 year at 30°C resulted in formation of reducible dimer and trimers.[62] Similarly, reducible aggregates of a monoclonal antibody (type not specified) were detectable

by SDS–PAGE after storage of the lyophilized formulation at 50°C for 6 months.[1] Such disulfide-linked covalent dimers and trimers were also observed during storage of spray-dried anti-IgE humanized monoclonal antibody.[71]

Nonreducible Cross-Linking

Nonreducible cross-linking was observed in several studies. Incubation of a human monoclonal antibody (C23) in solution at a basic pH at 37°C for 14 days resulted in formation of nonreducible bands above the light chains at pH 4 and 10.[72] Incubation of two mouse monoclonal antibodies—IgG2a (κ) and IgG1 (κ), between pH 3.0 and 10 at 37°C for 32 days led to formation of a nonreducible 88 kDa species by SDS–PAGE.[17] A species with the same molecular weight was observed after incubation of another monoclonal antibody, OKT3 (IgG2a), at 37°C for 2 months.[10,73] Further examination suggests that this species is a cross-linked product of H and L chains between the L46-52 and H99-121 regions. Although the exact chemical nature was not determined, an oxidative step is apparently involved as inhibition of oxidation delayed the formation of this species.[10,73]

A recent study shows that a nonreducible thioether bond can be formed between C223 of the heavy chain and the C-terminal Cys residue of the light chain in humanized IgG1 antibodies. This cross-linked product has a molecular weight of 75 kDa by MS but shows an apparent molecular weight of 92 kDa by SDS–PAGE.[74]

Nonreducible cross-linked products were also observed in solid dosage forms. Incubation of a lyophilized mouse monoclonal antibody [IgG2a (κ)] without lyoprotectant or in the presence of 5% sucrose, dextran, or hydroxypropyl-beta-cyclodextrin (HP-β-CD) at 56°C for 18 days led to formation of 100-kDa band under reduced conditions by SDS–PAGE.[59]

Deamidation

Antibody deamidation has been extensively reported in the literature. This common protein degradation pathway is so prevalent that purified antibody preparations may contain many deamidated forms. For example, several deamidated forms of recombinant humanized monoclonal antibody HER2 (rhuMAb HER2, Herceptin, trastuzuMAb) were found by cation-exchange chromatography, including (1) deamidated Asn30 (to aspartate) on one light chain, (2) deamidated Asn30 (to aspartate) on two light chains, and (3) deamidated Asn55 on the heavy chain (to isoaspartate).[37] The sole conversion of Asn30 to aspartate (not isoaspartate) suggests that the deamidation mechanism in cell culture may be different from that during storage.

Storage can easily generate large amounts of deamidated products. Incubation of a monoclonal antibody, OKT3 (IgG2a) in a PBS solution at pH 7 containing Tween 80 at 37°C for 2 months led to more than 90% deamidation at Asn H386.[10] By monitoring the ammonia generation, Poborji et al.[41] estimated that 4% of the total number of Asn and Gln residues in a chimeric antibody (L6) was deamidated after storing the solution at pH 7.2 at 50°C for 3 weeks.

Deamidation also occurs in solid state. In the evaluation of storage stability of a model recombinant humanized monoclonal antibody (rhuMAb HER2), Cleland et al. reported that incubation of a high-moisture formulation (8.4% moisture) for 12 months at 40°C resulted in a 19% drop in the main peak by IEX due to deamidation.[57]

Deamidation in proteins goes mostly through the intermediate of succinimide at Asn (more readily) and Gln (see reviews[75,76]).This seems to be the case for antibodies, even though alternative mechanisms were reported, such as deamidation of Asn30 on the light chain of recombinant humanized monoclonal antibody HER2 (rhuMAb HER2, Herceptin, trastuzuMAb).[37] A method has been developed to detect the formation of succinimide.[77]

The rate of deamidation via succinimidation in proteins can be affected by a variety of factors, such as pH, sequence, and steric effect (see review[44]). A recent report demonstrated a strong pH-dependency of deamidation at Asn55 (pH 7 > pH 6 > pH 5 > pH 4) in a fully human IgG1 in the pH range of 4–7 during storage at 25°C.[78] Using small peptide as a model, Tyler-Cross and Schirch[79] found that deamidation of Asn residues depends on the amino acid residue on the carboxyl side of Asn in neutral and alkaline solutions. Increasing the size and branching of these amino acids decreased the rate of deamidation by as much as 70-fold compared with the N–G sequence (greatest rate of deamidation) but under acidic conditions, these amino acids lost their protective effect.

Isomerization

The most common isomerization in antibodies is iso-aspartic acid formation, which results not only

from direct isomerization of Asp but also, mostly, from hydrolysis of succinimide intermediate. The pH-dependent formation of succinimide intermediate can occur either from Asn deamidation or Asp dehydration.[36] In neutral and alkaline condition, the isoAsp and Asp products formed in a peptide were at a ratio of about 3:1.[79]

Isomerization is also prevalent and difficult to control. Again, many purified proteins contain significant amount of isomerized products, either from deamidation or direct isomerization. A typical example is recombinant humanized monoclonal antibody HER2 (rhuMAb HER2, Herceptin, trastuzuMAb).[37] In addition, since isomerization occurred at Asp H102 in CDR regions, the activity of the isomerized species is only 9–21% of the major species.[37] Similarly, isomerization of Asp L32 on one light chain in a recombinant monoclonal anti-IgE antibody (E25) reduced the relative binding activity of $F(ab)'_2$ to 42% and the activity of isomerized form on both chains (iso-Asp-iso-Asp) to 15%.[36]

Since formation of the succinimide intermediate does not need participation of water, it can readily occur in solid state. Asp isomerization is one of the major degradation pathways for a lyophilized, monoclonal antibody (type not specified), and increases with increasing storage temperatures both above and below their Tg values.[1]

Like deamidation, the relative rate of isomerization can be influenced strongly by steric effect. For example, a recombinant monoclonal anti-IgE antibody (E25) contains two Asp-Gly sequences within its CDRs, but only one site was found to be labile to isomerization (Asp L32). The unreactivity of one Asp residue is suggested to be due to hydrogen bonding.[36]

Oxidation

The oxidizable residues in proteins include Met, Tyr, Trp, His, and Cys. Although oxidation is not as prevalent as deamidation and isomerization in antibodies, it can easily occur during storage of antibodies. For example, the major route of degradation of a monoclonal antibody, OKT3 (IgG2a), in solution during storage at 5°C is oxidation of a nondisulfide Cys and several Met residues.[10] Oxidation was also observed in rhuMAb anti-CD20 in solution during storage at 40°C for 2 months.[55]

Oxidation of CDP870, a pegylated one-half antibody (one light and one heavy chain) at a Met residue (M3) was also observed after incubation of the protein at 25°C or 40°C for 12 weeks (14% at pH 4.1 and 4.2% at pH 5.5).[80] Light exposure can accelerate baseline oxidation. For example, light exposure (20,000 lux for 2 weeks at 27°C) of recombinant humanized monoclonal antibody HER2, rhuMAb HER2 caused 5–10% increase in oxidation in various liquid formulations at Met H255 (primary site) and Met H431 at temperatures 30 and 40°C.[81]

Formation of Acidic or Basic Species

Antibodies can form acidic or basic species, which are often monitored by IEF or ion exchange chromatography. Among all the possibilities, deamidation is the most likely degradation pathway, leading to formation of acidic species. Therefore, formation of such species is used loosely as an indication of deamidation in the absence of other confirmatory studies. Strictly speaking, formation of such species can only be considered as an indication of chemical instability (see below). A pH-dependency is almost certain for this kind of degradation. In the stabilization of CDP870, a one-half antibody (one light and one heavy chain), which is linked through Cys to a branched (20 kDa each) PEG, Johnson et al.[80] found that the protein generated more acidic species (up to 17% at pH 4.1 and 41% at pH 5.5) by IEF as the solution pH was increased from 4.1, 4.5, 5.0, to 5.5 during storage at 25°C for 12 weeks.[80]

Another possible mechanism for generating acidic species in antibodies is the Maillard reaction, where reducing sugars can react with lysine residues. Such a reation will result in loss of positive charges and formation of apparently more acidic species. This is the case for an spray-dried anti-IgE humanized monoclonal antibody with lactose during storage.[71]

Formation of basic species was also observed in antibodies. Its formation can result from several possibilities, most likely as a result of succinimide formation, removal of sialic acids, or pyroGlu formation. Such a basic species was observed after incubation of a human monoclonal antibody (C23) at 37°C for 14 days in a solution at an acidic pH by IEF.[72]

C-Terminal Clipping

Terminal cleavages can easily occur during normal production of antibodies. For example, the majority of a purified chimeric protein rCD4-IgG lacks the heavy chain C-terminal Lys.[82]

This is also the case for recombinant humanized monoclonal antibody HER2 (rhuMAb HER2, Herceptin, trastuzuMAb)[37] and a recombinant monoclonal anti-IgE antibody (E25).[36] It was suggested that the cleavage was due to the action of basic carboxypeptidases during production.[37]

Fragmentation

The most likely sequences leading to fragmentation in proteins are Asp-Gly and Asp-Pro,[83] although other sequences can also experience cleavages, such as Asn-Ser.[79] Fragmentation can easily occur in antibodies even during production processes. A small amount of fragment (MW < LC) was detectable under reduced condition in a purified recombinant MAb IgG4 produced in Chinese hamster ovary (CHO) cells.[7] Half molecules should be monitored routinely for IgG4s.[84]

A variety of processing conditions may accelerate fragmentation of antibodies, such as acidic or basic treatment,[72] thermal treatment,[41,67] freeze-thaw,[41] and storage.[17,73,78] Fragments can simply include masses with a loss of a light chain (M-LC), a loss of a Fab arm (M-Fab), and separated heavy-chain (HC) and light-chain (LC), as well as species less than common antibody subunits, resulting from both peptide and/or disulfide bond cleavage.[67] Antibodies are also subject to radiation-induced cleavages.[85]

Maillard Reaction

Proteins may form adducts with sugars. In an investigative study, mouse MAbs were found to form glucose adducts after incubation with 0.5 M glucose at pH 7.4 for 14–21 days at 37°C and the glycated antibodies significantly increased the dissociation of the antibody-antigen binding complex.[86]

ANTIBODY FORMULATION

As mentioned above, antibodies, like other proteins, may generate a variety of degradants during production, processing, and storage both in liquid and solid states. Their tendency to generate such degradants depends very much on their individual sequence, pI, hydrophobicity, and carbohydrate content.[23] The antibody degradation products may have reduced activity and more importantly, increased immunogenicity.[14,87] A strong immune response (antibody formation) may lead to severe neutralization of the protein drug product and even endogenous proteins.

The major purpose of formulating antibodies as therapeutic agents is to control the rate of antibody degradation to provide an acceptable shelf life for worldwide shipping and storage. This can be done by choosing proper formulation excipients and other formulation conditions. It should be stressed that one formulation excipient stabilizing a specific antibody may not be suitable for another because of the differences in their sequence. A proper formulation composition should not only maximally preserve the activity of the antibody but also minimize the potential immune response, as excipients could alter the immune response by changing the presentation of the protein (such as the spacing of epitopes).[14] The following section will discuss critical factors in formulating both liquid and lyophilized antibody products. Advanced formulations will also be briefly described.

Liquid Formulation

Liquid dosage form is usually preferable to lyophilized products as it is easier to administer and less expensive to manufacture. Among all the commercial antibody products, about half are stable enough to be formulated in a liquid form (Tab. 1). Formulating a successful liquid product needs consideration of at least the following aspects.

Protein Concentration

Since the EC50 values of antibodies are often 10–1,000 times higher than for other proteins such as hormones and cytokines,[35] a relatively large amount of antibodies needs to be dosed to achieve any therapeutic effect. A large dose requires that an antibody product needs to be formulated/reconstituted at a high concentration to reduce dose volume, especially for subcutaneous injections; otherwise, a large volume of antibody preparation has to be infused. Since the aggregation process is usually concentration-dependent, antibodies at high concentrations have at least two potential issues: high tendency to aggregate during storage and likely high viscosity, leading to more difficulty during injection (injection time correlated with viscosity).[47] It has been suggested that the concentration-dependent protein aggregation is the greatest challenge to developing high-concentration

Samsung Bioepis Exhibit No. 1002
Page 5522

protein formulations.[47] On the other hand, moderate increase in protein concentration may not have a significant effect. In fact, increasing the concentration of antifebrin T2G1s Fab′ (IgG1 (κ)), from 0.05 to 5.0 mg/mL in solution resulted in a decrease in dimer formation during storage (73.2% to 22.9% after 13 weeks at 22°C).[61]

An effective way to reduce the viscosity of an antibody solution is to add sufficient amount of NaCl.[5,88] Adjustment of formulation pH could potentially reduce the viscosity of an antibody solution.[88] Although not very effective, histidine has been shown to reduce the solution viscosity of a fully human anti-IL8 monoclonal antibody (ABX-IL8; IgG2) from 20 to about 9 centistokes when its concentration was increased from 0 to 60 mM.[21] However, use of such excipients in reducing viscosity can be limited, as the excipient can adversely affect the stability of the antibody, such as MAb anti-CD20.[55]

Another potential issue in formulating high-concentration antibody products is their solubility limits, as presence of undissolved proteins are generally not acceptable for intravenous injection. Fortunately, the solubility of antibodies seems to be relatively high and solubility-limited formulation difficulty has rarely been reported to our knowledge. To increase the solubility of antibodies, excipients such as surfactants may be used. In a recent report, Golovanov[89] found that a combination of L-Arg and L-Glu at an equimolar concentration (50 mM) significantly increased the solubility of several proteins (either alone does not have a significant effect). In addition, the room-temperature storage stability (fragmentation) of proteins was significantly improved as monitored by SDS–PAGE.

One potential advantage of formulating a protein product at higher concentrations is the greater freeze-thaw stability. Often, proteins at high concentrations are more resistant to freeze-thaw-induced aggregation, as the interface-induced protein denaturation can easily reach a saturation point. However, it does not seem to be the case for antibodies. Increasing the concentration of a chimeric antibody (L6) did not inhibit freeze-thaw-induced aggregation.[41] In addition, surfactants such as Pluronic F68 and Tween 80 did not appreciably prevent antibody aggregation. In a different study, Tween 80 alone (or Pluoronic F68) at 0.1% failed to offer any protection against freeze-thaw-induced aggregation of a single-chain antigen-binding protein CC49/218 sF_v (MW 27,000 d) at 1.45 mg/rnL in sodium PBS at pH 7.3.[56] These results suggest that freeze-thaw-induced antibody aggregation may not be a surface phenomenon.

Effect of Formulation pH

Formulation development often starts with determination of a stable formulation pH. Like other proteins, the pH effect on the stability of antibodies depends on the formulation composition,[55] stress conditions,[41] and even antibody concentration.[84] Formulation pH may affect the physical stability of antibodies as it alters the number and distribution of charges on the protein surface. For example, low pH (below 3) caused a loss of tertiary structure in rhuMAb anti-CD20.[55] Low pH (pH 3 and 4) is also responsible for the precipitation of two mouse monoclonal antibodies IgG2a (κ) and IgG1 (κ) during incubation at 37°C for 32 days.[17] Susceptibility to pH stress varies among different IgG molecules.[68]

Regarding chemical stability, formulation pH may play a critical role in controlling many degradation pathways, such as disulfide bond formation/exchange, deamidation, fragmentation, isomerization, etc. Due to variable effects on different degradation pathways, the optimum pH value varies depending on both the sequence of an antibody and the analytical methods used in monitoring the stability. By IEF, Lam et al. found that rhuMAb anti-CD20 is most stable at pH 5 within the pH range of 3–6 irrespective of protein concentration in formulations containing 10 mM citrate, 8% trehalose, and 0.05% Tween 20.[55] By SEC-HPLC, Usami et al. found that pH 6 was the most stable condition during incubation of a human monoclonal antibody (C23) at 37°C for 14 days in a solution containing isotonic sodium chloride between pH 4 and 10.[72] Sometimes, the optimum pH values reside outside a certain range. In the study of the stability of a chimeric antibody (L6), minimum amount of dimmer formation was observed at pH below 5.5 or above 8.5 (less than 2%) during freeze-thaw.[41]

It needs to be mentioned that oxidation could be inhibited by adjusting formulation pH. In a formulation containing 40 mg/mL MAb anti-CD20, 150 mM trehalose, 0.9% benzyl alcohol, 0.02% Tween 20, and 50 mM histidine, reducing the pH from 7.5 to 5 virtually eliminated the oxidation during storage at 40°C for 2 months.[55] In a different study, oxidation (M3) of PEG-CDP870, a one-half antibody, in a formulation containing 160 mg/mL CDP870, 10 mM sodium acetate, and

125 mM NaCl was slower at pH 5.5 than at pH 4.1 (4.2% vs. 14% after 12 weeks at 40°C).[80]

Due to the simultaneous presence of multiple antibody degradation pathways, the formulation pH has to be adjusted to balance all the potential degradations. Generally, a weakly acidic condition seems to be the optimum for most antibodies (see Tab. 1).

Effect of Buffering Agents

It has been long recognized that both the type and concentration of a buffering agent may affect the stability of proteins (see review[44]). For example, phosphate buffer accelerated deamidation of an IgG1 relative to citrate buffer at both pH 6.5 and 7.0 during storage at 25°C.[78] Therefore, proper choice of a buffering agent can potentially achieve both pH control and stabilization of antibodies. In rare cases, a buffering agent good for one antibody may be harmful to another. One of these agents is histidine, which has a pKa of pH 6 and potentially good in controlling a weakly acidic condition. It has been shown to provide better protection against precipitation for a fully human anti-IL8 monoclonal antibody (ABX-IL8; IgG2) upon thermal incubation both at 50 and 40°C than citrate at pH 6.[21] Addition of a small amount of histidine (0.4 mM) also increased the freeze-thaw (4×) recovery of a single-chain antigen-binding protein CC49/218 sF_v (MW 27,000 d) at 1.45 mg/rnL in PBS solution at pH 7.3 from 37% to 90%.[56] On the contrary, histidine was found not only to increase the rate of aggregation of rhuMAb anti-CD20 during storage but also to induce coloration of the solution compared to a acetate-buffered antibody solution at pH 5.[55] In addition, oxidation of Fc was also faster in histidine solution.

The concentration effect of a buffering agent was demonstrated in a study where freeze-thaw-induced aggregation of a chimeric antibody (L6) was higher with increasing phosphate buffer concentration (0.01 vs. 0.05).[41] Therefore, both the type and concentration of buffering agents need to be examined.

Effect of Formulation Excipients

Use of formulation excipients remains a major and convenient approach in stabilizing antibodies in solutions. It is so at least in reducing antibody aggregation.[47] A variety of formulation excipients have been shown to stabilize antibodies under different processing conditions and during storage, including sugars,[41] polyols,[41] amino acids,[41,84] surfactants,[84] and polymers.[45,84]

Formulation excipients are effective generally in protecting the physical stability of an antibody. Among these excipients, sugars are the most commonly used. A variety of sugars, such as sucrose, trehalose, glucose, have been shown to reduce the freeze/thaw-induced aggregation of rhuMAb anti-CD20,[55] a chimeric antibody (L6),[41] a human anti-IL8 monoclonal antibody (ABX-IL8; IgG2),[21] and a single-chain antigen-binding protein CC49/218 sF_v (27 kDa).[56] Other excipients can be equally or even more effective in protecting antibodies. Histidine was shown to be equally effective as sucrose in reducing freeze-thaw induced aggregation of a human anti-IL8 monoclonal antibody (ABX-IL8; IgG2) and combination with Arg resulted in a better protection than sucrose in solution stability at different incubation temperatures.[21] Sugars are not always effective. In the evaluation of freeze-thaw stability of a single-chain antigen-binding protein CC49/218 sF_v (MW 27 kDa) at 1.45 mg/mL in sodium phosphate buffered saline (PBS) at pH 7.3, addition of 10 mM trehalose, lactose, glucose, or mannitol did not offer any protection.[56] In a rare case, sucrose has been shown to promote agitation-induced aggregation of an IgG1 antibody.[90]

Formulation excipients may inhibit chemical degradation of antibodies. Replacing 0.14 M NaCl with 8% trehalose or 4% mannitol in a rhuMAb anti-CD20 increased the chemical stability as measured by IEX during storage at 40°C.[55] Such an effect is likely a result of stabilization of antibody conformation. Some excipients may protect antibodies against oxidation. Replacing NaCl with 4% mannitol and 1% benzyl alcohol protected recombinant humanized monoclonal antibody HER2, rhuMAb HER2, against oxidation (primarily Met-255) at 30 or 40°C.[81] This effect is likely due to a function of mannitol as a free radical scavenger. Some commonly used antioxidants, such as thiosulfate and methionine, are effective in inhibiting antibody oxidation. The minimum effective levels (molar ratios of antibody to antioxidant) required to inhibit temperature-induced oxidation of rhuMAb HER2 were 1:5 and 1:25 for methionine and thiosulfate, respectively.[81] However, use of thiosulfate has led to formation of a thiosulfate adduct of rhuMAb HER2.[81] The safety of such a thio-containing adduct has not been reported.

One of the main excipients often used in antibody formulation is surfactants. They are usually

effective in reducing shaking/stirring-induced aggregation[91] but may have negative effect during long-term storage.[92] One obvious concern is that such compounds may contain a residual level of peroxides, which have been shown to cause protein oxidation.[93] Therefore, use of surfactants should be kept to a minimum in an antibody formulation if possible. For example, removing Tween 20 in a recombinant humanized monoclonal antibody HER2, rhuMAb HER2 solution reduced the oxidized antibody from 52% to <10% at 40°C.[81]

Effect of Shaking/Shearing

Both shaking and shearing can denature antibodies. It was found that shaking murine antibody-based products caused antibody precipitation and the shape of the precipitates are different depending on the fill volume.[53] In the evaluation of shear effect on the antigen-binding activity of a recombinant scFv antibody fragment in a buffer solution, Harrison et al.[45] found that the binding activity decayed at a mean rate constant of 0.83/h at a shear of approximately 20,000/s.

Effect of Preservatives

A limited number of studies have been conducted on the compatibility of preservatives with antibodies. Compatibility depends both on the antibody and the preservative. In the evaluation of the effect of several preservatives, including benzyl alcohol, chlorobutanol, methylparaben, propylparaben, phenol, and m-cresol, on the stability of a humanized monoclonal antibody, Gupta and Kaisheva[94] found that the antibody was most stable in the presence of methylparaben and propylparaben, compatible with benzyl alcohol and chlorobutanol at low concentrations, and not compatible with phenol and m-cresol, as measured by a variety of assays, including SEC-HPLC, DSC, light scattering, UV, and potency assay.

Effect of Processing Equipment

Another important issue in antibody formulation is the choice of proper processing equipment/containers. A few studies have shown that antibody stability can be significantly affected if an inappropriate choice is made. In the study of the stability of recombinant humanized monoclonal antibody HER2, rhuMAb HER2, in liquid formulations, Lam et al.[81] found that replacing the stainless steel filler with a nonstainless steel filler reduced the oxidized antibody (primarily Met-255) from 52% to 18% after storage for 2 weeks at 40°C. It was suggested that Fe ions, released from erosion of stainless steel by chloride ion at low pH, catalyzed the oxidation. In a similar case, Chen et al.[21] demonstrated that a fully human anti-IL8 monoclonal antibody (ABX-IL8) in aqueous formulations containing 60 mM His had a higher level of aggregates after freeze-thaw in a stainless steel vessel and subsequent storage at 40°C, possibly due to the effect of Fe ions generated from a similar mechanism. In addition, proteins have been shown to facilitate metal corrosion.[95]

Effect of Product Containers

The effect of product containers was clearly demonstrated in a recent study. McCormick et al.[96] reported that Tween 80, intended to replace human albumin in the original formulation, interacted with uncoated rubber stoppers in single-use Eprex syringes. The stoppers leached a small amount of plasticizers in the drug solution, which acted as an adjuvant, and caused a strong IgG response to the recombinant human erythropoietin. The immune response was significantly minimized later by switching to PTFE-coated rubber stoppers. In addition, headspace oxygen can accelerate oxidation of antibodies and replacement with an inert gas could minimize such a reaction.[73]

Accelerated Stability Studies

To accelerate formulation development process, accelerated stability studies are usually conducted. However, estimation of room temperature or 5°C stability based on accelerated stability studies for antibodies, like other proteins, may or may not be possible or accurate. The unpredictability is due to the presence of complex and multiple degradation pathways, which may have different degree of temperature dependencies.[10] Kroon et al.[10] found that relative to other degradation pathways of a monoclonal antibody, OKT3 (IgG2a), Asn deamidation was preferentially accelerated with increasing temperature. Therefore, caution needs to be applied in interpreting such high-temperature-induced deamidation. On the other hand, Poborji et al.[41] found that loss of monomeric chimeric antibody (L6) at pH 7.2 followed Arrhenius behavior in the temperature range of 30–50°C and extrapolation of the high temperature SEC data to 2–8°C at pH 7 and

Samsung Bioepis Exhibit No. 1002
Page 5525

5.5 resulted in comparable stability estimates with the available real-time data.

Lyophilized Formulation

Preparation of lyophilized protein formulations has been extensively reviewed.[97–101] Like most proteins, some antibodies are not stable enough in a liquid form and lyophilized dosage forms will have to be considered. Lyophilization was suggested to be one of the two approaches in solving antibody aggregation.[47] This section will discuss some critical issues in formulating a lyophilized antibody product.

Amorphous Versus Crystalline State

It has been commonly believed that proteins are more stable in an amorphous state.[103] This is mainly because proteins need another excipient(s) to replace water molecules to from the required H-bonds as water molecules are removed during drying and a crystalline matrix does not provide intimate H-bonding between the excipient and the protein.[104,105] It appears that if the protein itself can be crystallized during the lyophilization process (may be difficult practically), the protein could be more stable and might not need as much stabilizing agent as in the amorphous state. In a recent report, Shenoy et al.[106] compared the stability of two model proteins, glucose oxidase (GO) and lipase, and demonstrated that dry crystalline formulations are significantly more stable than their amorphous counterparts as monitored by size-exclusion chromatography. It remains to be seen whether this can be applicable to antibodies.

Effect of Formulation Excipients

To make a lyophilized antibody product, at least one formulation excipient, a bulking agent, is needed to confer an acceptable cake. If the antibody is not stable enough in the presence of a bulking agent during lyophilization or storage, a stabilizer(s) is included. The relative amount of formulation excipients (bulking and stabilizing agents) should be carefully chosen as these agents may affect each other's behavior, the tonicity, and stability of the lyophilized products.

Commonly used bulking agents include mannitol and glycine. Crystallization of these agents during lyophilization makes them wonderful bulking agents but poor stabilizing agents.[57] Although mannitol could stabilize an antibody as in the case for a monoclonal antibody-vinca conjugate in a PBS solution at pH 7.4 upon lyophilization,[60] many studies showed that mannitol could not stabilize effectively or even destabilizes antibodies. Such examples include the aggregation of a lyophilized model recombinant humanized monoclonal antibody (rhuMAb HER2) in the presence of mannitol during storage.[3,57] In the lyophilization of a human monoclonal antibody at 1 mg/mL, it was demonstrated that addition of 5% mannitol has no effect on the lyophilization-induced aggregation in PBS or physiological saline as measured by OD600 and addition of 10% mannitol actually accelerated aggregation during the lyophilization process.[58] On the other hand, when used in combination with sucrose or trehalose, similar degree of stabilization to that for sugar alone may be achieved such as the case for model recombinant humanized monoclonal antibody (rhuMAb HER2).[57] The disaccharide/mannitol formulations also inhibited deamidation of this antibody during storage to a greater extent than the lyoprotectant formulations alone.[57]

Sugars are the most common stabilizing agents used in lyophilized formulations. Among these, two nonreducing sugars, sucrose and trehalose, are usually the first choice. Both sugars protected the stability of a few lyophilized antibodies equally well. These antibodies include lyophilized recombinant humanized monoclonal antibody, rhuMAb HER2,[57] IgG1 (containing 12 intra and 4 interchain disulfides),[62] anti-IgG,[107] and chimeric monoclonal antibody.[64] However, sucrose seemed to be more effective in protecting a lyophilized IgG1 against aggregation during storage at 40°C at a sugar:protein weight ratio of 1:1 (Chang et al. 2005b).[102] In another study, sucrose at 5% failed to offer any protection to a lyophilized mouse monoclonal antibody (MN12) (IgG2a (κ)) during storage at 56°C for 18 days.[59] Again, as mentioned in the liquid formulation section, even the most commonly used stabilizers may not stabilize all antibodies in the solid state and antibodies need to be treated as individually different molecules.

When sugars are used as stabilizing agents (lyoprotectants), a sufficient quantity needs to be used to achieve a significant effect depending on the protein concentration. It has been suggested that a molar lyoprotectant:protein ratio of 300:1 or greater should be used.[47] Indeed, the available literature data indicate a ratio higher than 300 is needed for better stabilization. It was shown that sugars protect the stability (aggregation and Asp isomerization) of a lyophilized, monoclonal

Samsung Bioepis Exhibit No. 1002
Page 5526

antibody (type not specified) to a significant level when the sugar to protein molar ratio was increased to 500:1, which happens to the theoretical number of water binding sites in the molecule.[1] Similarly, Andya et al.[62] demonstrated that use of sucrose or trehalose inhibited the aggregation of a lyophilized antibody (IgG1—containing 12 intra and 4 interchain disulfides) during storage at 30°C to a maximum degree when the sucrose/trehalose:protein ratio was 500 or higher, which is roughly equivalent to the number of water-binding sites on the surface of the protein (550 estimated). Increasing the trehalose/anti-HER2 ratio from 60 mM (360 molar ratio) to 200 mM (1,200 molar ratio) significantly increased the stability (monomer by SEC-HPLC) of lyophilized formulation at 25 mg/mL at 40°C.[107] In a recent report, it was found that at least a weight ratio of 2:1 (molar ratio of about 800) was needed to achieve a maximum protection of a lyophilized sucrose:IgG1 formulation against both aggregation and chemical degradation during storage at 40°C (Chang et al. 2005a).[101]

Other formulation excipients were also used and some of these are effective in stabilizing certain antibodies. A recent study demonstrated that addition of sorbitol to a sucrose or trehalose formulation resulted in further enhancement of storage stability of a lyophilized IgG1 antibody (Chang et al. 2005b).[102] Either dextran or HB-β-CD (better) protected the activity of a lyophilized mouse monoclonal antibody (MN12) (IgG2a (κ)) during storage.[59] Glucose was shown to inhibit gamma irradiation-induced fragmentation of humanized monoclonal antibodies (hr3).[85]

Effect of Formulation "pH" and Buffering Agents

The pH of the liquid formulation can have a potential effect on the stability of the lyophilized formulation, even though the solid-state "pH" could not be well defined. In the evaluation of the stability of a lyophilized monoclonal antibody-vinca conjugate, aggregation seems to be lower at a neutral pH 7.1 than at either pH 8.5 or 6.1 when the lyophilized formulation was stored at 25°C.[60] In adjusting the formulation pH, use of a buffering agent may have effect on the antibody stability both during lyophilization and storage of the lyophilized products. For example, in the stability study of a fully human anti-IL8 monoclonal antibody (ABX-IL8; IgG2), Chen et al.[21] demonstrated that addition of histidine inhibited the formation of HMW species upon lyophilization and during storage at 37°C in a concentration-dependent manner. Among several excipients including His, Gly, mannitol, Glu, and polysorbate 20, His is the most critical in protecting solid-state stability of the antibody (minimizing aggregation). In a different study, the aggregation rate of a lyophilized anti-IgE at 5°C at 5 mg/mL in 10 mM buffer is affected both by pH and buffers in the following order: Na Phosphate, pH 7 > K Phosphate, pH 7 > Na Succinate, pH 6 or 5 > Histidine, pH 7.[107] It should be noted that some buffering agents may induce significant pH change due to selective crystallization during lyophilization, such as sodium phosphate. Such buffering agents should be avoided in preparing pH-sensitive antibody formulations.

Effect of Protein Concentration

In many cases, increasing the protein concentration increases the stability of the protein during lyophilization (see review).[98] However, antibodies do not seem to follow this trend and many antibodies have been shown to be less stable both during lyophilization and storage at high concentrations. For example, lyophilization of a human monoclonal antibody caused significant aggregation of MAb formulated in PBS or physiological saline as measured by OD600 and increasing the protein concentration from 1 to 2.5 or 5 mg/mL increased the OD600 from 0.194 to 0.311 or 0.427, respectively.[58] Similar trend was also observed during storage of lyophilized formulation. Increasing the concentration of a chimeric monoclonal antibody from 2.5 mg/mL to 25 mg/mL in a lyophilized formulation, containing 31 mg/mL sucrose, 15 mM NaCl, 0.02% Tween 80, 10 mM citrate, increased the aggregate level from 0.4% to 0.6% after 2 months at 5°C.[64] In the evaluation of the stability of a model recombinant humanized monoclonal antibody (rhuMAb HER2), Cleland et al.[57] demonstrated that increasing the protein concentration from 25 mg/mL to 50 mg/mL in a lyophilized formulation (containing 60 mM trehalose, 5 mM His at pH 6) increased the aggregate level from 8.5% to over 30% by SEC-after storage at 40°C for 44 months. Such behavior was also observed of anti-HER2 from 5 to 21 mg/mL containing 250 mannitol.[107]

Effect of Moisture Content

The effect of moisture on the stability of lyophilized antibodies has not been consistent and seem

Samsung Bioepis Exhibit No. 1002
Page 5527

to depend on the antibody and the degradation pathways being monitored.[57] Generally, increasing moisture content of a lyophilized antibody will gradually increase the rate of degradation during storage. Such negative effect of moisture was clearly demonstrated in a variety of degradation pathways, including dimerization of T2G1s Fab′ (IgG1 (κ)) in a moisture content range of 1.5–12.3%,[61] aggregation of TNF-MAb between 0.68% and 8%,[108] formation of acidic/basic species of rhuMAb HER2 between 1% and 8.4%[57] and fragmentation of a mouse monoclonal antibody (MN12) with and without secondary drying.[59]

In other cases, the effect of moisture is either complex or not significant. For example, Breen et al.[1] found that high-moisture formulations (8%) of a lyophilized monoclonal antibody (type not specified) showed a higher rate of aggregation, fragmentation, and Asp isomerization during storage than those containing 2% or 5% moisture but formulations containing intermediate moisture levels (2–3%) were less prone to aggregation than those containing a lower moisture levels (1%) as measured by SEC under accelerated storage conditions. Similarly, the aggregation of a lyophilized IgG1 was found to be minimal at an intermediate moisture level around 3% at different storage temperatures. (Chang et al. 2005b).[102] In a different study, Cleland et al.[57] found that using a model recombinant humanized monoclonal antibody (rhuMAb HER2), a residual moisture between 1 and 8.4% had no impact on the rate of aggregation during storage at 2–8 or 40°C for 12 months.

Accelerated Stability Studies

Like antibodies in a liquid state, those in a solid state may or may not undergo degradation that is characteristic of Arrhenius behavior. It is generally believed that stability studies should be conducted at least below the glass transition temperature of the formulation. Duddu et al.[64] found that accelerated stability data generated in the glassy state (40°C) seems to be a better predictor of the relative stability of formulations than the data generated at a higher temperature (60°C). In the study of the stability of a high-concentration, lyophilized, monoclonal antibody (type not specified), Breen et al.[1] demonstrated that chemical and physical degradation pathways followed Arrhenius kinetics during storage in the glassy state. Only Asp isomerization followed the Arrhenius model above the Tg value.

Advanced Formulations

In addition to the liquid and lyophilized dosage forms on the market, other dosage forms have been tried and limited success has been reported.

Spray-Dried Formulations

Spray drying is an efficient way of preparing solid dosage forms because of the rapid removal of a solvent(s) from a drug solution. It is a very attractive alternative to lyophilization in preparing solid protein formulations. So far, a number of studies have been conducted for proteins and partly because of the high-temperature exposure in the process, albeit very short, limited success has been achieved. Protein aggregation during spray drying seems to be the major challenge.[65,66,109,110]

Spray drying of antibodies has also been tried. As for other proteins, process-induced protein aggregation seems to be the major issue.[71,110] Careful selection of proper excipients could minimize this problem. In the evaluation of a spray-dried formulation for a rhuMAbE25, it was demonstrated that trehalose or lactose minimized the aggregation during spray drying (aggregate dropped from 5–6% to ≤1%). For trehalose, the molar ratio for significant inhibition of aggregation is at or above 300:1 to 500:1 (excipient: protein).[71] Such a ratio seems to be comparable with that used in the lyophilized formation. The addition of mannitol also reduced aggregation, but only up to a molar ratio of 200:1 and further increase in mannitol content resulted in crystallization, which had a detrimental effect on protein stability and aerosol performance.[71] In a different study, either trehalose or sorbitol could inhibit IgG aggregation during spray drying.[110]

Stabilized Antibodies via Mutagenesis

Antibody fragments, such as scFv, are alternative antibody therapeutics. However, since their stability is not as good as full-length antibodies, mutagenesis, or chemical modifications have been tried to improve its stability. For example, R71A mutagenesis in the heavy chain of an unstable scFv dramatically increased its stability when incubated in human serum while having only a minor effect on the binding affinity of the molecule.[111] A more stable heavy chain, made through mutation, can increase the stability of

Samsung Bioepis Exhibit No. 1002
Page 5528

the light chain, and thereby confer more stability to the whole scFv.[24]

Stabilized Antibodies via Chemical Modifications

The clinical performance of an antibody can be desirably changed by conjugation of the antibody to another entity. For example, McIntosh et al.[112] demonstrated that conjugation of carboxymethyl dextran to a tumor-antigen-specific monoclonal antibody (A5B7) increased the clearance of the antibody in mice but conjugation of cisplatin (*cis*-dichlorodiammine platinum II) with the antibody complex restricted its tissue distribution and reduced its systemic clearance.

PEGylated Antibodies

A common method of increasing plasma half-life of proteins is to increase the size of the protein by attaching a PEG moiety. Currently, there are at least four PEGylated commercial protein products on the market. However, the long plasma half-lives (as high as 7–23 days) of antibodies made much less appealing the development of such an antibody product. Since the long half-life was due to the binding of Fc to the Fc-receptor (neonatal Fc receptor, FcRn) in various tissues,[33,35] antibody fragments, such as Fab′ lacking the Fc portion have a much shorter half-life and could be good candidates for PEGylation. Such a modification was made on a humanized IgG Fab′ fragment via site-specific attachment to an additionally engineered Cys residue, as modification via the lysine residues led to substantial losses in binding activity.[29]

Controlled Release Systems

Controlled release systems have been extensively investigated for proteins in the past two decades.[113,114] However, limited work has been conducted in this area for antibodies, partly due to their relatively long plasma half-lives compared with other proteins. To achieve long-term effect, a controlled released system consisting of biodegradable poly (lactic-glycolic) acid (PLGA) was prepared for an anti-cocaine catalytic monoclonal antibody and an *in vivo* release of the antibody for up to 10 days was demonstrated in mice following subcutaneous injection.[115] It is anticipated that such systems could be useful if a minimum of 1-week duration of release is achieved.

SUMMARY

Antibodies have similar tertiary structures. The presence of a significant number of disulfide bonds and intimate domain–domain interactions in antibodies make them relatively stable and more resistant to moderate thermal stress compared to other proteins. Nevertheless, antibodies do experience a variety of instabilities similar to most proteins. These physical and chemical instabilities include denaturation, aggregation, surface adsorption, deamidation, oxidation, isomerization, fragmentation, etc. Due to the significant difference in the primary sequence among different antibodies, the relative severity of these degradation pathways can be significantly different. On the average, aggregation, deamidation, and isomerization seem to be more prevalent in antibodies.

Partly due to the stability difference, some antibody products are made into a liquid form while others are lyophilized (Tab. 1). The solution pH of these commercial products is either neutral or weakly acidic, suggesting that such a pH range is probably optimal for most antibodies. Surfactants have been used in most of these antibody products, presumably to inhibit antibody aggregation, and will likely be used in other antibody drug candidates. Two most commonly used formulation excipients in these antibody formulations are sucrose and NaCl. For liquid formulations, either one can be used if they offer the same level of stability protection. For lyophilized formulations, sucrose is preferable as NaCl does not form intimate H-bonds with the antibody and also reduce the glass transition temperature of formulation, making lyophilization less efficient. All the formulation excipients and buffering agents used in commercial antibody products or discussed in the article should be evaluated individually for each antibody drug candidate through stability studies before they are chosen as part of the product, as antibodies are structurally different.

The available stability data in the literature and the information on the commercial antibody products suggest that antibody formulation seems challenging but relatively manageable, compared to other protein drugs. Three major issues in antibody formulation are apparently challenging and need significant attention in the coming years: (1) development of stable high-concentration formulations, (2) management of the high-concentration-associated viscosity of these formulations,

Samsung Bioepis Exhibit No. 1002
Page 5529

and (3) minimization of antibody-formulation-induced immune response.

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Samsung Bioepis Exhibit No. 1002
Page 5531

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Declaration by Dr. Michael Treuheit

I, Michael Treuheit, Ph.D., declare that:

1. I am and have been employed by Amgen Inc. since February, 1993. Since 2006, I have held the title of Director Process Development. In this role, I am responsible/oversee formulation development of large molecule therapeutics, such as cytokines, antibodies, and antibody fragments. During my career, I have been directly involved in the formulation of at least 30 clinically tested and/or marketed therapeutic antibodies. A current copy of my CV is provided as **Appendix D**.

2. A study was conducted to demonstrate the stability of eculizumab over a range of concentrations. Eculizumab was formulated at 2 mg/ml (2PBST22), 5 mg/ml (5PBST22), 10 mg/ml (10PBST22), 30 mg/ml (30PBST22), and 50 mg/ml (50PBST22) in 10 mM Na Phosphate, 150 mM NaCl, 0.022 % polysorbate 80 at pH 7.0. Soliris® (eculizumab) is 10 mg/ml eculizumab formulated in 10 mM Na Phosphate, 150 mM NaCl, 0.022 % polysorbate 80 at pH 7.0. The EMA label indicates Soliris should be stored between 2-8°C.

3. Like Soliris®, the eculizumab formulations used in the study were sterile and preservative free.

4. The stability of the 2 mg/ml, 5 mg/ml, 10 mg/ml, 30 mg/ml, and 50 mg/ml eculizumab formulations was monitored over a period of 13 weeks at storage conditions of (4°C) and at elevated temperature (25°C). Storage at the elevated temperature is used to evaluate the product specific routes of degradation in an accelerated manner as compared to recommended storage conditions. Physical and chemical stability were analyzed by size-exclusion ultra high-performance liquid chromatography (SE-UHPLC), anion exchange high-performance liquid chromatography (AEX-HPLC), and reduce sodium dodecyl sulfate capillary electrophoresis (rCE-SDS).

5. Aggregation of the antibody results in the formation of high molecular weight (HMW) species that can be visualized by SE-UHPLC. A large degree of HMW formation is a sign of low long-term stability. All of the eculizumab solutions stored at 4°C and 25°C were sufficiently stable throughout the study, i.e., there was no significant loss in main peak for any of the protein concentrations examined. The results are summarized in **Appendix A**.

6. The storage of antibodies in solution can result in chemical degradation, leading to the formation of acidic species (e.g. by deamidation of asparagine residues), which can be detected by AEX-HPLC. A high degree of acidic species is a sign of low chemical stability. Analysis of the eculizumab solutions stored under label conditions and the elevated temperature showed that all tested antibody concentrations were essentially equivalent. At the elevated temperature, all showed some chemical degradation, which increases over time upon storage, but there is no significant difference in chemical stability between protein concentrations. The results are summarized in **Appendix B**.

7. rCE-SDS offers high resolution separation of most types of chemically or structurally altered species from the main (intact) species for both heavy chain (HC) and light chain (LC) of the antibody. Analysis of the eculizumab solutions by rCE-SDS shows that a detectable decrease of the main species (HC+LC) occurs at both tested temperatures for all samples. However, in agreement with the AEX-HPLC measurements, the antibody solutions were equivalent at the recommended storage conditions and elevated temperature. The results are summarized in **Appendix C**.

8. The results of the SE-UHPLC, AEX-HPLC, and rCE-SDS analyses demonstrate eculizumab is stable at the range of concentration tested, 2-50 mg/ml, and there is no significant difference in stability amongst the concentrations tested. Thus, the 10 mg/ml formulation does not demonstrate superior stability compared to the less- or more-concentrated formulations of eculizumab.

I certify that the above statements are correct to the best of my knowledge and belief.

Amgen Thousand Oaks, CA, Aug 3, 2017

Place and Date

Ph.D.

Dr. Michael Treuheit

Attachments
Appendices A-D

Samsung Bioepis Exhibit No. 1002
Page 5536

CURRICULUM VITAE

Michael Treuheit, Ph.D.

Education

Doctor of Philosophy in Chemistry, October 1990, University of Cincinnati.
Thesis Title: *Disease-Coupled Specificity In The Glycan Structures of Orosomucoid.*

Bachelor of Science in Biology, June 1980, University of Cincinnati.

Work Experience

Amgen, Inc., 1993-present

Director of Drug Product Formulation Technology in the Department of Product Development leading a group of approximately 30 scientists (15 at the PhD. Level). The group has responsibility for developing FIH and commercial formulations, and for the CMC sections of our large molecule MA's.
Notable past accomplishments include the submission of the Development Pharmaceutics Section in the BLA's for Neulasta , Palifermin and Denosumab along with a variety of IND's and INDa's for our pipeline molecules

University of Cincinnati, College of Medicine, Department of Pharmacology and Cell Biophysics. Postdoctoral Fellowship in the Protein Chemistry Core Laboratory of Dr. Terence L. Kirley, 1990 to 1993.

University of Cincinnati, Department of Chemistry Teaching Assistant, 1985 to 1990.
Taught freshman, Bio-Organic and Biochemistry laboratory and selected lecture courses.

The Christ Hospital, Research Technologist in the Tumor Research Laboratory, experienced in tissue culture techniques (Human Tumor Clonogenic Assay), 1975 to 1989.

Abstracts and Presentations

Kim, S.B., Distler, C. and Treuheit, M. Human Tumor Clonogenic Assay (HTCA) In Patients With Metastatic Adenocarcinoma, Primary Unknown. Presented at the Annual meeting, Central Society for Clinical Research, Chicago, 1984.

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Samsung Bioepis Exhibit No. 1002
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Samsung Bioepis Exhibit No. 1002
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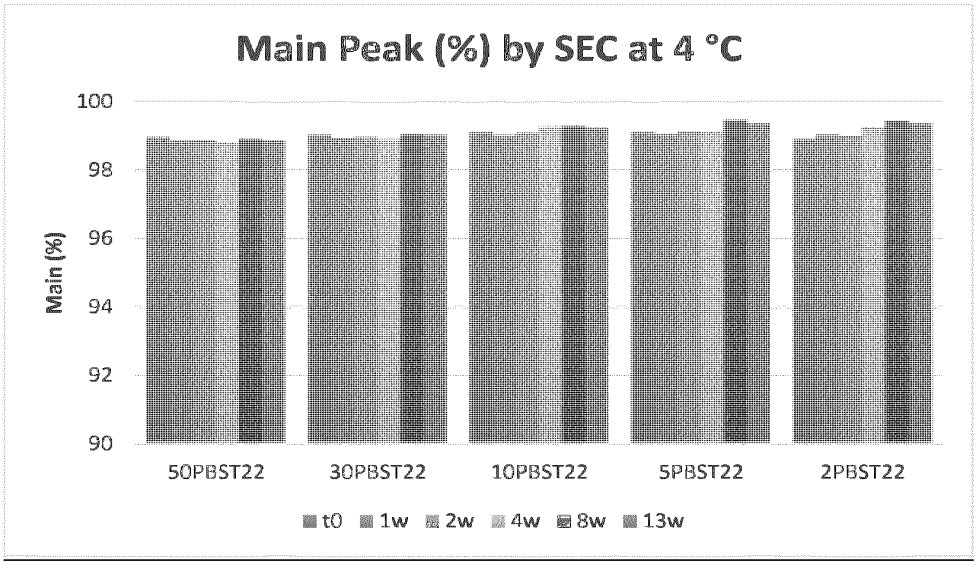
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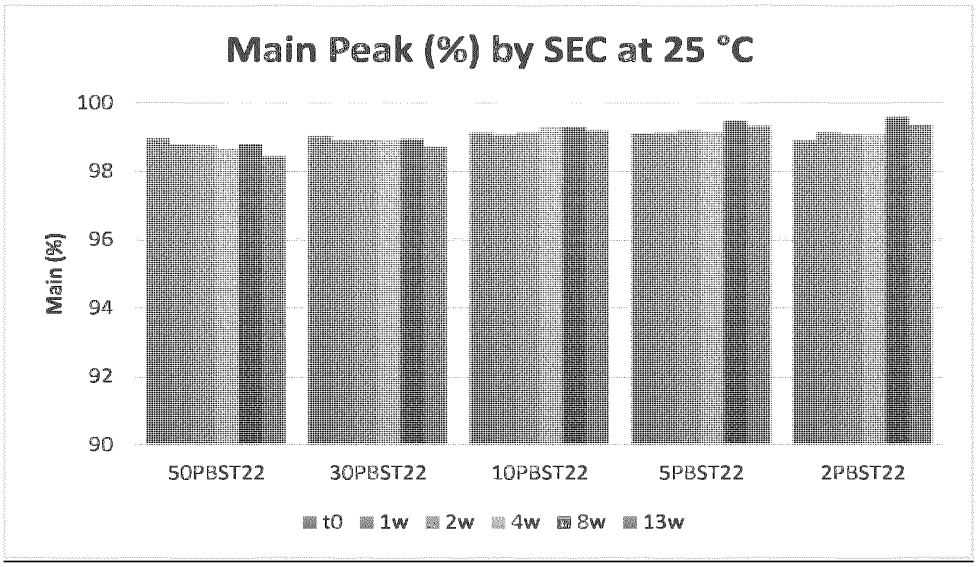
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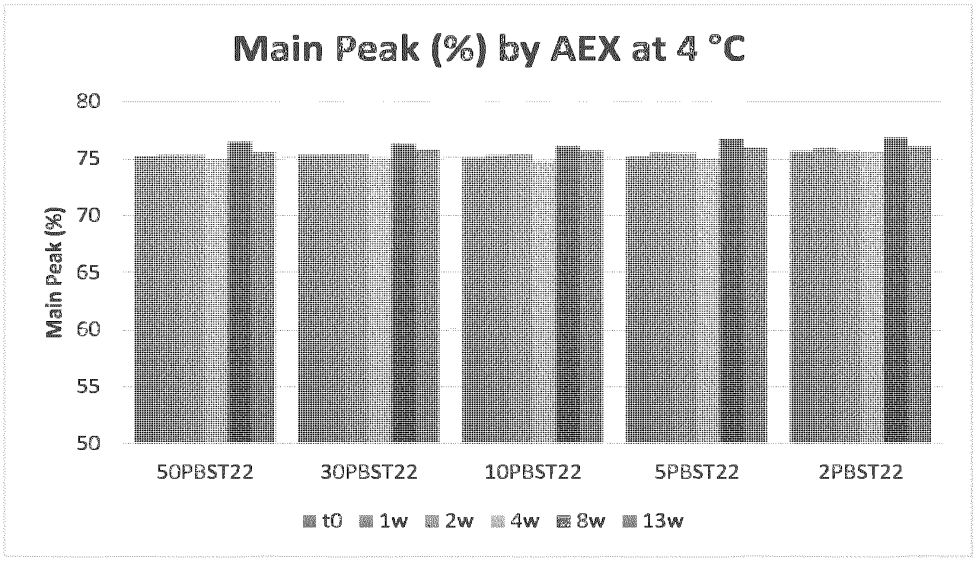
Samsung Bioepis Exhibit No. 1002
Page 5543

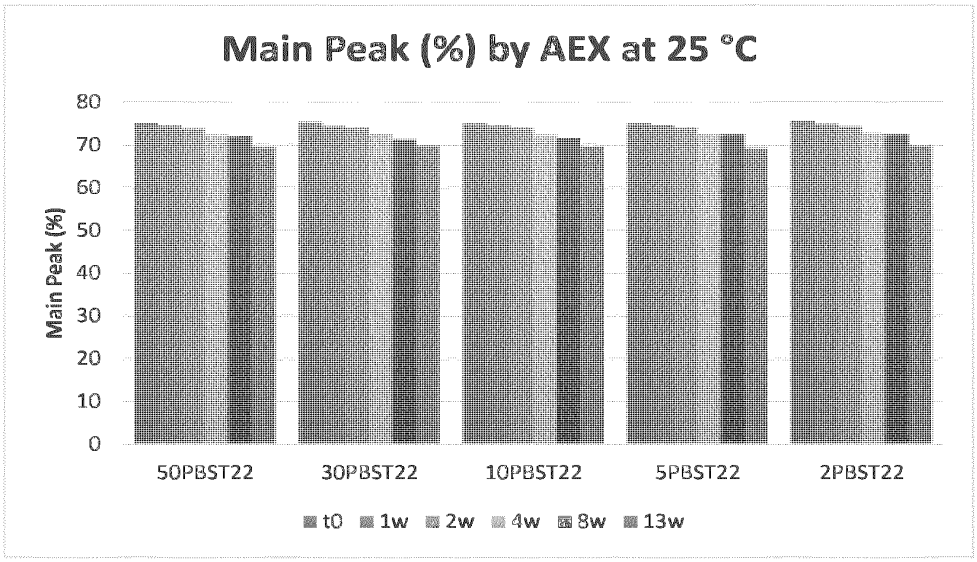
Appendix A


Main Peak (%) by SEC at 4 °C
100
98
96
94
92
90
Main (%)
50PBST22
30PBST22
10PBST22
5PBST22
2PBST22
t0
1w
2w
4w
8w
13w


Main Peak (%) by SEC at 25 °C
100
98
96
94
92
90
Main (%)
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13w

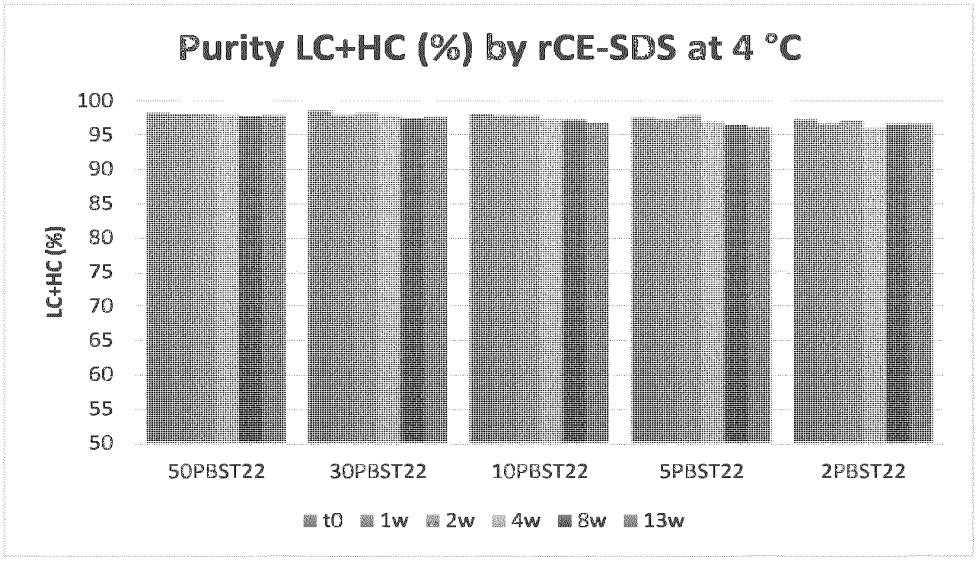
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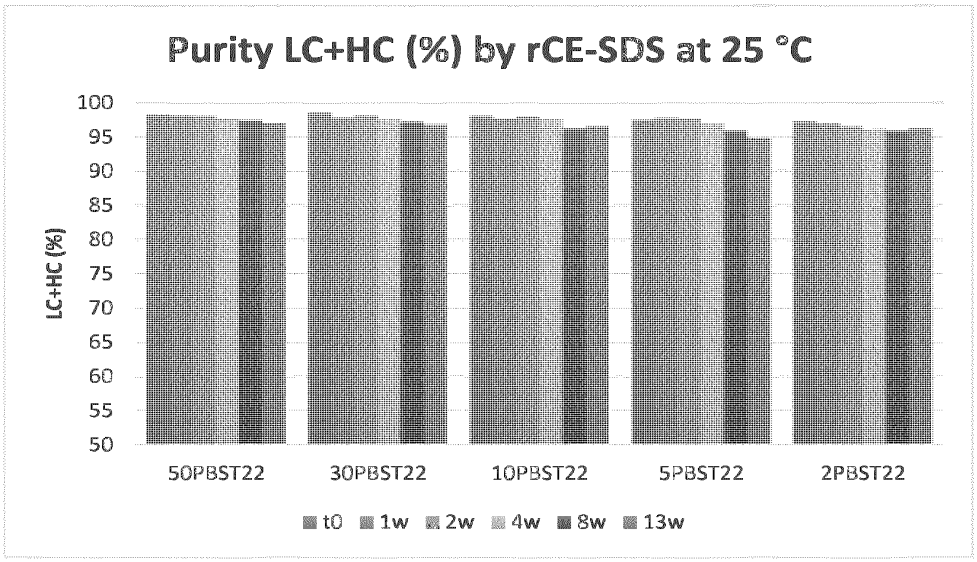




Samsung Bioepis Exhibit No. 1002
Page 5545

Appendix C





Samsung Bioepis Exhibit No. 1002
Page 5546